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VALIDATED STABILITY INDICATING METHODS FOR *THYMOQUINONE* USING HPLC AND HPTLC

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ABSTRACT

A stability indicating assay method was developed and validated according to the ICH guidelines for estimation of Thymoquinone using HPLC & HPTLC. HiQSil C18 column was used with Acetonitrile as mobile phase. Wavelength selected was 253 nm. HPTLC analysis was done using precoated silica plates. Toluene was used as mobile phase & detection wavelength was 253 nm. Drug was subjected to forced degradation studies. Validation was carried according to ICH guidelines.

KEY WORDS

Thymoquinone, HPLC, HPTLC, Stability indicating.

INTRODUCTION

Among various medicinal plants, *Nigella sativa* (*N. sativa*) (Family Ranunculaceae) is emerging as a miracle herb with a rich historical and religious background since many researches revealed its wide spectrum of pharmacological potential. *N. sativa* is commonly known as black seed. *N. sativa* is native to Southern Europe, North Africa and Southwest Asia and it is cultivated in many countries in the world like Middle Eastern Mediterranean region, South Europe, India, Pakistan, Syria, Turkey, Saudi Arabia (1). Thymoquinone chemically is 2-Isopropyl-5-methyl-1,4-benzoquinone. It is very important constituent in *Nigella sativa* seeds (2). Besides being used as a spice and a condiment, *N.S.* seeds have been used for medicinal purposes in many Middle Eastern and Far Eastern countries for more than two thousand years. It is very popular in various traditional systems of medicine like Unani and Tibb, Ayurveda and Siddha. Seeds and oil have a long history of folklore usage in various systems of medicines and food. The seeds of *N. sativa* have been widely used in the treatment of different diseases and ailments. In Islamic literature, it is considered as one of the greatest forms of healing medicine. It has been recommended for using on regular basis in Tibb-e-Nabwi (Prophetic Medicine). It has been widely used as antihypertensive, liver tonic, diuretic, digestive, anti-diarrheal, appetite stimulant, analgesics and in skin disorders. Literature survey reveals that certain pharmacological studies (3,4) have been reported on *Nigella sativa* (5-7). But no stability indicating HPLC & HPTLC methods are reported for marker Thymoquinone.

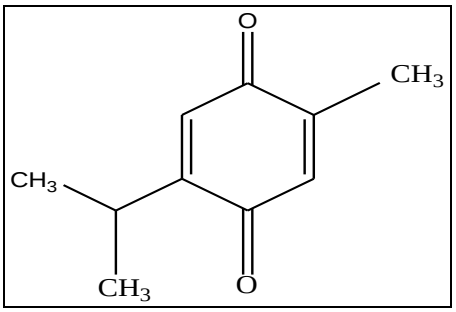

Fig 1. Structure of Thymoquinone

MATERIAL AND METHOD

Plant material

Seeds of *NIGELLA SATIVA* (BLACK CUMMIN) was purchased from Vishal-Chem Mumbai.

Chemicals

Acetonitrile, Methanol& Toluene were purchased from LOBA Chemie (Mumbai).

INSTRUMENTS

HPLC

JASCO-PU 2080 PLUS intelligent HPLC pump.
MD 2010 PDA detector
Borwin- PDA software (version 1.5)
Rheodyne injector port with 20µl loop size
Hamilton Syringe (100 µl)

HPTLC

CamagLinomat 5 (Semiautomatic Spotting device)
Camag Twin trough chamber (10 x10 cm)
Camag TLC Scanner-3
Camag WINCATS Software (version 1.4.3.6336)
Hamilton Syringe (100 µl)
All weighing were done on electronic analytical balance. (Shimadzu AY 120)

Extraction procedure

50 gm of seeds were soaked overnight in 100 ml methanol in stoppered flask. Filtered through whatmann paper.

Optimised HPLC Chromatographic Conditions:

The method consisted of mobile phase of Acetonitrile (100 % v/v) with HiQSil C18 HS column (250×4.6mm; 5 µm particle size). The mobile phase was filtered through 0.45 µm membrane filter followed by sonication for 10 min using bath sonicator. The optimum wavelength selected for quantification was 253 nm with a total run time of 10 min & flow rate of 1.0 ml/min.

