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Development and validation of analytical methods for the estimation of trioxsalen in pharmaceutical dosage form by using uv-spectrophotometry and HPTLC

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ABSTRACT

Analytical monitoring of a pharmaceutical product or 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 specifications elaborated 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. Determination of Trioxsalen in a fixed dosage form was carried out by UV Spectrophotometric and HPTLC method. The absorbance values were observed for different dilutions of drug at 248 nm and which are used for the dilution in Ethanol. This method obeys Beer's Lambert's Law in the concentration range of 1-5µg/ml. The results have been validated statistically and the recovery studies confirmed the accuracy of this proposed method.

Keywords: UV -Ultraviolet visible, µg -Micro gram, ml- Milliliter, nm- Nano meter, GIT -Gastro intestinal tract, RS - -Resolution, CSF - Cerebro spinal fluid.

INTRODUCTION

Vitiligo is a disorder in which asymptomatic whitish patch or macule with sharply demarcated margins appears on different parts of the skin. It occurs due to the systemic destruction of *melanocytes,* especially in the mucous membranes, eyes, and the membranous labyrinth of the inner ear. Vitiligo can develop at any age; however, in 70% - 80% of cases, it arises before the age of 30. By far, vitiligo is bethought as the most frequent disorder of pigmentation and India shows its highest incidence all over the world (up to 8.8%). [4, 5]

Vitiligo can be classified majorly into two clinical forms.[7]

- Non-segmental vitiligo (NSV)
- Segmental vitiligo (SV)

NSV is an acquired chronic pigmentation disorder characterized by white patches, often symmetrical, which usually increase in size with time, corresponding to a substantial loss of functioning epidermal and sometimes hair follicle *melanocytes.* NSV is further divided into

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acrofacial, universal, mucosal and so on based on their corresponding locations.

Analytical method development[4]

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.

High performance thin layer chromatography

High Performance Thin Layer Chromatography (HPTLC) is a modified TLC technique which has been reported to provide excellent separation, qualitative and quantitative analysis of a wide range of compounds, such as herbal and botanical dietary supplements, nutraceuticals, traditional western medicines, traditional Chinese and Ayurvedic (Indian) medicines and determination of radio labeled substances in chemical, biochemical, biological, pharmaceutical, and medicinal samples.

HPTLC is superior to other analytical techniques in terms of total cost and time for analysis. It is an offline process in which various stages arc carried out independently.

Drug Profile of Trioxsalen

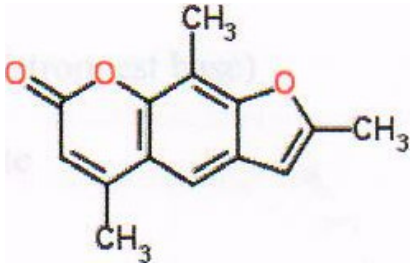


Fig 1: Chemical structure of Trioxsalen

Trioxsalen is a, a synthetic derivative of Psoralen, obtained from several plants mainly *Psoraleacorylifolia*. ltis a furanocoumarin (7H-Furo [3, 2-g] chromen-7-one) derivative in which positions 2, 5, and 9 are substituted by methyl groups. Trioxsalen causes photosensitization of the skin. It is administered orally

Mechanism of Action

After photo activation, Trioxsalen creates interact and cross-links in DNA, which can cause programmed cell death.

MATERIALS AND METHODS

Uv-visible spectroscopy

Reagents and chemicals

- Trioxsalen Reference Standard (RS) obtained IromSankalpMealthcare and Allied products (P) Ltd.
- Methanol HPLC grade obtained from Merck specialties (P) Ltd. Mumbai.
- Commercially available Trioxsalen tablets (TROID-25, contains 25 mg of Trioxsalen) marketed by Resilient Cosmeceuticals (P) ltd.

Preparation of standard solution

Weighed accurately l0mg of Trioxsalen RS, transferred into a 100ml standard flask, dissolved, and made up to the volume with methanol. The

final solution had a concentration of 1 00mcg/ml (solution A).

Accurately pipetted out 10ml of solution A into a 100ml standard flask and the volume was made up to 100ml using methanol to get a concentration of l0mcg/ml (solution B).

Study of spectral characteristics of Trioxsalen RS in methanol

THERMO SCIENTIFIC UV-2600 double beam Spectrophotometerwas used for scanning Trioxsalen RS in methanol (solution A and B) from 200-400nm after enabling blank correction in the above region. An absorption band ranging from 200-400 was observed with maximum absorption at 248nm.Using solution A, the absorption intensity was beyond the limits of the instrument. The spectrum obtained with solution B is shown in the figure.

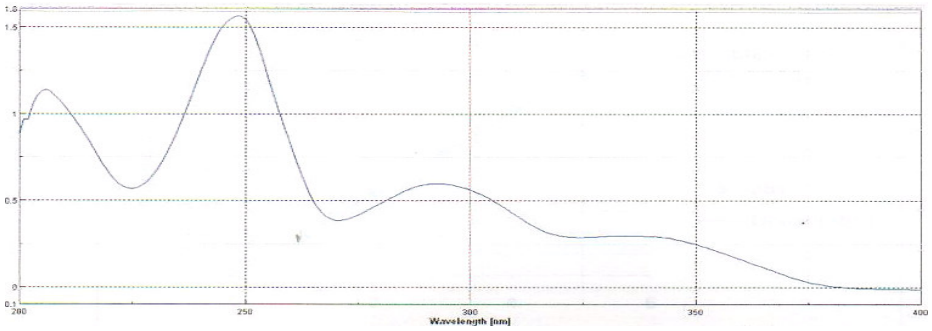


Fig 2:UV absorption spectrum of Trioxsalen in methanol with absorption maxima at 248nm

Statistical evaluation of calibration plot

The calibration curve was plotted with Absorbance in the Y-axis and concentration in the X-axis. A linear plot was obtained within this concentration range. The correlation coefficient was obtained as 0.998 and the regression equation was found to be Y=0.18Sx+0.002(Y = M x + c).

