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Development and validation of an RP-HPLC method for estimating nutraceutical lutein

Shah Esha Bhavin
Krishna School of Pharmacy and Research, Krishna Edu Campus, KPGU, Vadodara, Gujarat, India and Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, Changa, Gujarat, India.
Corresponding author email: eshuu13@gmail.com

Gajjar Anuradha
L.M. College of Pharmacy, Ahmedabad, Gujarat, India.

***Abstract*---**Background: Nutraceuticals have long been thought of as natural, safe supplements that can help prevent disease, replace prescription medications, compensate for poor diet, and boost overall health. One such nutraceutical is Lutein, a pigmented xanthophyll carotenoid, which offers a plethora of uses and health benefits. Lutein, like many carotenoids is a lipid soluble pigment which faces major solubility and bioavailability issues owing to its lipophilicity, due to which its estimation is indeed challenging. And the analysis of a simple solvent extract of Lutein is complicated and lacks any detailed method for its estimation. Additionally, literature review indicates use of very complicated mobile phases and tedious solvent extraction procedures involved in the estimation of Lutein. Objective: The manuscript describes the development and validation of a rapid, sensitive and specific Reverse Phase-High Performance Liquid Chromatographic (RP-HPLC) method involving Ultraviolet (UV) detection for the determination and quantification of nutraceutical Lutein in bulk and capsule dosage form. Methods: Chromatography was carried out on an Inertsil ODS C_{18} (150 x 4.6 mm), 5µ, using a filtered and degassed mixture of water: acetonitrile (10:90% v/v) as the mobile phase at a flow rate of 1 mL /min with isocratic elution method and effluent was monitored at 442 nm. Results: The method was linear in the range of 50-150 µg mL^{-1} of Lutein. The method was validated with respect to precision, accuracy, specificity and robustness. The run time of the analyte was 7.91 ± 0.02 min. Conclusion: The developed method is simple, specific, precise, accurate, and robust and shows no interference with diluents which proves the adaptability of the method for routine analysis of the nutraceutical Lutein in bulk and marketed formulations.

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***Keywords*---**Lutein, analytical method development, validation, nutraceutical, RP-HPLC.

***Abstract*---**Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words. Put your abstract here. Use single spacing and don't exceed 200 words.

***Keywords*---**3-10 keywords separated by commas.

Abbreviations
HPLC: High Performance Liquid Chromatography
ICH: International Council for Harmonization
LC: Liquid Chromatography
LOD: Limit of Detection
LOQ: Limit of Quantification
LUT: Lutein
RP-HPLC: Reverse Phase – High Performance Liquid Chromatography
RSD: Relative Standard Deviation
UV: Ultraviolet

Introduction

A Nutraceutical also referred to as a 'bioceutical' is gaining immense interest in the present-day healthcare and research sector owing to its natural origin and the enormous health benefits which it has to offer. The term originates from two words i.e., 'Nutrition' and 'Pharmaceutical' which has conquered substantial attention owing to the supplementary health benefits they offer alongside the basic nutritional value (Huang *et al.*, 2010). These functional foods aid in disease prevention in addition to being explored as potent health supplements. They can be categorized as food or part of food which offers a plethora of medicinal or health benefits (Télessy, 2019). Nutraceuticals are broadly classified into dietary fibre, prebiotics, probiotics, polyunsaturated fatty acids, antioxidants and other variable types of herbal natural foods which have a substantial role to play in a variety of disease conditions ranging from obesity, diabetes, osteoporosis to even cancer (Polidori *et al.*, 2002). Understanding the intricate link between nutraceutical/phytopharmaceutical dose composition and disease prevention necessitates the quantitative measurement of food phytochemicals (Zhang *et al.*, 2015). Analytical approaches that measure nutraceuticals/phytochemicals in a single step, without the need for complicated extraction procedures, would therefore prove to be important tools, drawing a slew of researchers and analysts from around the globe.

