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Research Article

IN-VITRO ASSIMILATION OF TRIMYRISTIN IN THE SEEDS OF *MYRISTICA FRAGRANS* AND IN POLY HERBAL AYURVEDIC FORMULATION BY UFLC METHOD

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ABSTRACT

The present study was aimed to develop a simple and validated chromatographic method for the estimation of Trimyristin in the API and in poly herbal Ayurvedic formulations by UFLC method, along with stress induced degradation studies of the drug and validate the method as per the ICH guidelines. The analysis was carried out under isocratic conditions using dichloromethane and acetonitrile in the ratio of 95:5 as mobile phase with a flow rate of 1.0 ml/min at 210nm. The method was simple, specific, precise, and accurate with retention times 0.904 and 0.906 minutes for the API and isolated product. The system obeys the Beer's law in the concentration range of 10-90µg/ml. The percentage recovery studies performed showed a percentage recovery of 98.3 -101.6%w/v and found to be linear with a correlation coefficient (r2) of 0.9993. The method was found to be precise with relative standard deviation of less than 2%, detection limit and quantitation limit was estimated to be 4.58µg/ml, 15.27µg/ml respectively. The method was found to be robust even by change in mobile phase ratio of ±5%, change in wavelength and change in flow rate of ±0.1 ml/min. This validated method was sensitive and reproducible enough to be used for routine analysis of Poweromin tablet and Makaradhwaj vatti in time and cost effective manner.

Keywords: Trimyristin, Poweromin, Makaradhwaj vatti, UFLC, isocratic elution, validation and stress degradation.

INTRODUCTION

The herbal medicinal science is an ancient system of medicine that is gaining a prominent importance in the global market. The herbal plant *Myristica fragrans* also called as nutmeg, has many uses in Ayurvedic system of medicine. *Myristica fragrans Houtt,* traditionally known as Jatiphal and Javitri in India, belongs to the family Myristicaceae. It mainly produces two spices, nutmeg seed and mace the thick fiber like red aril on the kernel [1]. The herbal drug yields two kinds of oils i.e., the compressed fatty oil and the steam distilled essential oil. The main constituents of the oils of *M. fragrans* have been found to be alkyl benzene derivatives terpenes, alpha-pinene, beta-pinene, phenyl propanoids like myristicin, elemicin, safrole and fatty acids Trimyristin, myristic acid, tripalmitin etc. [2]. The triglyceride Trimyristin (fatty oil) was given less importance when compared to the essential oil part of the nutmeg mostly myristicin as it was believed to be responsible for the various pharmacological actions of the herb. So, several studies were confined to the isolation and usage of Trimyristin as a lipid polymer in formulations. But to the fact, the triglyceride Trimyristin was proven to have anxiogenic activity which, the principle reason of using the herbal drug in various polyherbal formulations [3]. Apart from this it also has other activities like anti-inflammatory and anti-bacterial [4-6]. Trimyristin is an example of fundamental type of fat known as a triglyceride. Hydrolysis of one mole of a triglyceride affords one mole of 1, 2, 3-propanetriol (glycerin) and three moles of fatty acids, which are carboxylic acids containing the functional group at the end of a long alkyl chain. Chemically Trimyristin is called as 1, 3 Di (tetradecanolyloxy) propan-2-yl tetradecanoate [7] [Fig. 1].

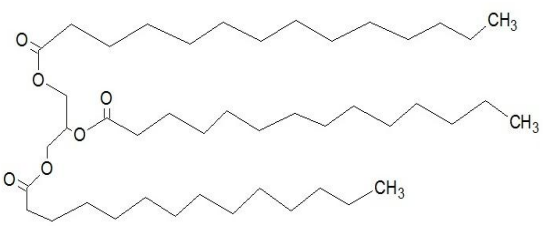

Fig. 1: Chemical structure of Trimyristin

The previous works reveal no specific analytical method for the estimation of Trimyristin quantitatively in poly herbal formulations. The literature survey reported various works on Trimyristin characterization by IR and NMR spectroscopy apart from the isolation of the components from the seeds and mace of *Myristica fragrans*. The previous reported works include the quantification of the components by HPLC, HPTLC [8-11] in various vegetable oils and in nutmeg seed extract and the standardization of the polyherbal formulations [12-13]. So, considering the need of developing a validated method for the estimation of trimyristin the present work was intended to quantify the trimyristin in the API and in poly herbal formulations by UFLC method and to validate the method according to the ICH guidelines.