Estimation of Trioxsalen in dosage forms

The details of the drug to be analyzed are given below: Trioxsalen tablets USP in the brand name TROID-25
Label claim: Trioxsalen tablets USP
Batch No: STH1501
Mfg.Date:JAN.2018
Exp.Date:DEC.2020
Mfd. by: Resilient CosmeceuticalsPvt.Itd

Procedure

Ten tablets were taken from the pack and peeled out the film coat over the tablets. Accurately weighed the tablets and the average weight calculated. The tablets were finely powdered in a glass mortar and weighed an amount equivalent to l0 mg of Trioxsalen..Carried out the dry extraction with 3 x 15 ml portions of methanol in a stoppered bottle. Then, the combined extracts were sonicated for 10 minutes, filtered using whatmann no. 1 filter paper and transferred into a 100ml standard flask. Washed down the residue left after using methanol and made up the volume with the same. The resultant solution had a concentration of 100mcg/ml. Accurately pipetted out 10 ml of the above solution to a 100ml standard flask and made up to the volume using methanol. The final solution had a concentration of l0mcg/ml. Standard solution of Trioxsalen was prepared in the similar manner, accurately weighed 10mg of Trioxsalen RS and transferred into a 100 ml standard flask, sonicated for 10 minutes (100mcg/ml), 10 ml of the resultant solution was pipetted into a 100ml standard flask and made up the volume to 100ml using methanol (l0 mcg/ml).

Measurement of absorbance

Accurately pipetted out final dilutions of both standard and sample solutions; 2,3 and 4 ml respectively to neatly labeled 10ml standard flasks. The resultant solutions had concentrations of 2,3 and 4 mcg/ml respectively. The absorbance of each

solution were measured at 248nm using methanol as blank. Absorbance measurements obtained were given in the table below.

Estimation of the content

Amount of drug per tablet
= [{absorbance of sample -r- absorbance of standard) x (dilution factor(for sample) -f- dilution factor(for standard) } x (weight of standard -r- weight of sample}] x average weight
Average weight of 10 tablets = 0.3089g
Weight of sample taken, equivalent to 10 mg = 0.1235g
Weight of standard taken = 0.0l00g
Label claim of the tablet = 25mg

Table 1.Assay result of Trioxsalensample

Conc. mcg/ml)	Abs. of sample	Abs. of standard	Amount in tablet	Label claim	% label claim
2	0.2891	0.2899	0.0249	0.0250	99.6
3	0.4375	0.4270	0.0256	0.0250	102.4
4	0.5809	0.5791	0.0250	0.0250	100

Average content per tablet = 0.0252g
Percentage label claim = 100.66%

Validation of the proposed method

Accuracy

The accuracy of the method is the closeness of the measured value to the true value for the sample. Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts to tablet. It was estimated by preparing concentration of 80%, 100% and 120% of the label claim of the tablet. Three samples were prepared for each recovery level. The solutions were then analyzed, and the percentage recoveries were calculated from the corresponding absorbance obtained.

Procedure

Ten tablets were taken from the pack, peeled out the film coat over the tablets. Accurately weighed the tablets and the average weight were calculated. The tablets were finely powdered in a glass mortar and weighed an amount equivalent to l0 mg of Trioxsalen. This is considered as 100% concentration of the test. Weight equivalent to 10 mg is taken in triplicate. Then, it is spiked with 5mg (80%), 10mg (100%) and 12mg (120%) of standard drug substance, respectively. Drug mixture, taken in a stoppered flask was extracted with 3x15ml of methanol. the solutions were sonicated for 10 minutes. The resultant solutions were filtered into 3 separate neatly labeled standard flasks, volume made up to 100ml using methanol. Then 2 more consequent dilutions were carried out for each of them (1ml of the stock was diluted to 10ml and again, 1ml of the obtained solution, diluted to 10ml) to obtain concentrations within the linearity range viz. 1.8, 2, 2.2mcg/ml respectively. Standard Trioxsalen solutions of similar concentrations were also prepared.

Measurement of absorbance

Absorbance of each solution was measured at 248nm using methanol as the blank. The data obtained is furnished in the table given below:

Table 2 .Recovery study results

Concentration of Trioxsalen (mcg/ml)	Standard absorbance at 248nm	Sample absorbance at 248nm	% recovery
1.8	0.2517	0.2470	98.09%
	0.2519 * 0.2517	0.2470 0.2471	
	0.2518	0.2470	
2	0.2869	0.2815	98.12%

	0.2868	0.2817	
	0.2870	0.2816	
	0.2869	0.2815	
2.2	0.3127	0.3086	98.68%
	0.3128	0.3088	
	0.3127	0.3085	
	03127	0.3086	

* Average percentage recovery = 98.29%

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility, In this work, repeatability and intermediate precision studies have been performed.

Repeatability/Intraday precision

Repeatability expresses the precision under the same operating conditions over a short interval of time, intraday precision. It is usually expressed as die standard deviation of a series of measurements. The repeatability /intraday precision of the method was Studied using three different concentrations of Trioxsalen (2mcg/ml, 3mcg/ml and 4mcg/ml respectively), taken from within the linearity range of the drug solution. The absorbance was measured at 248nm against methanol! as blank. The absorbance was measured in triplicate for each concentration and the mean values were calculated and the data is shown in the table given below.