Optimised HPTLC Chromatographic Conditions:

Aluminium sheets precoated with silica gel G60 F_{254}(10 x10 cm) of layer thickness 0.2mm were used. TLC plates prewashed with Methanol & activated in oven at 60°C for 10 mins. Mobile phase consisting of Toluene with detection wavelength 253 was used. TLC chamber was saturated for 15 min.

Preparation of solutions

Preparation of standard stock solution of drugs

Standard stock solution of drug Thymoquinone of concentration of 1000 µg/ml (1000ppm) was prepared in methanol by dissolving 10 mg of drug in 10 ml of methanol. The standard stock solution was diluted with the mobile phase to get solution in concentration ranging from 5 µg/ml to 30 µg/ml.

Forced degradation studies (8)

To provide an indication of the stability-indicating ability and specificity of the proposed method forced degradation studies were performed. Hydrolysis under acidic, alkaline & neutral, oxidative, thermal and photolytic degradation studies were conducted on Thymoquinone.

Acid degradation

Thymoquinone working standard was weighed accurately & dissolved in MeOH & volume was make up to obtain 100 µg/ml. From that 1.5 ml of sample was pippeted out. 1ml of 0.1 N HCL was added. Volume was made up to 10 ml & sample aliquots were kept for instant degradation & examined. Sample was neutralized using 0.1 N NaOH& volume was make up with methanol.

Alkaline degradation

From 100 ug/ml stock solution 1.5 ml of sample was pippeted out. 1ml of 0.1 N NaOH was added. Volume was made up to 10 ml & sample aliquots were kept for instant degradation & examined. Sample was neutralized using 0.1 N HCL before injection.

Oxidative degradation

From 100 ug/ml stock solution1.5 ml of sample was pippeted out. 1ml of 0.6% H_2O_2 was added. Volume was made up to 10 ml & sample aliquots were kept for instant degradation & examined.

Neutral degradation

From 100 ug/ml stock solution1.5 ml (15ug/ml) of sample was pippeted out. 1ml of distilled Water was added. Volume was made up & sample aliquots were kept for overnight & instant degradation & examined.

Thermal Degradation

Thymoquinone was exposed to 50 ^{0}C for 4 & 8 hours. 10 mg of sample was weighed & dissolved in methanol & diluted approximately to get 20 ug/ml of sample was pipetted out examined.

Photolytic degradation

Standard of thymoquinone was exposed to UV light for 200-watt hours/square meterand for fluroscence 1.2million Lux/hours. The samples were allowed to cool. 10 mg of standard thymoquinone was and volume was makeup with methanol. Further diluted with methanol to attain the working standards of 15 µg/ml concentration.

RESULTS AND DISCUSSION

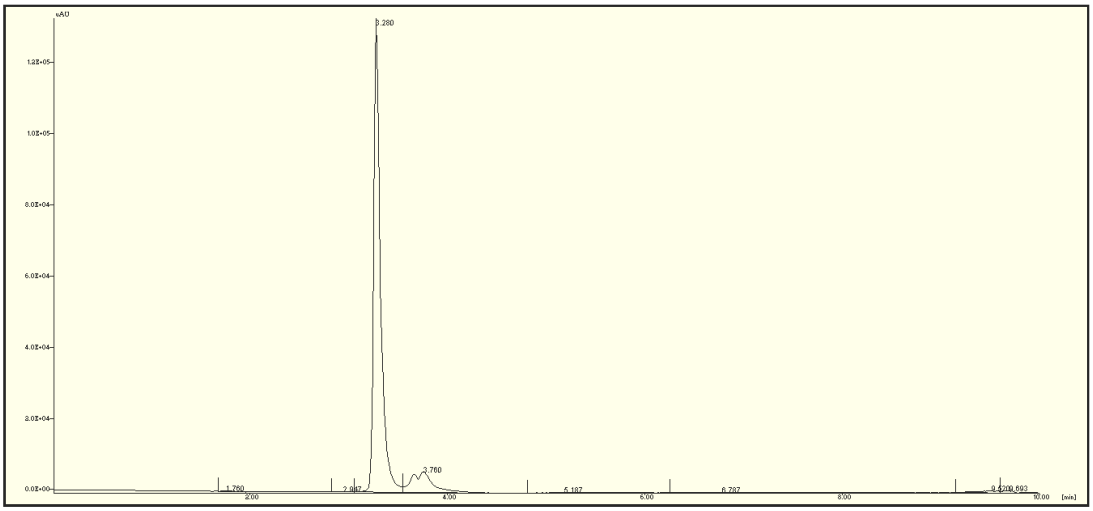

Figure 2. Standard chromatogram of thymoquinone (5ug /ml)