The herbal formulations include a compressed tablet named Poweromin and a mould named Makaradhwaj Vatti. Analytical validation is the cornerstone of process validation. Without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purport to do. Hence, there is a need to validate the new methods developed. So the present is being validated for various parameters according to the ICH guidelines. The stability of the drug in various environments was also studied by the stress degradation studies as per the ICH guidelines [14, 15].

MATERIALS AND METHODS

Plant material

The seeds of *Myristica fragrans* were collected from the local drug store. The seeds were crushed to fine powder and stored at 25^{0}C.

Instrumentation

The method was developed using Shimadzu LC 20 AD UFLC system with 20 µL loops, LC 20 AD pump, SP-20AD Photo- Diode array detector, Waters C8 (350µm, 4.6 x 150mm) column was used in the analysis. LC solutions software was utilized for instrument control, data collection and data processing using a Pentium (R) Dual –Core Processor. The analysis was carried out under isocratic conditions using dichloromethane and acetonitrile in the ratio of 95:5 as the

mobile phase with a flow rate of 1.0 ml/min at 210nm. All solvents were filtered through a 0.45 µm Millipore filter. A volume of 20 µL of the standard and samples solutions was injected into the column.

Chemicals

Standard Trimyristin was obtained from Himedia (RM 1301-1G), India. Acetonitrile and Dichloromethane were purchased from Merck Chemicals Ltd. Hydrogen peroxide, Sodium Hydroxide was purchased from SD FCL (SD Fine Chem. Limited) and Mio Chem. Pvt. Ltd respectively. Hydrochloric acid was obtained from Merck Chemicals. All other chemicals and reagents were of HPLC grade. The polyherbal tablets Poweromin (Apex Laboratories Pvt. Ltd.) and Makaradhwaj vatti (Godavari Ayurvedic Pharmacy) were obtained from the local ayurvedic store.

Extraction of Trimyristin

Crushed whole nutmeg seeds of 5gms was weighed and transferred to a 50ml round-bottomed flask. 50ml of hexane was added to the flask which was attached to a water-cooled condenser, the mixture was heated under a gentle reflux for approximately 4 hours at 60^{o}C. The mixture was cooled to room temperature. The hexane layer was separated out with pipette without disturbing the settled solids. The hexane layer was evaporated by using rotavapour apparatus which on evaporation yields a white crystalline powder. The crude extract was recrystallised by using 95% ethanol to yield pure trimyristin. The extracted trimyristin was characterized by IR [Fig. 2].

Preparation of standard and extract stock solution

An accurately weighed quantity of 10mg API and extract were transferred into two separate 10ml volumetric flasks and dissolved into 10ml dichloromethane to give a concentration of 1000µg/ml. The final concentration was brought to 10µg/ml by diluting the stock solution with dichloromethane, filtered through 0.45 µ 6, 6 membrane nylon filter.

Assay of marketed Polyherbal Ayurvedic Poweromin tablet

Twenty tablets were weighed and ground to a fine powder using a mortar and a pestle. An accurately weighed portion of the powder, equivalent to 5mg of drug was transferred into a 250ml round bottomed flask and 30ml of petroleum ether HPLC grade was added to it, the above solution was refluxed for 30min at 70°C. The petroleum ether layer was separated, evaporated completely and diluted to 50ml with dichloromethane. From the above stock solution the dilutions were prepared using dichloromethane such that the final concentration of the solution was 10µg/ml.

Assay of polyherbal Ayurvedic Makaradhwaj Vatti tablet

Twenty tablets were weighed and ground to a fine powder using a mortar and pestle. An accurately weighed portion of the powder, equivalent to 5mg of drug was transferred into a 250ml round bottomed flask and added with 10 ml of water, 30ml of dichloromethane respectively. The above solution was sonicated for 20min at 60°C and centrifuged for 5 minutes. The dichloromethane layer was separated and diluted to 50ml with the same solvent. This solution was further diluted to 10µg/ml with dichloromethane.

Method Validation

The method was validated for specificity, linearity, accuracy, precision, LOD, LOQ, robustness and ruggedness as per the ICH guidelines [14, 15].