Nutraceutical Lutein (LUT), is one such versatile phytonutraceutical which is proven efficacious against an array of acute and chronic disease conditions ranging from age related macular degeneration, cancers of skin and lung, atherosclerosis etc. to name a few (Mitri *et al.*, 2011). It is a reddish orange coloured crystalline powder which is lipophilic in nature and chemically constitutes a C40 isoprenoid backbone structure (Huang *et al.*, 2015) (Chen *et al.*, 2019). Lutein or [(3R,3_R,6_R)-beta, epsilon-carotene-3,3_-diol] is popularly known as an 'eye vitamin' that is naturally occurring and originated from plants (Figure 1). It coexists alongside its isomeric counterpart, Zeaxanthin and has two cyclic end groups (beta- and alphaionone rings) (Shegokar & Mitri, 2012). It is soluble in fats and lipophilic solvents but not in water and has the molecular weight of 568.87 g/mol.

The mechanisms by which a xanthophyll carotenoid like LUT exerts its miraculous health benefits are attributed to its ability to scavenge reactive oxygen species directly, preventing them from causing damage to DNA and protein molecule (Slavin & Yu 2012). Also, Lutein which is naturally present in the macula of the human retina exerts its effect by filtering out potentially phototoxic blue light and near-ultraviolet radiation from the macula (Sumantran *et al.*, 2000) (Barlow *et al.*, 1997).

Therefore, LUT is one of the most popular nutritional supplements (Dwyer *et al.*, 2001). Literature review revealed various complicated chromatographic methods for the estimation of LUT involving gradient system and lengthy analysis procedures and lacked reports of any simple, direct and economical RP-HPLC method for the estimation of LUT. And owing to advantages like short analysis time, high resolution and good reproducibility, high performance liquid chromatographic analysis has been widely applied to the study and analysis of carotenoids as Lutein. Thus, it was thought of interest to develop a simple, direct and economical method in terms of time and cost for the estimation of LUT. The developed method was then validated with the help of stringent validation protocols for the routine estimation of the nutraceutical LUT in bulk and marketed dosage forms.

Experimental

Chemicals and reagents

Lutein working standard was obtained as a gift from OmniActive Health Technologies, Thane, Maharashtra. HPLC grade methanol and acetonitrile were purchased from Loba Chemie Pvt. Ltd. HPLC grade water was obtained from Milli-Q water purification system (Millipore, Milford, USA) for the experimentation. Capsule formulation (Zipvit LUT 50 mg Softgel Capsule) containing labelled amount of 50 mg of LUT was procured from the local market. All chemicals were of HPLC grade. Membrane filters (Nylon *66, Axiva) of 0.45* µm were utilized for filtration of the mobile phase.

Instrumentation and Chromatographic Conditions

The RP-HPLC instrument used was Model-LC20AT Shimadzu with LC Solution software and chromatographic separations were performed on Inertsil C_{18} (150 x 4.6 mm), 5µ column. The mobile phase was filtered through 0.45 µm nylon membrane filter. The injected volume corresponded to 20 µL using a 40°C column temperature. Sartorius electronic balance was used for weighing the samples accurately.

Chromatographic system

The chromatographic separation was performed using Inertsil ODS C_{18} (150 x 4.6 mm), 5µ column with the column oven temperature of 40˚C. The degassed mixture of water: acetonitrile: 10:90% v/v was used as the mobile phase after filtering through 0.45 µm nylon membrane filter. The flow rate was maintained at 1 mL/min throughout the analysis. The elution was monitored with UV detector at 442 nm, while the injection volume was set at 20 µL.

Samples

Preparation of Stock Standard Solution and Working Standard Solution

The mobile phase of water: acetonitrile: 10:90% v/v was used for the preparation of the stock and working standard solutions as diluent.

Standard stock solution (1000 µg mL^{-1}): 100.00 mg of LUT was accurately weighed and transferred to a 100 mL volumetric flask, to which about 60 mL of diluent was added and sonicated to dissolve the contents with intermittent shaking for about 10 minutes at room temperature. The volumetric flask was then cooled and its volume was made upto the mark with diluent.
Standard working solution (100 µg mL^{-1}): 1 mL of standard stock solution was accurately transferred to 10 mL volumetric flask and volume was made up to the mark with diluent.