Table 3 -Results of intraday precision study

Concentration (mcg/ml)	Time in hours				Mean
	0	1	2	3	
2	0.3437	0.3410	0.3411	0.3485	0.3435
	0.3437	0.3411	0.3410	0.3484	
	0.3438	0.3410	0.3411	0.3485	
	0.3437	0.3410	0.3411	0.3485	
3	0.4667	0.4667	0.4636	0.4797	0.4686
	0.4668	0.4666	0.4635	0.4797	
	0.4667	0.4667	0.4636	0.4796	
	0.4667	0.4667	0.4636	0.4797	
4	0.6146	0.6222	0.6245	0.6257	0.6217
	0.6145	0.6221	0.6245	0.6258	
	0.6146	0.6222	0.6246	0.6257	
	0.6146	0.6222	0.6245	0.6257	

Table 4 - Statistical results of intraday precision study

Concentration (mcg/ml)	Standard deviation	Relative standard deviation (%)
2	0.0035	1.02
3	0.0074	1.60

4	0.0049	0,80

Mean percentage relative standard deviation was found to be 1.14%

Intermediate precision (Inter-day precision)

Intermediate precision, expresses within laboratory variations including different days, different analysts, different equipment's etc. The inter-day precision study of Trioxsalen was carried out by estimating the absorbance of 3 different concentrations of Trioxsalen (2, 3 and 4 mg/ml) taken during 3 consecutive days. The data obtained from the study is given below:

Table 5 - Results of inter-day precision study

Concentration (mcg/ml)	Absorbance			Mean absorbance
	Day 1	Day 2	Day 3	
2	0.3466	0.3336	0.3429	0.3410
	0.3466	0.3335	0.3429	
	0.3466	0.3436	0.3429	
	0.3466	0.3336	0,3429	
3	0.4730	0.4663	0.4601	0.4666
	0.4932	0.4649	0.4602	
	0.4727	0.4689	0.4600	
	0.4730	0.4667	0.4601	
4	0.6436	0.6472	0.6346	0.6418
	0.6436	06472	0.6345	
	0,6434	0.6471	0.6346	
	0.6435	0.6472	0.6346	

Table 6 -Statistical results of inter-day precision study

Concentration (mcg/ml)	Standard deviation	Relative standard deviation (%)
2	0.0066	1.96
3	0.0064	1.38
4	0.0064	1.01

Mean percentage relative standard deviation was found to be 1.45%

Detection limit (LOD) and Quantitation limit (LOQ)

- The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated.
- The quantitation limit of an individual analytical procedure is the lowest concentration of analyte in a sample, which can be quantitatively determined with a suitable level of precision and accuracy.
- The LOQ and LOQ of the developed method has been found out by plotting a minimum of 5 calibration curves and estimating the slopes and the standard deviation.

Table 7: Linearity data for determination of LOD and LOQ.

	Y-intercept	Slope
Curve 1	0.003	0.199
Curve 2	0.023	0.173
Curve 3	0.005	0.187
Curve 4	0.000	0.146

Curve 5	0.039	0.167
	S.D of Y-intercept = 0.016	Mean = 0.1 74

The LOD and LOQ were found as 0.303 and 0.92 mcg/ml respectively

Linearity Range

The linearity of an analytical procedure is its ability to obtain test results which are proportional to the concentration of analyte in the sample. The range of an analytical procedure is the interval between upper and lower concentrations of the analyte in the sample for which it has been demonstrated that the analytical procedure has suitable level of precision, accuracy and linearity. The calibration curve of Trioxsalen was found to be linear over the range of 1-5 meg/ml.

High Performance Thin Layer Chromatographic Determination of Trioxsalen (HPTLC)

Reagents and Chemicals

1. Trioxsalen RS obtained from Sankalp Healthcare and Allied Products Pvt Ltd, Mumbai
2. Chloroform: Toluene:.Methanol: HPLC grade, from Merck Specialties (P) Ltd. Mumbai.

Trioxsalen tablets USP in the brand name TRO1D-25
Label claim: Trioxsalen tablets USP
Batch No: STH1502Mfg.Date:JAN,2018Exp,Date:DEC.2020M fd. by; Resilient CosmeceuticalsPvt.ltd

Equipment used

- Development mode: CAMAG Twin trough chamber
- Scanner: TLC Scanner with WINCATS Software
- Visualization: CAMAG UV Cabinet
- Quantification: CAMAG Video Densitometer

Stationary phase

TLC plates (15xl0cm) with 250um thickness; E.Merck, Darmstadt, Germany

Preparation of standard solution of Trioxsalen

Weighed accurately 10mg of Trioxsalen RS and transferred to a 100ml standard flask. Added about 50ml of methanol, sonicated for 10 minutes and then made up to the volume using methanol. This solution had a concentration of 100 mcg/ml.

Development of solvent system

The mobile phase was selected based on the polarity of analyte and adsorption property of silica gel plates. The solubility of drug played a significant role in the selection of mobile phase. The suitable solvent system was selected by a series of trial and error process. Different solvent systems were used in different proportions and the summary is listed in the table below:

Table 8: Solvent system selection trial and error data

Toluene	-	No movement of spot
Methanol	-	Spot moved
Chloroform	-	Spot moved up to solvent front
Methanol: Toluene	9:1	Spot moved upto solvent front
Methanol :Toluene	5:5	Moved near to solvent front
Chloroform: Toluene	5:5	Spot moved with little resolution

Optimization of mobile phase

Chloroform: Toluene mobile phase system was optimized by changing the ratio of solvents. Table below shows the different ratio that has been tried.

Table 9.Optimization of mobile phase

1	Chloroform: Toluene 5:5
2	Chloroform: Toluene 6:4
3	Chloroform: Toluene 7:3

Mobile phase

Chloroform: Toluene (7:3, v/v) was chosen as the mobile phase, which gives a chromatogram with good resolution for Trioxsalen.

Development of Chromatogram

Selection of chromatographic layer

HPTLC pre-coated plates of silica gel G60F254 were employed for the spotting of standard solutions.

Preparation of mobile phase and saturation of Twin trough chamber

Mobile phase (Chloroform: toluene in the ratio 7:3, v/v) was freshly prepared and transferred into a clean and dried twin trough chamber. The chamber was then allowed to saturate for 30 minutes.