Table 1. System suitability of 5 ug/ml Thymoquinone chromatogram

Sr.No.	Conc (ug/ml)	Rt	Area	Plates	Resolution	Asymmetry
1.	5 ug/ml	3.280	646714.429	14937.012	1.77	1.52

Fig 3. Standard densitogram of thymoquinone (500 – 2500 ng/band)

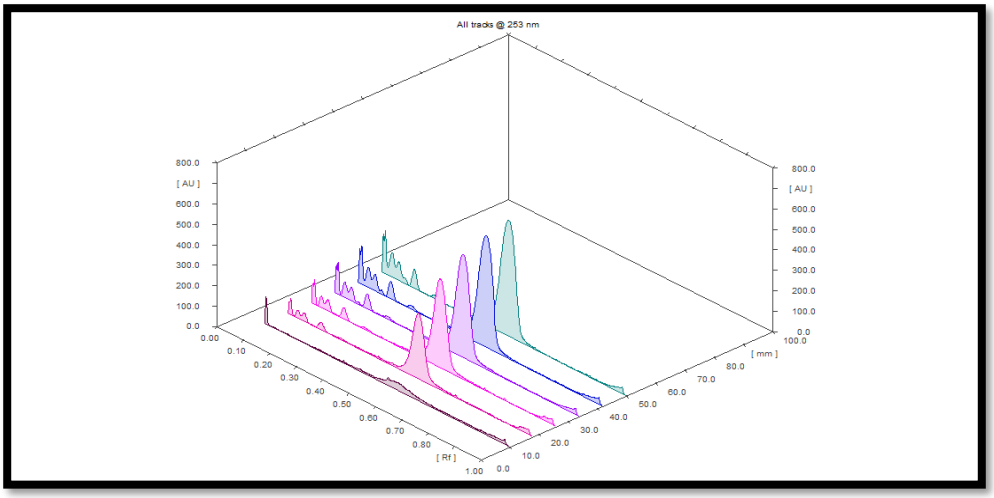

Table 2. Linearity of Thymoquinone

Sr. no.	Amount spotted (ng/band)	Area
1	500	12130.4
2	1000	18473.1
3	1500	22927.6
4	2000	25020.4
5	2500	26195.3

Table 3. Forced degradation studies of Thymoquinone (HPLC)& (HPTLC)

Sr. No.	Conditions	HPLC			HPTLC		
		% rec	Peak tail	Peak front	% rec	Peak tail	Peak front
1.	15 ug/ml	-	633.67	991.94	-	0.996	0.995
2.	0.01 N HCL (INST)	63.34	735.77	977.93	73.23	0.995	0.994
3.	0.01 N NaOH (INST)	49.57	574.66	799.80	46.02	0.992	0.996
4.	0.6% H_2O_2 (INST)	52.84	644.96	521.75	60.50	0.993	0.992
5.	Neutral	82.88	773.45	705.65	85.16	0.998	0.995
6.	Thermal 50 ^{0}C (4hrs)	44.53	525.33	982.61	49.24	0.999	0.998
7.	UV (200 watt/ hrs)	60.53	700.09	883.65	61.38	0.995	0.994
8.	Fluroscence (1.2 million lux hours)	63.30	538.73	940.22	66.09	0.998	0.997

STATISTICAL TREATMENT OF ANALYTICAL DATA:

Chromatographic methods validated statistically by applying student t test, to check statistical difference in these method and results were found as follows,

Student t test

Comparison between % recovered after stress degradation by HPLC and HPTLC methods for **Thymoquinone** done by applying paired t test using formula,

t = [đ/(s/√n)].

Table 1.18: Statistical treatment for comparison of HPLC and HPTLC method for estimation of Thymoquinone

Parameters	HPLC (x)	HPTLC (y)	d = (y - x)	(d- đ)	(d- đ) ^{2}
Acid hydrolysis	67.34	73.23	5.89	2.94	8.67
Base hydrolysis	49.57	46.02	-3.55	-6.50	42.25
Oxidation	52.84	60.50	7.65	4.70	22.17
Neutral	82.88	85.16	2.28	-0.66	0.43
Thermal	44.53	49.24	4.70	1.75	3.09
UV	60.53	61.38	0.85	-2.09	4.37
Fluroscence	63.30	66.09	2.78	-0.15	0.02
			đ= 2.94		**∑ (d- đ) ^{2} = 81.02**

Null hypothesis (H_0) i.e. there is no significant difference between mean results by two methods.