Specificity

For a chromatographic method developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all interferences. Therefore the samples were analyzed in the presence of interfaces and the analyte peak was evaluated for peak purity and resolution from the nearest eluting peaks.

Linearity

Nine point linearity plots were constructed for API using the concentrations ranging from 10µg/ml-90µg/ml. The linear relationship of the system was calculated through the coefficient of regression.

Precision

Precision was assessed through repeatability and intermediate precision. Assay precision was calculated using the formula **%RSD = (SD/M) × 100** where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M.

Accuracy

The accuracy of the method was established by using analyte at low, medium and high concentrations of 8µg/ml, 10µg/ml and 12µg/ml for trimyristin API. The percentage assay was calculated for 2 consecutive days by preparing fresh solutions each day.

Recovery studies

Percentage recovery studies were performed for 80%, 100% and 120% concentrations for both the tablets respectively for 8µg/ml, 10µg/ml and 12µg/ml. The percentage recovery of trimyristin of the spiked samples of herbal formulation was calculated.

Robustness

Robustness of the method was determined by slight deviation in the method parameters. The parameters deviation wavelength, flow rate, and mobile phase were selected. Robustness was done by changing the flow rate (±0.1ml/min), changing the wavelength (±1nm), and organic composition of mobile phase (±5%).

System Suitability

The chromatographic parameters such as selectivity, theoretical plates and tailing factor were determined for trimyristin with five replicate injections.

LOD and LOQ

Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LOD) and limit of quantitation (LOQ) according to a signal to noise (S/N) ratio of 3:1 and 10:1 respectively.

Stress degradation studies

The stress degradation studies such as hydrolytic (in acidic & alkali medium), photolytic, oxidation and dry heat induced degradation studies were performed for API as per ICH guidelines. All the solutions are injected after filtering through 0.45 µ 6, 6 membrane nylon filter.

Hydrolytic degradation under acidic conditions

Hydrolytic degradation studies under acidic conditions were performed by using 2ml (10µg/ml) of stock solution of trimyristin API in three sets of separate volumetric flasks, to this 1ml of 1N, 0.1N, 0.01N methanolic HCl was added into each flask and volume was made to 10ml with dichloromethane, these solutions were kept at 25^{0}C, 60^{0}C for 20 min. 5ml of the above solution was further diluted to 10ml with dichloromethane and injected into the system.

Hydrolytic degradation under alkaline conditions

Hydrolytic degradation studies under alkaline conditions were performed by taking 2ml (100µg/ml) of stock solution of trimyristin API in three sets of separate volumetric flasks, to this 1ml of 1N, 0.1N, 0.01N methanolic NaOH was added into each flask and the volume was made up to 10ml with dichloromethane, these solutions were kept at 25^{0}C and 60^{0}C for 20 min, from this 5ml of solution was diluted to 10ml with dichloromethane and injected into the system.

Dry heat induced degradation

Dry heat induced degradation studies were performed by subjecting 10 mg of Trimyristin API in a standard volumetric flask to a temperature of 80^{o}C for 48 hours in a hot air oven. After 48 hrs the drug was taken out and diluted with the dichloromethane such that the final concentration was 5µg/ml and injected into the system.

Oxidative degradation

Oxidative degradation studies were performed by taking 1.5 ml (100µg/ml) of stock solution of Trimyristin API in a 10 ml volumetric flask, to this 1ml of 3% hydrogen peroxide (which was stored for overnight) was added and the volume was made up to 10ml with dichloromethane. This solution was kept at room temperature for 90 minutes and then diluted to a concentration of 5µg/ml. The final solution was injected into the system.

Photolytic degradation

Photolytic degradation study was performed by exposing the trimyristin API to near UV light for 30 minutes in an UV chamber and to the daylight for 2 days. After the exposure 10 mg of drug was taken and diluted to a concentration of 5µg/ml using dichloromethane for the purpose of analysis.

RESULTS AND DISCUSSION

The extracted trimyristin was characterized using the FTIR spectroscopy by its characteristic peaks at 2900 -2700 cm-1 (C-H stretching), 1729 cm-1 (C=O stretching vibration of ester), 1388 cm^{-1} (symmetrical deformations of CH_3group), 1226cm-1, 1172 cm^{-1}, 1111 cm^{-1}(asymmetrical stretching of ester C-O-C, common to all long chain triglycerides of long chain fatty acids), 1035 cm^{-1} (symmetrical stretching of ester C-O-C), 714 cm^{-1} (wagging of CH2 group) [Fig. 2].