Activation of plate and sample application

A single track was selected on the activated pre-coated HPTLC plate and spotting was done by using CAMAG Linomat IV sample applicator in the form of bands. Trioxsalen sample was applied on the track. Volume of sample application was selected according to the volatility of the solvent used for preparing the sample solution. Concentration was selected as 100mg/ml by trial and error method. The applied band was sharp when the volume was 2ml.A band width of 4mm was selected for the entire experiment.

The following manual adjustments were done in the linomat applicator.

Plate size: 10x3cm Start position: 12mm

Band width: 4mm

Application volume: 2ul

Flow rate: 2ml/sec.

After application, the plate was taken out and the position of the spot was visualized and confirmed under the LTV cabinet at 254nm.

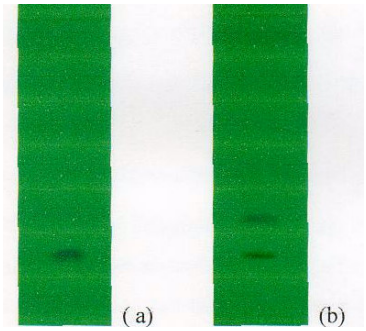


Fig 3:TLC plates, before and after spotting visualized in a UV cabinet

Development of spot

The plate was developed in the saturated twin trough chamber containing the mobile phase. The plate was dried after development and viewed tinder UV cabinet to evaluate the spots obtained.

Determination of R_f value of Trioxsalen RS

Detection and visualization (b)

The developed plate was mounted on the CAMAG HPTLC Scanner IV and scanned from 200-400nm.The spot showed good response at 254nm.The Rf value of the drug was found to be: 0.22

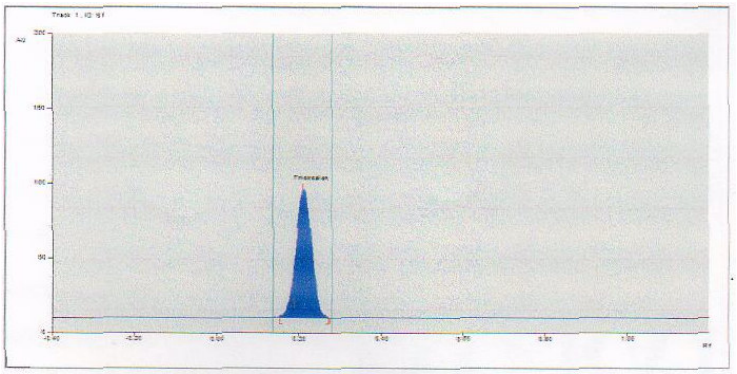


Fig 4.: chromatogram of Trioxsalen with R_f0.22.

Preparation of calibration curve and quantitative estimation of drug in the dosage form

Preparation of standard solution of Trioxsalen in methanol Weighed accurately l0mg of Trioxsalen RS and transferred to a 100ml standard flask. Added about 50ml of methanol, sonicated for 10 minutes and then madeup to the volume using methanol. This solution had a concentration of 100mcg/ml.

Preparation of sample solution of Trioxsalen in methanol Details of analyzed dosage Form:

Trioxsalen tablets USP hi the brand name TRO1D-25

Label claim	:	Trioxsalen tablets USP
Batch No	:	STHI501
Mfg.Date	:	JAN.2018
Exp.Date	:	DEC.2020
Mfd. By	:	Resilient CosmeceuticalsPvt ltd

Accurately weighed 10 no's of TROID-25, Trioxsalen tablets arid peeled off the outer film coating. Then, it was finely powdered and a weight equivalent to 10 mg was taken.

The weighed amount of drug was transferred into a stoppered flask, about 50ml of methanol was added and sonicated for 10 minutes, and then the aggregate solution obtained was transferred into a 100ml standard flask. The stoppered flasks as well as the residue obtained were washed out through the filter paper into the standard flask, using methanol and the volume was made up to 100ml with the same. The final solution constituted a concentration of 100□g/ml.

- Development of chromatogram
- Selection of chromatographic layer

HPTLC pre-coated plates of silica gel G60$F_2$54 were employed for the spotting of sample solutions. Preparation of mobile phase and saturation of Twin Trough Chamber (TTC)

Mobile phase containing chloroform: Toluene in the ratio 7:3, v/v was freshly prepared and transferred into a clean and dried twin trough chamber. The chamber was then allowed to saturate for 30min.

Activation of plate and sample application

Thirteen tracks were selected on the activated pre-coated HPTLC plate and spotting was done by using CAMAG Linomat IV automatic sample applicator in the form of bands. Sample solutions were applied in duplicate on first 3 and last four tracks, in order to have a comparison. Standard drug solutions were applied on 4-8 tracks and correspondingly on the last track together with the sample (over spot).

The following manual adjustments were done in the Linomat applicator.

Plate size: 20x10cm Start position: 15mm

Band width: 6mm

Application volume: 2ml

Flow rate: 2ml/min

Space: 10mm

After application the plate was taken out and the position of the spots were visualized and confirmed under UV cabinet at 254nm.

Development of spots

The plate was developed in the saturated twizi trough chamber containing the mobile phase. The plate was dried after development and viewed under UV lamp to evaluate the spots obtained. The spots were uniform without tailing.

Scanning and integration of chromatogram

The developed plate was mounted on the CAMAG HPTLC Scanner TV and scanned at 254nm.The results are furnished in the table below. The calibration plots of concentration v/s peak height and concentration v/s peak area were plotted and shown in figure below. The overlay spectrum and the plate developed are shown in the figures below. The chromatograms of standard and samples were given respectively in the fig below.