Method validation

The validation of the developed RP-HPLC method was carried out in-line to International Council on Harmonization (ICH) guidelines Q2 (R1) (Guideline IH, 2005) for specificity, accuracy, precision, repeatability and robustness. The linearity of standard curve was expressed in terms of the determination coefficient (r^2) from plots of the integrated peak area vs concentration (µg/mL). Method precision, as repeatability, was evaluated on the basis of the relative standard deviation (RSD) of LUT while six replicate analysis of the same sample were also made on different days to determine reproducibility. The quantitative validated method developed for the determination of nutraceutical LUT considerably reduced the time required for sample preparation procedures, and in addition is precise and accurate, confirming its utility for routine analysis.

Linearity of calibration curves

Linearity of an analytical method is its ability, within a given range, to obtain test results that are directly or through a mathematical transformation, in direct proportion to the concentration of analyte. The linearity of the method was evaluated by constructing calibration curves at six concentration levels over a range of 50 - 150 µg mL^{-1} of LUT. The calibration curves were developed by plotting peak area versus concentration of LUT (n=6). The linearity of the calibration graphs was validated by the high value of correlation coefficients of the regression graph.

Accuracy

The accuracy of the method was determined by calculating the recoveries of LUT by the method of standard additions. Three levels 80, 100 and 120% of the target concentration of the sample were evaluated for the determination of accuracy for the developed method. Calculated amount of standard solution of LUT was spiked with added sample solution to prepare 80, 100 and 120% of the working concentration.

Precision

Intra-day precision refers to the use of an analytical procedure in a laboratory by the same operator with the same equipment over a short period of time, whereas inter-day precision refers to the estimation of differences in analysis when a method is performed in the laboratory on different days. The precision of the developed method was evaluated in terms of repeatability, intra-day and inter-day precisions. Repeatability was performed by preparing six different sample preparation of 100 µg mL^{-1} and finding the AUC for each. The %RSD was then calculated. Intra-day precision was determined by analysing the sample solutions of LUT at three levels including low, medium, and high concentrations of the calibration curve three times on the same day while Inter-day precision was determined in a similar manner by analysing the low, medium, and high concentration samples over a period of three consecutive days. The peak areas were obtained and the relative standard deviation (RSD) values were determined. The precision of the method was evaluated by calculating the % RSD of the mean peak areas obtained from injection of the samples.

Specificity

The specificity ensured the identity of the analyte of interest, that is LUT. The specificity of the method was ascertained by analysing LUT in presence of excipients commonly used for capsule formulations.

Sensitivity

The lowest concentration of analyte that can be reliably distinguished from background noise is known as the limit of detection (LOD). The lowest amount of analyte that can be quantitatively measured with sufficient precision and accuracy is the limit of quantification of an analytical technique. Calibration

curve was replicated five times and the standard deviation (SD) of the intercepts was calculated. The limit of detection (LOD) and limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise (i.e., 3.3 for LOD and 10 for LOQ) ratio using the following equations designated by International Council for Harmonization (ICH) guideline:

$\mathrm{LOD} = 3.3 * \frac{\sigma}{S}$ and $\mathrm{LOQ} = 10 * \frac{\sigma}{S}$
Where, σ = Standard deviation of the response,
S = Slope of the calibration curve.

Robustness

The robustness of the analytical procedure was evaluated to study the ability of the method to remain unaffected by small, but deliberate variations in method parameters and provide indication of its reliability during normal usage. Robustness of the method was determined by minor changes in flow rate, mobile phase ratio and column temperature and the effects on the results were examined. Robustness of the analytical procedure was determined in triplicate and the mean and RSD of the peak areas were calculated.

Analysis of Marketed Formulation

20 capsules were accurately weighed and the average weight was calculated. Quantity of capsule contents equivalent to about 50 mg of LUT was accurately transferred into a 100 mL volumetric flask. About 50 mL of the diluent was added and the solution was sonicated for approximately about 20 minutes before filtration. The final volume was made up with diluent. Further dilutions were made to get a final concentration of about 50 mg of LUT. All the experimental procedures were performed in triplicates.