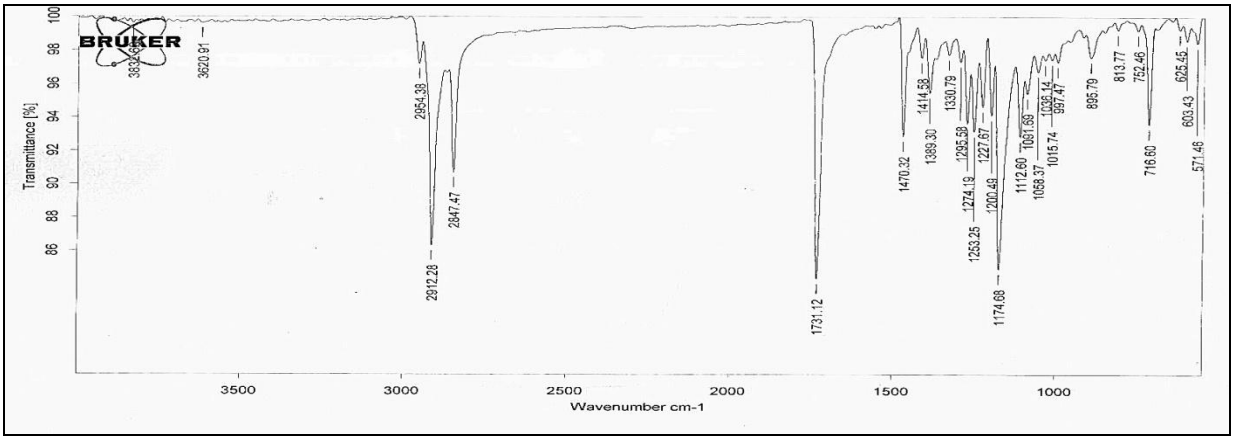

Fig. 2: IR Spectrum of Sample showing the characteristic peaks

Preliminary experiments were carried out to optimize the experimental parameters affecting chromatographic separation of Trimyristin. To obtain chromatograms with good separation various columns, mixtures of the mobile phase and different flow rates were investigated. For the assay of Trimyristin in polyherbal formulation Waters Prepacked C_8 column with various mixtures of dichloromethane and acetonitrile were used as mobile phase and results indicated that dichloromethane and acetonitrile in a ratio of 95:5 v/v gives the best resolution. Also using different flow rates (in the range of 0.8-2 ml/min) revealed that the best separation was achieved on a flow rate of 1.0 ml/min of mobile phase. Trimyristin was well separated with retention time of 0.904 for standard API and 0.906 for recrystallised extract [Fig. 3, 4]. The drug in polyherbal formulations Makaradhwaj vatti and Poweromin after extraction showed the prominent peak at 0.906, 0.905 respectively indicating the specificity of the method [Fig. 5, 6]. The percentage assay was determined to be 99.89%, 99.65% for Poweromin and Makaradhwaj vatti respectively.

Method Validation

The method was validated for specificity, linearity, accuracy, precision, robustness, system suitability, LOD, LOQ as per the ICH guidelines.