Table 10.Chroma to gram analysis data

Track	Substance	Concentration (ng/spot)	Height	Area
1	Sample	400	129.85	3598.41
2	Sample	600	200.17	5198.05
3	Sample	800	253.91	6951.39
4	Standard	200	65.29	1760.35
5	Standard	400	130.58	3602.7
6	Standard	600	201.34	5281.05
7	Standard	800	254.15	7041.4
8	Standard	1000	324.45	8761.5
9	Standard	1200	393.32	9867.8
10	Sample	400	128.89	3506.62
11	Sample	600	199.30	5125.70
12	Sample	800	252.90	6860.36
13	Sample+Standard	200+200	131:32	3154.00

Table 11. Linearity data

Concentration of standard (ng/spot)	Peak height	Concentration of standard (ng/spot)	Peak area
200	65.29	200	1760.35
400	130.58	400	3602.7
600	201.39	600	5281.05
800	254.15	800	7041.4
1000	324.45	1000	8761.5
1200	393.32	1200	9867.8

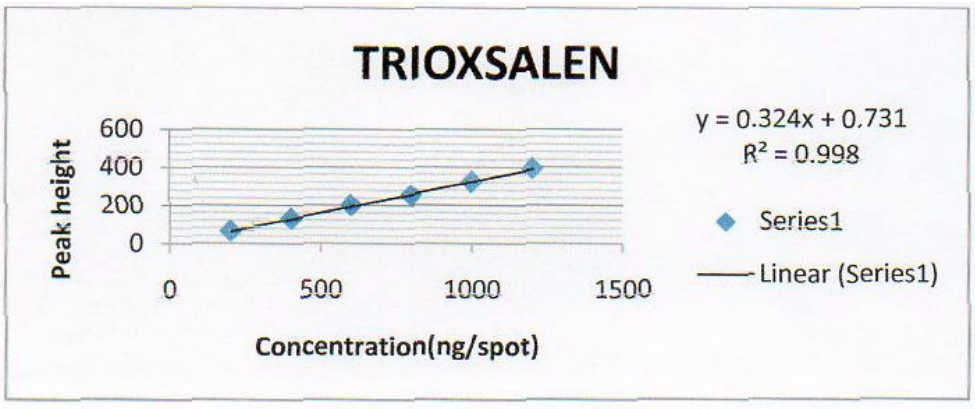


Fig 4: Calibration plot of Trioxsalen [Concentration v/s Peak height]

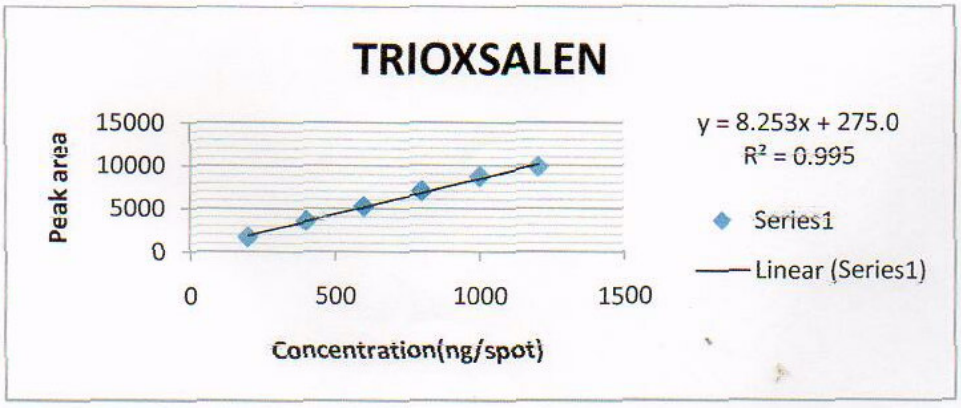


Fig 5: Calibration plot of Trioxsalen [Concentration v/s Peak area]

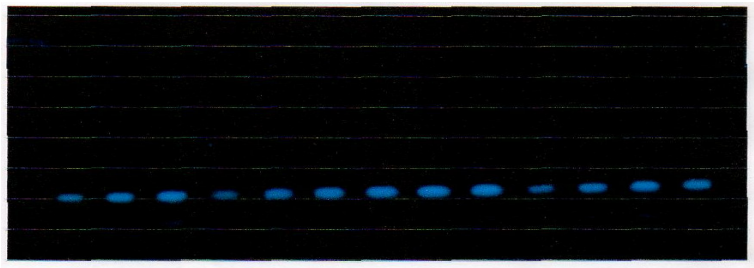
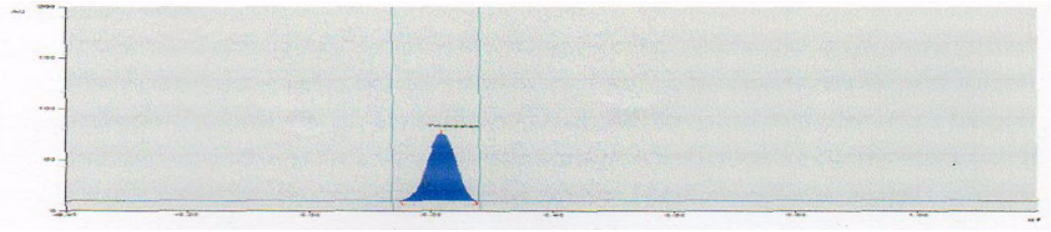
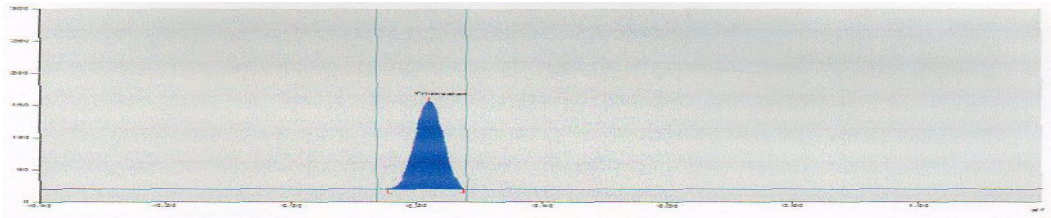