Results

Optimization of the chromatographic conditions

Mobile phase for separation of LUT was selected on the basis of literature review and trial and error method. Since the most technically sound and accurate method for determining the LUT content of a product was RP-HPLC analysis, LUT was subjected to preliminary trials by using different proportion of water and acetonitrile as mobile phase. Therefore, trials were taken for method development and the optimized chromatographic conditions involved Inertsil ODS C_{18} (150 x 4.6 mm), 5µ, column with a column temperature of 40˚C using a filtered and degassed mixture of water: acetonitrile (10:90% v/v) as mobile phase at a flow rate of 1 mL/min with isocratic elution method at detection wavelength of 442 nm. The optimized chromatogram for nutraceutical Lutein is shown in the Figure 2. Method development involved preparation of working standards of various concentrations and taking aliquots of standard solution and diluting to get required concentration for calibration plot, which was injected and analyzed.

Method validation
Linearity and calibration curves

Linear correlation was obtained between area versus concentrations of LUT in the concentration range of 50 - 150 µg mL^{-1}. The correlation coefficient was found to be 0. 9996. The peak areas obtained were directly proportional to the LUT concentration in the sample. The method can, hence be regarded as linear in the range considered. The results of Linearity study are shown in Table 1 and the overlain chromatograms showing the linearity of LUT are depicted in Figure 3. The Figure 4 illustrates the calibration curve of LUT.

Accuracy

Accuracy of the method was computed at different concertation levels by the standard addition method. The accuracy was observed by taking 80 %, 100 % and 120 % of concentration levels with respect to target assay concentration. Each concentration level was precisely injected thrice into the HPLC system and % recovery was found to be within 98-102 % at all the three levels studied. The results of accuracy study for nutraceutical LUT are shown in the Table 2, which clearly depict that the methods were accurate.

Precision

Values of % RSD less than 2 % indicates that the method is precise in accordance with the ICH guideline. The results of precision study and repeatability are shown in the Table 3. The results of the precision studies expressed in % RSD obey allowable standards (< 2%) of ICH guidelines, which demonstrate strong repeatability, indicating an excellent precision of the developed method.

Limit of detection and limit of quantification

The limit of detection and quantification were determined based on the signal to noise ratio and were evaluated by applying statistical calculation to the linearity. Under the optimised chromatographic conditions used, the lowest amount of LUT that could be detected accurately and precisely was 3.34 µg mL^{-1} and the lowest amount that could be detected and quantified was found to be 10.13 µg mL^{-1}.

Robustness

The low values of % RSD obtained after introducing small but deliberate changes in the parameters of the developed RP-HPLC method confirmed the robustness of the method. In this method, flow rate, column temperature and mobile phase composition were the parameters which were varied to ± 2 % to study the robustness of the method, while evaluating the retention time and peak area. The % RSD values were within the limits (i.e <2 %) and found to very low which indicated that the proposed method was robust. The results of robustness study are depicted in the Table 4.

Specificity

Specificity is the ability of an analytical method to determine the analyte unequivocally in the presence of the sample matrix. Specificity of the method was proven by spectral scan and peak purity correlation for LUT in the marketed formulation. There is no co–eluting peak with LUT. The representative chromatogram for specificity study of LUT is shown in Figure 5.

Analysis of marketed formulation

The marketed formulation was analysed using the developed and validated method which gave percentage recovery for LUT 98.90%. The results of assay are shown in the Table 5 while the representative chromatogram for assay of LUT is shown in the Figure 6.

Discussions

Summary of validated method

The optimized chromatographic condition consisted of Inertsil ODS C_{18} (150 x 4.6 mm), 5µ, analytical column with water: acetonitrile (10:90% v/v) as the mobile phase at a flow rate of 1 mL/min and 442 nm as the detection wavelength. The retention time for LUT, was found to be 7.91 ± 0.02 minutes. The method was validated as per ICH Q2 (R1) guideline. The method was linear with r^2 value greater than 0.995. The precision, accuracy and robustness values were also within the prescribed limits (<2%). The summary of results of validation parameters are illustrated in Table 6. Benefits of this method are its simplicity, direct analysis and use of economic solvent in addition to shorter runtimes.

