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Research Article

RP HPLC Method Development and Validation for Estimation of Orlistat in Pharmaceutical Dosage Forms

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Abstract: Orlistat is an anti-obesity drug that inhibits the secretion of gastric and pancreatic lipases. Normally lipase breakdown triglyceride in the intestine as a result of metabolism. Despite the numerous advantages of this drug, researchers are facing problems with estimation by HPLC because limited methods are available; only United State Pharmacopoeia (USP) method is most reliable and effective. The present work aimed to develop an RP- HPLC method for determining orlistat in floating microcapsules. Considering the virtual reality in drug testing, quality control of dosage forms is the main criterion to be followed in the Pharmaceutical industry, which is to establish the procedures undertaken to ensure the identity and purity of a particular dosage form. Syncronis C18, 150mm x 4.6mm, 3µm column were used. Isocratic elution was performed with ACN:Water mixture. The flow rate was 2.0 mL min^{-1} and UV detection was at 205 nm. Caffeine was used as an internal standard. The developed method was validated according to the ICH guidelines and found to be linear within the range 150-600 ppm of Orlistat. In addition, this method was validated for its suitability, accuracy, precision, selectivity, robustness, and ruggedness per the ICH guidelines. The results HPLC analytical method validation was suitable for the quantitative and qualitative analysis of orlistat and it showed that this method could be successfully utilized for the identification and quantification of orlistat in any dosage form, with high resolution, accuracy, and precision.

Keywords: HPLC, Orlistat, Suitability, Precision and Stability.

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1. INTRODUCTION

Obesity is a chronic condition linked to a high morbidity and mortality rate. Over 300 million people are obese worldwide, with another 800 million being overweight. This incidence ranges from around 30% to over 70% in the United States, the Eastern Mediterranean, and the Pacific Islands. Orlistat is rapidly excreted and expelled mostly in the feces with oral absorption of less than 1% of the administered dose and no indication of build-up after long-term use. Two metabolites are produced by bile excretion[1]; M1 and M3 have half-lives of 2 and 3 hours, respectively, and have no pharmacological effects. Orlistat is an anti-obesity drug that inhibits the secretion of gastric and pancreatic lipases. Normally lipase breakdown triglyceride in the intestine as a result of metabolism[1]. When Orlistat blocks lipase activity, triglycerides from the diet are not hydrolyzed to form free fatty acid that remains unchanged in the intestine and excreted. Orlistat was approved for market by USFDA in the year 2006 for the treatment of obesity[2].

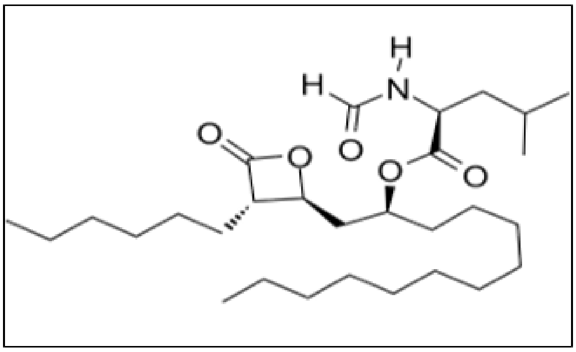

Fig 1: Structure of Orlistat

Orlistat is a semisynthetic derivative of lipstatin, which is a potent inhibitor of gastric and pancreatic lipase, which is used for the digestion of dietary fat. Orlistat is a carboxylic ester resulting from the formal condensation of the carboxy group of N-formyl-leucine, which is practically insoluble in water and slightly soluble in methanol.[3] It also improves insulin sensitivity and reduces body fat and serum leptin levels. Orlistat is a semisynthetic derivative of lipstatin; it is a potent and selective natural inhibitor of gastric and pancreatic lipases, which play an essential role in the digestion of dietary fat. When administered along with fat-containing foods, orlistat partially inhibits the hydrolysis of triglycerides. Therefore, it decreases the subsequent absorption of monoacyl glycerides and free fatty acids. Many formulations of orlistat are available in the market and so many are in clinical phase and under development. Despite numerous advantages of this drug, researchers are facing problems for estimation by HPLC because limited methods are available; only United State Pharmacopoeia (USP) method is the most reliable and effective. In the literature, there is an HPLC method for the determination of Orlistat in human plasma for pharmacokinetics studies[3]. However, there is no method for quantifying Orlistat in pharmaceutical dosage forms, especially in the floating drug delivery system. Considering the virtual reality in drug testing, quality control of dosage forms is the main criteria to be followed in Pharmaceutical industry, which establishes the procedures undertaken to ensure the identity and purity of a particular dosage form.[4]

2. MATERIALS AND METHODS

Orlistat working standard was procured from Alli capsules (GSK) - Only USFDA Approved drug under "OTC". Analytical grade of acetonitrile was procured from Merck.

2.1 Apparatus and Chromatographic Condition

HPLC analyses were performed on Thermo -Ultimate CAD 300 with UV Detector. Separations were carried on Syncronis C_{18} 150mm x 4.6mm, 3µm LC column. The column temperature was maintained to 40°C, and the flow rate was set at 2.0 $mLmin^{-1}$. The run time of the analysis was fixed at 15 minutes, while the injection volume was maintained at 25 µL. The isocratic elution method was followed by acetonitrile: water mixture as needle wash, where methanol and acetonitrile were used as organic modifiers. The drug was analyzed at 205nm using UV spectrometry. The data were interpreted using chrome Leon 7.2 version RS4 to tabulate the findings.