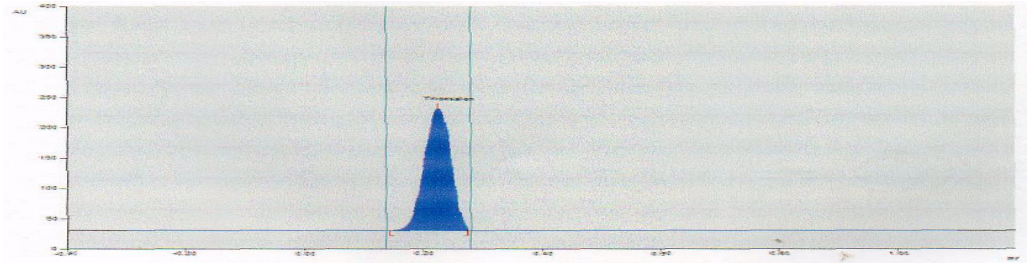
Fig 6:Photograph ofdeveloped HPTLC plate



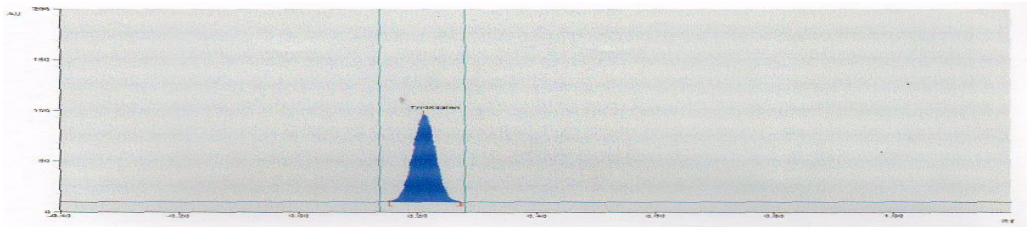
a) Trioxsalen 200ng/spot



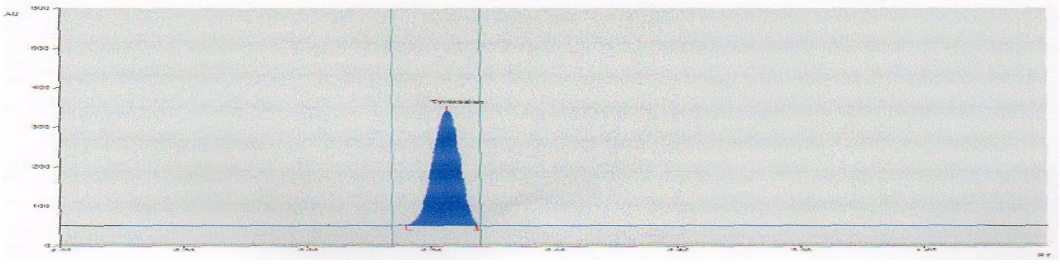
b)Trioxsalen 400ng/spot



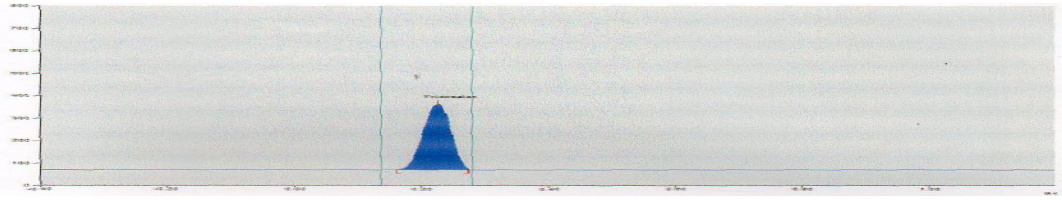
c) Trioxsalen 600ng/spot



d) Trioxsalen 800ng/spot

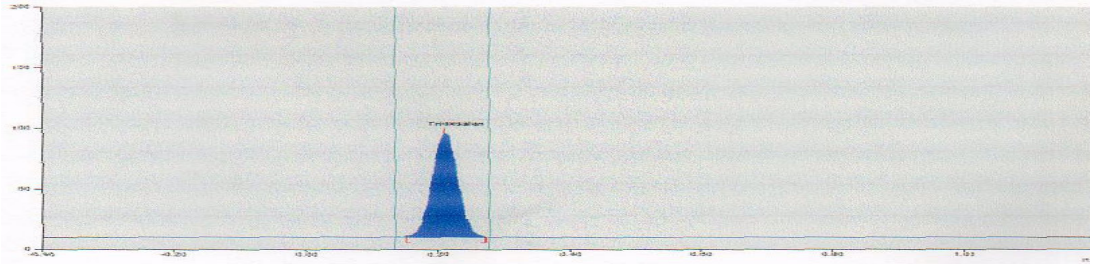


e)Trioxsalen 1000mcg/spot

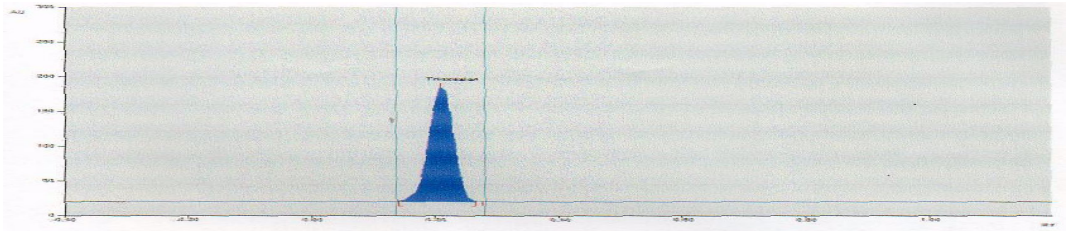


f) Trioxsalen 1200ng/spot

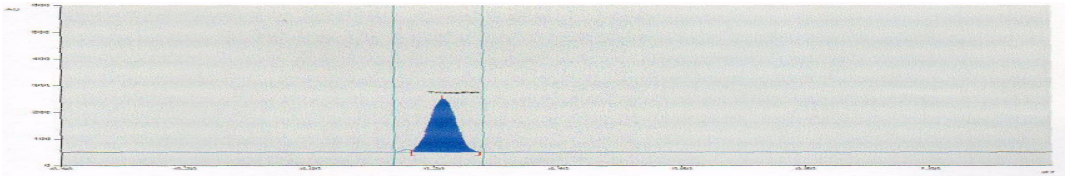
Fig.7 a,b,c,d,e,f: Chromatograms of Trioxsalen standards