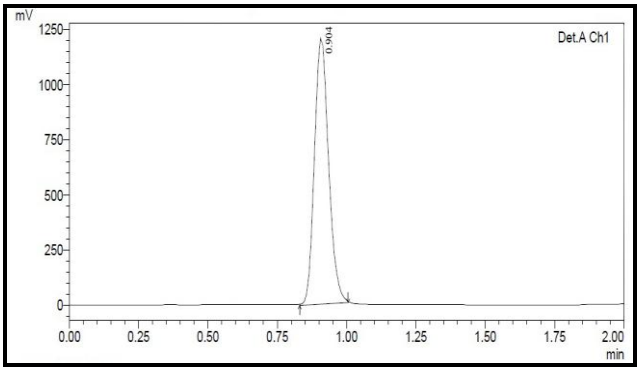

Fig. 3: UFLC Chromatogram of Trimyristin API

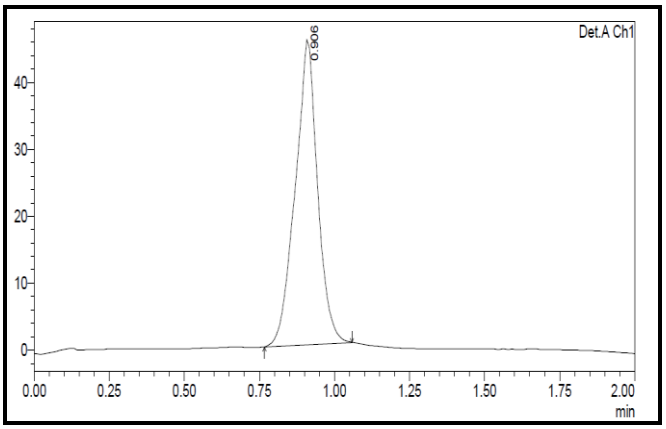

Fig. 4: UFLC Chromatogram of Trimyristin extracted from the seeds of nutmeg

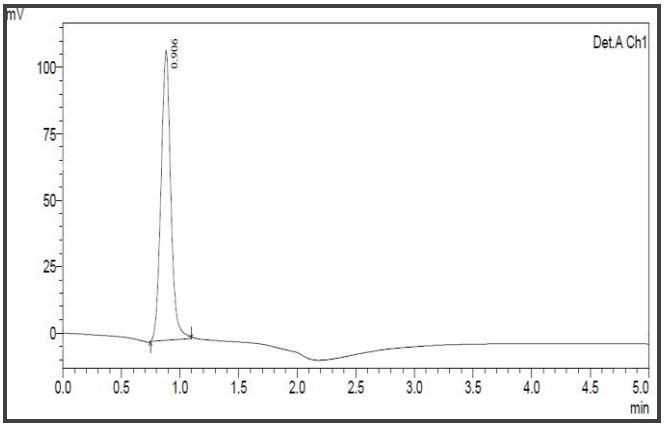

Fig. 5: UFLC Chromatogram of Trimyristin extracted from the polyherbal formulation Makaradhwaj Vatti

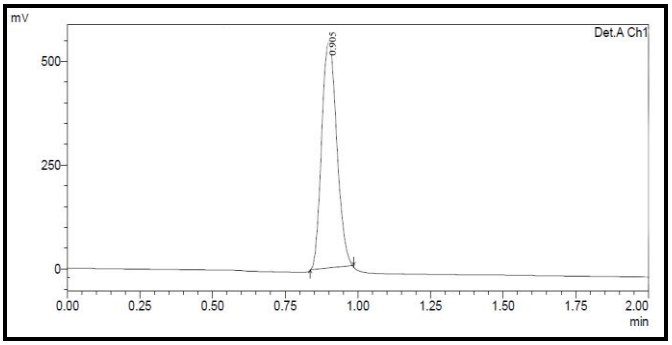

Fig. 6: UFLC Chromatogram of Trimyristin extracted from the Polyherbal formulation Poweromin

Linearity

The linearity was observed to obey the Beer's law in concentrations ranging from 10-90µg/ml. The linear plot plotted with concentration against absorbance with correlation coefficient (r^2) of 0.9993 for both the API and extract for 3 successive days [Fig. 7].

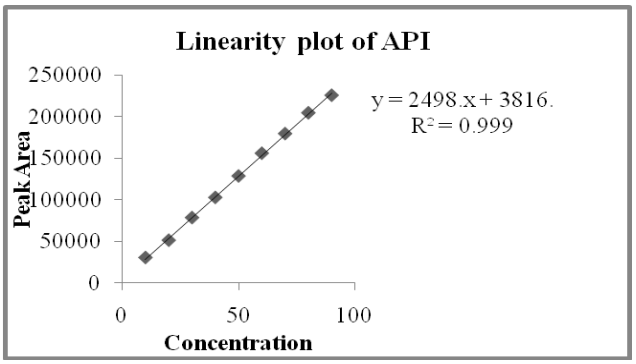

Fig. 7: Linearity plot of Trimyristin API.

Precision

The precision was performed for the Trimyristin API standard solutions of concentration of 10µg/ml, 20µg/ml, 40µg/ml by injecting five times and basing on the area for all six injections the precision of the method was determined. The % RSD for the area of six replicate injections was found to be within 2% [Table 1, 2].