Conclusions

Estimation of nutraceuticals like LUT through HPLC requires tedious extraction procedures and use of complicated mobile phases. Hence a simple, accurate and precise RP-HPLC method has been developed for the estimation and quantification of LUT. This sensitive and selective isocratic RP-HPLC developed method is direct, precise, specific, robust and accurate for the estimation of LUT and was successfully validated in accordance with ICH guidelines. This method can be suitable for routine analysis of the nutraceutical in bulk and commercially available dosage forms without any interference from excipients.

Consent For Publication
The research manuscript has no data which needs consent prior to publishing.

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Conflict Of Interest
The authors have no conflicts of interest that are directly relevant to the content of this research article.

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Legends
Figures
Figure 1: Structure of LUT
Figure 2. Representative chromatogram of LUT using the developed HPLC Method
Figure 3. Overlain chromatograms showing the Linearity of LUT
Figure 4. Calibration curve for LUT
Figure 5. Representative chromatogram for Specificity Study of LUT
Figure 6. Representative chromatogram for Assay of LUT

Tables
Table 1. Regression Analysis of Calibration Curve
Table 2. Accuracy Study
Table 3. Precision Study and repeatability
Table 4. Robustness Study
Table 5. Analysis of Marketed Formulation
Table 6. Summary of Validation Parameters