S^2 = (1/n-1) ∑ (d- đ) ^{2}

S^2 = 13.50

t = [đ/(s/√n)]

t = 0.4684

Calculated |t| is less than tabulated t (2.447), H0 may be accepted at 5% level of significance and conclude that results by two methods do not differ significantly.

METHOD VALIDATION (9)

Both the methods were validated in terms of linearity, range, precision, accuracy, specificity, limitof detection, limit of quantitation and robustness as per ICH guidelines. The system

suitability parameters were evaluated as specified in Indian Pharmacopoeia 2014.

Specificity:

The specificity of the method was confirmed by analyzing standard marker Thymoquinone & methanolic extracts. The spot for Thymoquinone in the sample was confirmed by comparing the Rf and spectra of the spot with that of sample. The peak purity of Thymoquinone was examined by comparing the spectra at peak start, peak middle and peak end positions of the bands.

Linearity& Range

Six different concentrations of (5, 10, 15, 20, 25 and 30 µg/mL) were preparedfrom standard stock solution of 1000 µg/mL of Thymoquinone and was analysed. For HPTLC, different volumes of 100 ug/ml standard solutions were spotted to obtain 500 to 2500 ng/ band. Linearity equation of HPTLC was found to be y = 7.0575x + 10653 r^2 = 0.940 & of HPLC was found to be y = 81153x – 34654 r^2 = 0.959 respectively.

Precision:

The precision was evaluated with respect to both repeatability and intermediate precision. Repeatability was evaluated by injecting six replicate injections of test solution of the drug Thymoquinone (5 µg/ml). The studies were repeated for three different days to

determine intermediate precision. Peak areas of the drugs were determined and % RSD was calculated

.

Table 4. Precision for Thymoquinone (HPLC & HPTLC)

Sr. No.	Method	Amount conc ug/ml & ng/band		Mean	SD	% RSD
1.	HPLC	5 ug/ml	**Interday**	660588.43	14652.27	2.20
			Intraday	421115.36	9031.95	2.14
2.	HPTLC	500	**Interday**	4081	90.29	2.21
		ng/band	**Intraday**	5807.1	122.09	2.10

Limit of detection (LOD) and Limit of Quantitation (LOQ):

From the linearity data the LOD and LOQ was calculated, using the formula LOD = 3.3 σ/S and LOQ = 10 σ/S where, σ = standard deviation of the y-intercept of linearity equations and S = slope of the calibration curve of the analyte.

Table 5: LOD & LOQ for Thymoquinone

PARAMETERS	LOD	LOQ
HPLC	0.59 ug/ml	1.80ug/ml
HPTLC	0.11 ng/band	0.33ng/band

Robustness:

Robustness was evaluated by deliberate variation in parameters like mobile phase and flow rate for HPLC & variation of mobile phase & time from spotting to development for HPTLC. The proposed method was found to be robust under given experimental conditions.

Table 6: Summary of validation parameters for Thymoquinone

Sr.no.	Validation Parameters		HPLC		HPTLC	
1.	**Specificity**		Specific		Specific	
2.	**Linearity**		y = 81153x – 34654		y = 7.0575x + 10653	
			r^2 = 0.959		r^2 = 0.940	
	Range		(5 -30 ug/ml)		(500- 2500 ng/band)	
3.	**Precision**	**Interday**	**5 ug/ml**	1.85	**500 ng/band**	0.59
		Intraday	**5 ug/ml**	1.15	**500 ng/band**	1.12
6.	**Limit of Detection (LOD)**		0.59 ug/ml		0.11 ng/band	
7.	**Limit of Quantitation (LOQ)**		1.80ug/ml		0.33ng/band	
8.	**Robustness**		Robust		Robust	

CONCLUSION:

Thymoquinone was found be prone to degradation in acidic, alkaline, oxidation, neutral & photolytic conditions. The developed method is simple rapid & stability indicating.

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