Table 1: Shows the peaks results for method precision

S.No	Concentration	Retention Time		Peak area	
		Mean	%RSD	Mean	%RSD
1	10µg/ml	0.9042	0.16	447320.625	0.5
2	20µg/ml	0.9023	0.25	447596.65	0.61
3	40µg/ml	0.9012	0.03	447465.23	0.52

Table 2: Intermediate Precision and Ruggedness Studies of Trimyristin

Analyte	Intraday		Interday	
	Mean±SD	%RSD	Mean±SD	%RSD
API				
10µg/ml	9.91± 0.05	0.5	9.83±0.02	0.4
20µg/ml	19.95±0.04	0.2	19.83±0.08	0.72
40µg/ml	39.81±0.02	0.13	39.64±0.02	0.05
Extract				
10µg/ml	9.8±0.01	0.15	9.62±0.05	0.61
20µg/ml	19.92±0.03	0.4	19.59±0.09	0.53
40µg/ml	39.93±0.06	0.35	39.39±0.03	0.64

API: Active Pharmaceutical Ingredient (Standard Trimyristin) (±SD) = Standard deviation

Accuracy studies

The accuracy of the method was established by using analyte at low, medium and high concentrations of 8µg/ml, 10µg/ml, and 12µg/ml of trimyristin API, extract and polyherbal formulations. The percentage assay calculated for 2 consecutive days by preparing fresh solutions each day was found to be in the range of 98 – 102 % [Table 3].

Recovery studies

Percentage recovery studies were performed for 80%, 100% and 120% concentrations for both the tablets by keeping the dilutions constant that is for 8µg/ml, 10µg/ml and 12µg/ml**.** The percentage recovery of trimyristin from the spiked sample was found to be in between 98.3 -101.6%w/v [Table 4]**.**

Table 3: Accuracy Studies of Trimyristin

Analyte	Concentration (µg/ml)	Day 1 Amount present (µg/ml)	Percentage (%)	%RSD n=3	Day 2 Amount present (µg/ml)	Percentage (%)	%RSD n=3
API	8µg/ml	7.92	99.6	0.9	7.64	97.9	1.27
	10µg/ml	10.2	101.4	1.08	9.82	98.3	0.52
	12µg/ml	11.93	99.6	0.94	11.62	97.7	0.45
Extract	8µg/ml	8.14	101.3	0.83	8.07	100.9	0.93
	10µg/ml	9.92	99.6	0.23	10.06	101.02	0.82
	12µg/ml	11.82	98.8	1.18	11.97	99.8	0.91
Poweromin	8µg/ml	7.94	99.3	0.82	7.74	98.64	1.04
	10µg/ml	10.03	101.3	0.65	9.7	98.06	1.21
	12µg/ml	11.97	99.8	0.91	11.71	98.6	0.93
Makaradhwaj Vatti	8µg/ml	8.04	100.6	0.63	7.85	96.9	0.68
	10µg/ml	9.92	98.02	0.67	9.83	98.6	0.17
	12µg/ml	12.12	100.8	0.44	11.93	99.8	0.81

Table 4: Recovery Studies of Trimyristin

Tablet	Concentration	Amount Added (µg/ml)	Amount Recovered (µg/ml)	Percentage	%RSD n=3
Poweromin	Low	7952	8104	101.62	0.8
	Medium	9940	9930	101.09	1.23
	High	11928	11885	99.97	0.74
Makaradhwaj vatti	Low	7984	7942	99.53	0.42
	Medium	9980	10080	101.23	0.83
	High	11976	12011	100.2	1.2

ROBUSTNESS

Robustness of the method was determined by a slight deviation in the method parameters. The parameters selected were deviation in column chemistry, wavelength, flow rate and mobile phase. The retention time of trimyristin was determined and % RSD using system suitability parameters was observed [Table 5].

Table 5: Robustness Studies of Trimyristin

Parameters	%RSD
Flow rate	0.552
Mobile phase	0.59
Wavelength	0.593

System Suitability

The chromatographic parameters such as selectivity, theoretical plates and tailing factor were satisfactory for trimyristin. The peak asymmetry was calculated in terms of tailing factor, 1.09 which was less than the approved limit. The theoretical plates were above 2000 (4332) for every peak of the drug.

Limit of Detection and Limit of Quantization

Detection limit and quantification limit was found to be 4.58µg/ml and15.27µg/ml by using the S/N ratios of 3:1 and 10:1 respectively.