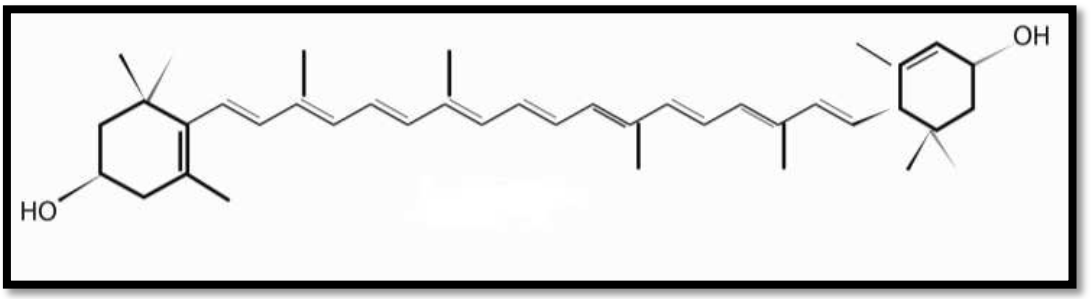

Figure 1. Structure of LUT

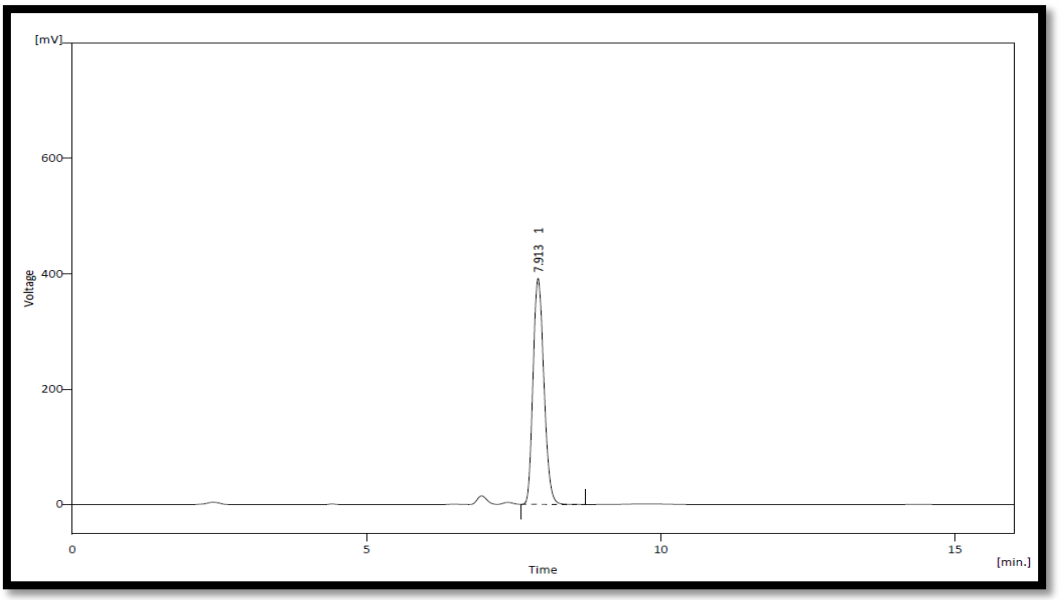

Figure 2. Representative chromatogram of LUT using the developed HPLC Method