a) Trioxsalen 400ng/spot



b) Trioxsalen 600ng/spot



c) Trioxsalen 800ng/spot

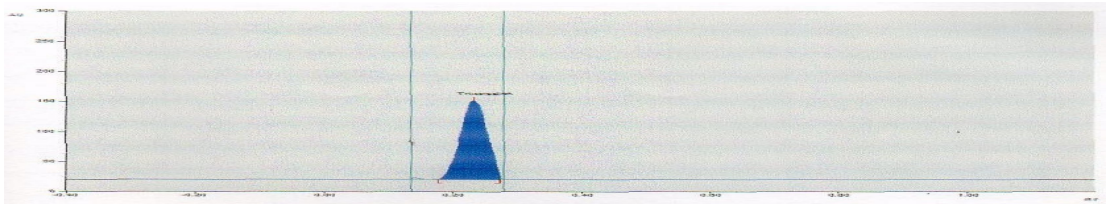


Fig 8.Trioxsalen sample over spot on standard (200ng each/spot)

Quantitative estimation of trioxsalen in tablet dosage forms

Quantitative estimation of drug was performed by comparing areas and heights of chromatograms obtained for the sample drug solution taken in three different concentrations with that of the standard.

Amount of drugper tablet
= [{area or height of the chromatogram of sample
-r area or height of the chromatogram of standard]
x {dilution factor(for sample) -r- dilution factor(for standard)}
X (weight of standard -^ weight of sample}] x average weight
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 = 0-0100

Table 12: Assay results of Trioxsalen sample.

Conc,	Sample		Standard		Avg. content		% label claim	
(ng/spot)	Ht.	area	Ht.	area	Ht.	area	Ht.	area
400	129.85	3598.41	130.58	3602.7	0.0248	0.0249	99.2	99,6
600	200.17	5198.05	201.34	5281.05	0.0248	0.0246	99.2	98.4
800	253.91	6951.39	254.15	7041.4	0.0249	0.0247	99.6	98.8

Table 13 . Assay results of Trioxsalen sample.

Average content/tablet (in gm)		% Label Claim	
Area wise	Height wise	Area wise	Height wise
0.0248	0.0247	99.2	99.6

Validation of the proposed method

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 1Omg 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 al 00ml 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.A chromatogram was developed using the above solution (3ml each is being spotted in order to get the concentration within the linearity range)

A standard solution of the same concentration (180fig/ml) was prepared and chromatogram was developed in order to determine the height and area respectively. 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 and statistically evaluated data are given below.

Table 14:Recovery study results

Cone. (ng/spot)	Sample spiked with standard		Standard		% Recovery	
	Ht.	Area	Ht.	Area	Ht.	Area
540	201.34	5281.05	200.17	5196.05	99.4	98.39
	201.36	5281.09	200.18	5198.08	99.4	98.43
	200.98	5282.01	200.19	5196.04	99.6	98.37
600	187.19 187.19	5076.58 5075.97	187.14 187.18	4978.12 4979.92	99.97 99.99	98.00 98.10
	187.06	5976.46	186.98	4978.19	99.95	98.06
660	243.59	6689.83	243.16	6600.72	99.82	98.66
	243.59	6689,84	243.15	6601.96	98.68	98.68
	243.69	6688.98	243.17	6601.87	99.78	98.69

Table 15:Statistical evaluation of recovery data

Level of recovery		mean	Standard deviation	%RSD
80%	Height wise	99.46	0.11	0.12
	Area wise	99.97	0.02	0.02
100%	Height wise	99.43	0.64	0.65
	Area wise	98.05	0.05	0.05
120%	Height wise	99.09	0.63	0.64
	Area wise	98.68	001	0.02

Precision

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

Repeatability

The repeatability of the method was studied using 100% test concentration of Trioxsalen prepared separately, and the peak area and peak height were determined six times at 254nm.The data is shown in table below;

Table16: Results of repeatability study

Sl. No.	Cone of Trioxsalen	Peak height	Peak Area
1	600	201.60	5281.59
2	600	203.00	5281.68
3	600	201.80	5281.85
4	600	201.20	5281.01
5	600	201.20	5281.02
6	600	201.00	5281.26

Table17: Statistical results of repeatability study

Mean	Standard deviation	Relative standard deviation (%)

Height wise	Area wise	Height wise	Area Wise	Height wise	Area wise
201.3	5281.40	0.33	0.35	0.16	0.01

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 18: results of inter-day precision study

Conc, of Trioxsalen (ng/spot)	Peak area and Peak Height					
	1st day		2nd day		3rd day	
	Height	Area	Height	Area	Height	Area
400	130.7	3601.3	129.8	3601.5	130.2	3600.7
400	130.6	3603.1	129.9	3606.2	130.1	3606.7
400	130.9	3604.6	130.1	3604.1	130.4	3601.1
600	201.4	5282.1	201.1	5282.7	201.8	5282.9
600	201.8	5282.3	201.6	5281.8	204.5	5282.5
600	201.9	5283.2	201.3	5282.2	203.6	5281.6
800	254.4	7042.4	254.1	7041.8	253.8	7040.9
800	254.4	7041.9	254.S	7040.6	253.6	7041.8
800	254.2	7044.1	254,4	7044.2	253.4	7042.4