Stress degradation studies

The standard API trimyristin when subjected to 0.01N, 0.1N HCl in case of acid induced hydrolytic studies no degradation was observed at 25^{0}C whereas the other set of same solutions when heated to 60^{0}C showed 15.6%, 28.9% degradation respectively with an additional peak at 0.852. 7.2% degradation was observed in case of 1N HCl at 25^{0}C after 20 minutes with an additional peak at 0.823. The drug totally degraded on heating with 1N HCl mixture for 20 minutes at 60^{0}C along with the same additional peak. In case of alkaline hydrolysis 0.01N, 0.1N, 1N NaOH slight degradation of 0.01%, 0.64% and 2.52 %was observed at 25^{0}C respectively after a period of 20 minutes with an additional peak at 0.732 with 0.01N, 0.1N NaOH and 1.037 with 1N NaOH. The other set of same solutions when heated at 60^{0}C for 20 minutes the drug degraded up to 8.25%, 23.2% and 52.4% respectively, the chromatogram showed additional peaks at 0.803, 1.215 apart from 0.732. The dry heat induced degradation studies indicated the stability of the drug at 80^{0}C for a period of 48 hours. The solid drug was melted when subjected to a temperature of 80^{0}C which showed the change in the physical stability of the drug (M.P- 54^{0}C) without any chemical degradation. The oxidative degradation showed 100% degradation with degraded peaks at 1.156 and 1.490. Under photochemical conditions the drug completely degraded with degraded peaks at 0.826, 1.156 and 1.356 under UV exposure where as the daylight exposure does not show any prominent degradation changes. The degradation studies confirmed that trimyristin was more susceptible to dry heat induced degradation studies. A total of seven degraded peaks were observed [Table 6]. Peaks with higher R_t values apart from acid hydrolytic degradation indicated less polar nature than Trimyristin.

Table 6: Summary of Stress Degradation Studies

Degradation conditions	% Degradation		R_t of degraded products	
Acidic Hydrolysis	At 25^{0}C	At 60^{0}C	At 25^{0}C	At 60^{0}C
HCL 0.01N	No degradation	15.6%	-	0.852
HCL 0.1N	No degradation	28.9%	-	0.852
HCL 1N	7.2%	100%	0.823	0.832
Alkaline Hydrolysis	At 25^{0}C	At 60^{0}C	At 25^{0}C	At 60^{0}C
NaOH 0.01N	0.01%	8.25%	0.732	0.732, 0.803, 1.215
NaOH 0.1N	0.64%	23.2%	0.732	0.732, 0.803, 1.215
NaOH 1N	2.52%	52.4%	1.037	0.732, 0.803, 1.215
Dry Heat Induced Degradation	No degradation		No degradation	
Photolytic Degradation UV Exposure	100%		0.826, 1.156, 1.356	
Day light	No degradation		No degradation	
Oxidative Degradation	100%		1.156, 1.490	

CONCLUSION

Recent investigations have confirmed that Trimyristin has vulnerable and wide-ranging pharmacological properties such as anxiogenic, anti-inflammatory properties. So the present cost-effective method can be used for the market analysis of trimyristin in polyherbal formulations and this particular stability indicated UFLC method was found to be simple, accurate and precise. The stability indicating stress degradation studies crams various physical and chemical stability properties of the drug in the

formulation which helps in the better formulation development. The current study demonstrates the degradation susceptibility of drugs to different stress conditions and thus helps in determining the changes in chemical, physical and microbiological properties of the drug samples with time. It also helps in understanding the mechanism and pathway of degraded product formation and in developing a profile reflecting the changes in identity, purity and potency of the product. Hence, the method can be recommended for routine analysis of Poweromin tablet and Makaradhwaj vatti poly herbal formulation containing the drug trimyristin. This method was very effective for the estimation of Trimyristin in API and in seed extract in particular with the impetus for the future development of quality monographs for dosage forms containing Trimyristin. It appears suitable for routine analysis of the drug selectively in the presence of their degraded products in individual and in combined pharmaceutical formulations in a time efficient manner.

ABBREVATIONS

API: Active Pharmaceutical Ingredient (Standard Trimyristin).

%RSD: Relative Standard deviation.

ICH: International Conference of Harmonization

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