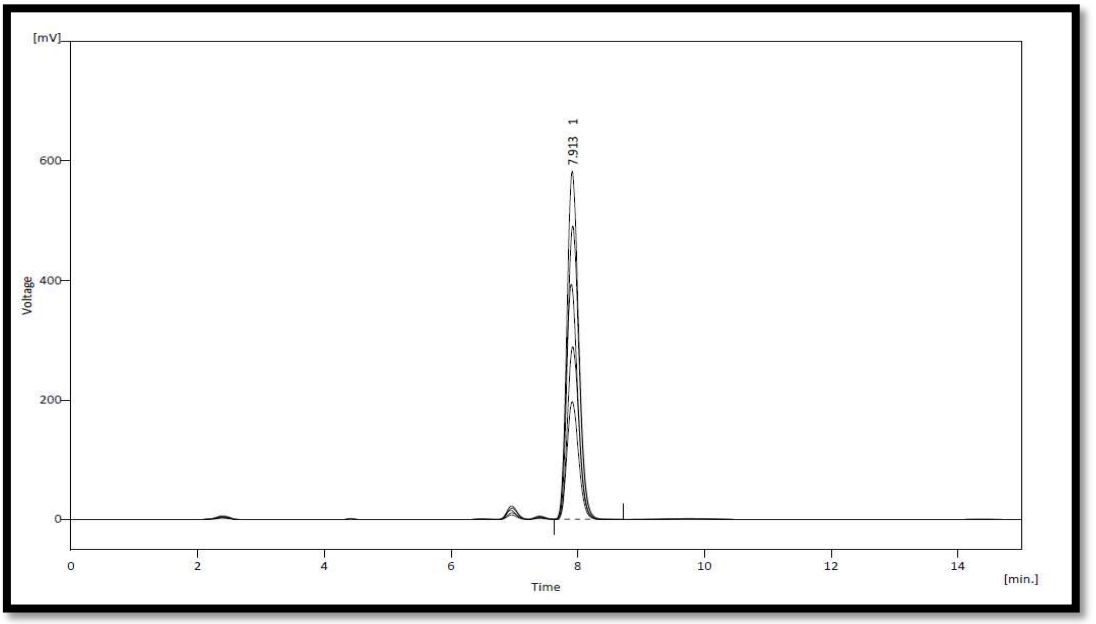

Figure 3. Overlain chromatograms showing the Linearity of LUT

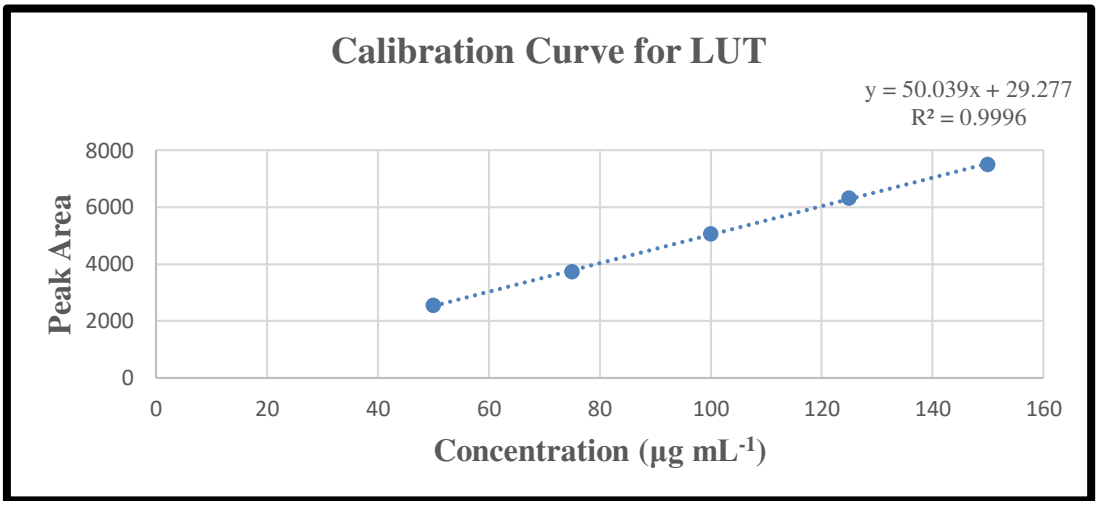

Figure 4. Calibration curve for LUT (50-150 µg mL^{-1})