Table 19 :Results of inter-day precision study

Concentration of Trioxsalen (ng/spot)		Mean		
		1st day	2nd day	3rd day
400	Height wise	130.73	129.93	130.23
	Area wise	3603	3603.93	3602.83
600	Height wise	201.7	201.33	203.3
	Area wise	5282.53	5282.23	5282.33
800	Height wise	254.33	254.43	253.6
	Area wise	7042.8	7042.2	7041.7

Table 20 : results of inter-day precision study

Conc, of Trioxsalen (ng/spot)		Standard deviation(SD)			%RSD		
		1st day	2nd day	3rd day	1st day	2nd day	3rd day
400	height	0.1527	0.1527	0.1527	0.01	0.12	0.12
	area	2.2502	1.5947	0.5033	0.05	0.07	0.04

600	height	0.2645	0.2516	1.3747	0.13	0.12	0.68
	area	0.8144	0.4509	1.3747	0.02	0.01	0.01
800	height	0.1155	0.3512	0.2000	0.05	0.14	0.08
	area	1.1532	1.833	0.7549	0.02	0.03	0.01

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 and peak height was determined by scanning at 254nm.Calibration graphs (concentration v/s peak height and concentration v/s peak area) were plotted for the drug and from this, the linearity was determined.

Table 21: Results of linearity data

Method parameters	Trioxsalen	
	Height wise	Area wise
Linearity range	100-600 ng/ml	100-600ng/ml
Slope	0.324	8.253
Intercept	0.731	275
Correlation coefficient(r^2)	0.998	0.995

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.

LOQ=10a/s

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

Table 22- LOD and LOQ data

Trioxsalen	Area wise (ng/spot)	Height wise (ng/spot)
LOD	0.95	12.65
LOQ	2.87	38.33

RESULTS AND DISCUSSION

Trioxsalen is a Psoralen derivative drug which causes photosensitization of skin and is administered orally in conjunction with UV-A for phototherapy treatment of vitiligo. This drug as such is pharmacologically inactive, but when exposed to UV or sunlight it is converted into its active metabolite to produce a beneficial reaction affecting the diseased tissue.

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.

UV Spectroscopic method

The UV spectroscopic method was developed using THERMO SCIENTIFIC UV-2600 double beam SpectrophotometerThe drug was soluble in methanol and also gave excellent UV detection in

methanol. So methanol was chosen as a desirable solvent for this estimation technique.UV response of the drug was checked by scanning from 200-400nm.Trioxsalen shown maximum absorbance at 248nm.Calibration curve for Trioxsalen was plotted using different concentrations and shown a linear relationship between concentration and absorbance in the range of l-5mcg/ml with a good correlation of 0.998.A marketed tablet dosage form containing 25mg of Trioxsalen was then analyzed by the developed method and the amount present per single tablet was estimated to be 0.0252g with a percentage label claim of 100.66%. *S*The validation of the developed method was conducted as per ICH guidelines (Q2B validation of analytical procedures: Methodology).Accuracy was determined by recover study employing standard addition method at three levels (80%, 100%, 120%). The percentage recovery was found to be 98.29%,*S*Precision was studied by 2 methods; repeatability and intermediate precision.

The percentage relative standard deviation was found to be < *2%.*The linearity range of the drug was found to be l-5ug/ml.The limit of detection and limit of quantitation were established and found to be 0.303 and 0.92 mcg/ml respectively.

HPTLC METHOD

HPLC grade Methanol was used for the preparation of sample and standard solutions. HPTLC pre-coated silica gel G60 F254 plates were used as stationary / phase. Suitable mobile phase, chloroform:methanol (7:3,v/v), was developed, employing a trial and error process. Spotting was done by using CAMAG Linomat IV automatic sample applicator with a band width of 6mm, space 10mm and an application volume of 2ul The developed plate was scanned at 254nm. The Rf value of Trioxsalen was 0.22.

Calibration curve for the drug was plotted using two parameters; concentration v/s Peak area and concentration v/s peak height. The linearity range of Trioxsalen was found to be between 100-600ng/ml, by both area wise and height wise.TROID-25, the marketed product was analyzed by the developed method and gave good results. The percentage label claim for Trioxsalen was estimated to be 99.2% (area wise) and 99.6% (height wise) and the amount of drug present per tablet with respect to peak area and peak height was found respectively as 0.0248 and 0.0247gm.The validation of the developed method was conducted as per ICH guidelines (Q2B Validation of analytical procedures: Methodology).

Accuracy of the method was determined by recovery study employing standard addition method at three levels (80%, 100% and 120%). The percentage recovery of the drug was found to be more than 98%.Precision was studied by two methods; repeatability and intermediate precision.The percentage RSD was found to be < 2, both area wise and height wise.The LOD and LOQ were determined and satisfactory results were obtained.

The developed method was found to be simple, economic and accurate. So it can be used for the routine analysis of Trioxsalen in tablet dosage forms.

CONCLUSION

The UV spectrometric and HPTLC methods demonstrated herein are applicable for the estimation of Trioxsalen in tablet dosage form without any pretreatments. Developed methods were validated according to ICH guidelines. The results obtained by these methods including recovery studies were comparable which proves the repeatability and suitability of the method. In order to ensure the integrity of the methods, all the procedures were conducted in calibrated equipment's and used good quality reagents. The capabilities of the methods are complimentary to each other and were found to be accurate, reproducible and reliable and at the same time simple and rapid. The common excipients and other additives usually present in the tablet dosage form do not interfered in the analysis of the drug in these methods from the above study double beam UV-Spectrophotometry method is conveniently accustomed for the routine quality control analysis of the drug in Pharmaceutical formulation.

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