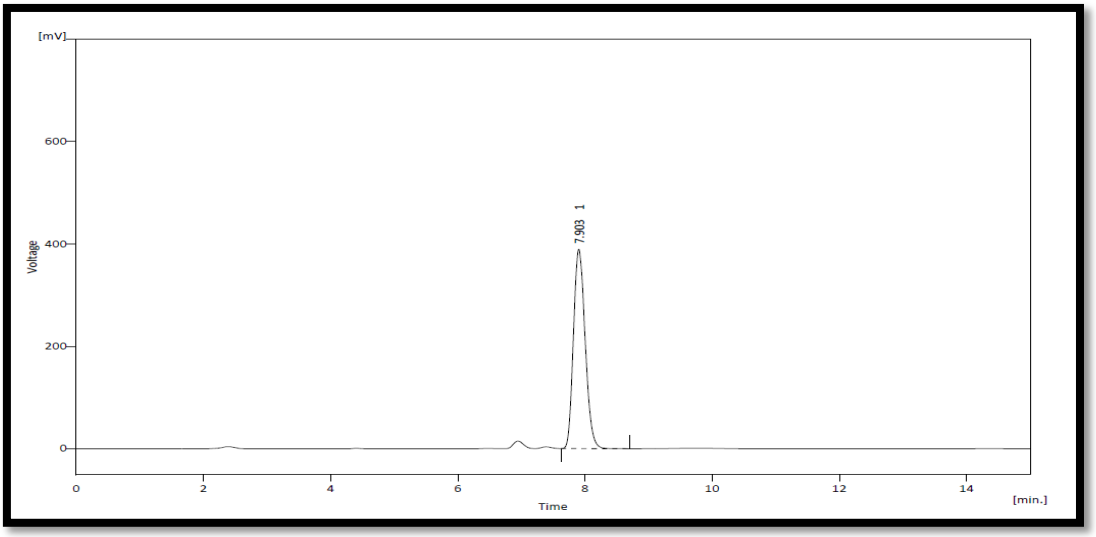

Figure 5. Representative chromatogram for Specificity Study of LUT

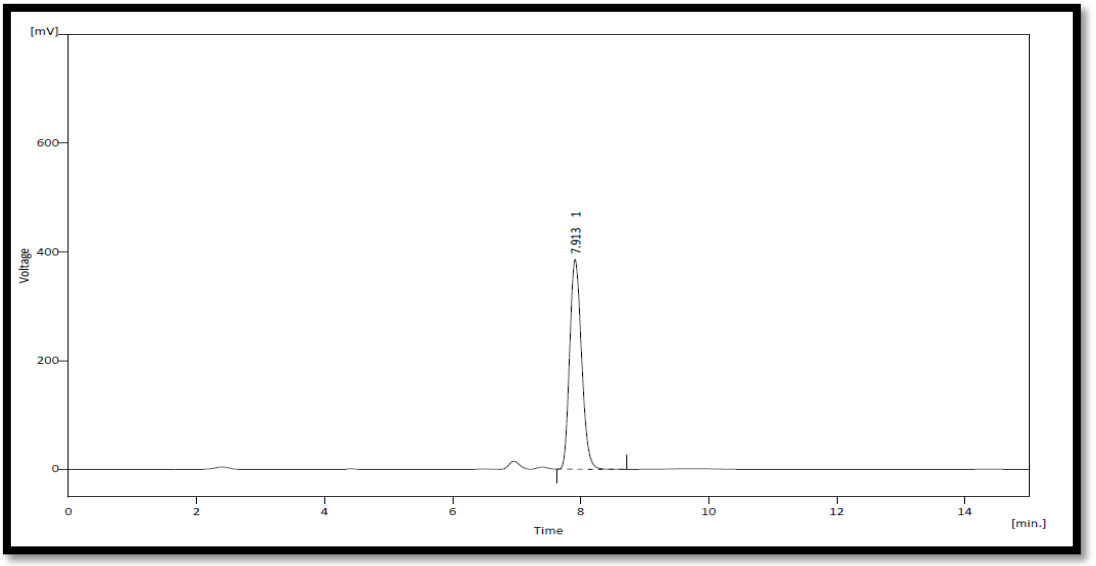

Figure 6. Representative chromatogram for Assay of LUT

TABLES

Table 1. Regression Analysis of Calibration Curve

Sr. No.	Parameter	LUT
1.	Linearity Range	50 - 150 µg mL^{-1}
2.	Regression equation	y = 50.039x + 29.277
3.	Correlation Coefficient	0.9996
4.	Intercept	29.277
5.	Slope	50.039

Table 2. Accuracy Study

Level (%)	Sample conc. (µg mL^{-1})	Conc. of Std. Added (µg mL^{-1})	Total conc. (µg mL^{-1})	Amount recovered (µg mL^{-1})	% Recovery	Mean % Recovery
80	50	40	90	39.74	99.35	99.45
				39.98	99.95	
				39.62	99.05	
100	50	50	100	49.01	98.02	98.74
				49.30	98.60	
				49.79	99.59	
120	50	60	110	58.92	98.20	98.98
				59.33	98.88	
				59.92	99.87	

Table 3. Precision Study and repeatability

Intraday Precision

Conc. (µg mL^{-1})	Area	Peak Mean Area ± SD	%RSD
50	2536.04	2537.72 ± 7.77	0.31
	2546.19		
	2530.92		
100	5052.05	5039.75 ± 21.26	0.42
	5052.01		
	5015.19		
150	7527.95	7522.79 ± 30.30	0.40
	7490.23		
	7550.18		

Interday Precision

Conc. (µg mL^{-1})	Area	Peak Mean Area ± SD	%RSD
50	2528.48	2533.56 ± 18.16	0.72
	2553.74		
	2518.50		
100	5062.07	5063.73 ± 12.72	0.25
	5051.91		
	5077.19		
150	7563.0410	7593.05 ± 40.18	0.53
	7638.7050		
	7577.4260		

Repeatability Study

Concentration	Injection No.	Area (AUC)
	1	5021.98
	2	5067.19

LUT 100 (µg mL^{-1})	3	5036.74
	4	5031. 67
	5	4976.38
	6	5011.16
	Avg.	5024.18
	SD	30.07
	% RSD	0. 60

Table 4. Robustness Study

Parameter	Factor	Mean Area ± S. D	%RSD
Flow Rate	0.8 mL	5165.25 ± 44.30	0.86
	1.2 mL	4883.86 ± 14.78	0.30
Mobile Phase Composition	(-2%)	5116.29 ± 44.43	0.87
	(+2%)	4960.17 ± 20.72	0.47
Column Temperature	(38 ˚C)	5169.22 ± 41.30	0.86
	(42 ˚C)	4889.16 ± 15.74	0.30

Table 5. Analysis of Marketed Formulation

Parameter	Area of sample	%Assay
Reading 1	4958. 64	98.54
Reading 2	5008.25	99.53
Reading 3	4963.18	98. 63
Average		98.90
SD		0.54
%RSD		0.55

Table 6. Summary of Validation Parameters

Sr. No.	Parameters	Result
1.	Linearity Range	50 - 150 µg mL^{-1}
2.	Interday Precision (% RSD)	0.25 - 0.72
3.	Intraday Precision (% RSD)	0.31 - 0.42
4.	Accuracy	98.74 - 99.45
5.	Repeatability (% RSD)	0. 60
6.	LOD	3.34
7.	LOQ	10.13
8.	Mean % Assay ± SD	98.90 ± 0.55