2.2 System Suitability

25.0 µ L of the orlistat standard solution was injected six times into the column to determine the test parameters. The final concentration of orlistat was maintained to be 120.0 µ g/mL. The standard chromatogram parameters, such as injection precision for the standard solution, its tailing factor and the standard solution's theoretical plate, were calculated.[5]

2.3 Preparation of Standard Solution

The standard solution with a concentration of 0.12 mg/mL, 12 mg USP orlistat, and the reference standard (RS) was placed in a 100 mL volumetric flask to which 80 mL acetonitrile: water was added; the mixture was stirred and sonicated to dissolve the drug, and the solution was diluted to total volume using methanol. (The solution will be stable for 1 week at 4°C.)[6]

2.4 Preparation of Sample Solution

The sample solution was prepared by reconstituting 5ml of the stock solution in a 100mLvolumetric flask, in which 75ml of the diluent was added. The mixture was made to dissolve rapidly and sonicated for 30 minutes. The solution thus prepared was made up to the final volume using the same diluent. Thereafter the mixture was kept undisturbed to separate the 5ml of supernatant into 50ml volumetric flask and made up to the final volume by using the mobile phase. A part of this mixture was filtered using a nylon syringe whose pore size is less than 0.45 µ m. The mixture of equivalent quantities of floating microparticles containing 120 mg of orlistat was prepared using ultrasound, which is sonicated for 15 minutes in addition to 80 mLof methanol. When the solution containing this mixture returns to room temperature, methanol was further added to make up the volume of 50mL

2.5 *Preparation of Analytical Placebo Solution*

Common inactive ingredients such as compritol 888 ATO (40%), Beta Cyclodextrin (1%), PVP S360 (1%), Magnesium stearate for lubrication, Eudragit RSPO, Stearic acid, Eudragit S100 and Poly vinyl pyrrolidone XL10 were weighed according to the ratios used in the formulation of microparticles to achieve 5g of bulk. Approximately 1g was taken from the bulk, which was used to produce the placebo by the same procedure employed for the formulation of microparticles. Synthetic capsules were formulated by adding a known amount of orlistat standard solutions to the placebo solutions.[7]

2.6 *Forced Degradation*

2.6.1 *High Temperature*

1000 µ L of orlistat solution was prepared by diluting 100 µ L standard stock solution with water. The diluted solution was transferred to a centrifuge tube and kept in a water bath maintained at 80 °C for 2 hours. Then the solution was cooled to room temperature (25 ± 5 °C). The solution that subjected to elevated temperature was further diluted to 20 µ g/mL by using mobile phase and injected into the HPLC system.[8]

2.6.2 *Acid and Alkali Hydrolysis*

100 µ L of orlistat standard stock solution was diluted to 1000 µ L by adding 0.1 N HCl, 0.1 N NaOH, and 3.0%w/v H_20_2. The concentration of orlistat was maintained to be 100 µ g/mL in the final solution. The prepared solutions were transferred to a separate centrifuge tube and kept in a water bath at 40°C for 2 hours. Then the solution was cooled to room temperature (25 ± 5 °C). Then the solution was neutralized with a suitable amount of neutralizing agent. The solution that was subjected to acid and alkali stress was further diluted to 20 µ g/mL by using mobile phase and injected into the HPLC system.[9-12]

2.6.3 *Irradiation with UV*

1000 µ L of orlistat solution was prepared by diluting 100 µ L standard stock solution with water. The concentration of orlistat was maintained to be 100 µ g/mL in the final solution. The solution was exposed to UV light of 205 nm combined with a tungsten lamp for 24 hours at room temperature. The solution, exposed to UV irradiation, was further diluted to 20 µ g/mL with the mobile phase and injected into the HPLC system.[11]

2.6.4 *Limit of Detection (LOD) And Limits of Quantification (LOQ)1*

The least concentration of analyte that can be detected in a sample under specific experimental conditions (LOD) was determined by using the y-intercepts of regression lines in the linearity curve. This can be calculated by using the equation

LOD= 3.3⌠ /s

Where,
⌠ is the standard deviation of the response,
s is the slope of the linearity curve

Limits of quantification (LOQ) is the lowest analyte concentration in a sample that can be determined quantitatively within the specified level of precision and accuracy under the standard specified operational condition. The LOQ can also be determined using the y-intercepts of regression lines in the linearity curve. This can be calculated by using the equation

LOQ = 10⌠ /s

Where,
⌠ is the standard deviation of the response,
s is the slope of the linearity curve

2.7 *Dissolution Study*

FDA has reported a standard dissolution method for conducting *in vitro* dissolution testing of orlistat capsules. For this testing, USP (type II) apparatus were used, which is maintained at 37°C at a paddle speed of 75 rpm. The dissolution medium used for this testing was 900 mL, 3% sodium lauryl sulfate in 0.5% sodium chloride at pH 6.0. One capsule of orlistat containing 120 mg of microparticles was placed in each bath, and 10mL of samples were withdrawn concerning time and replaced with fresh medium to maintain the sink condition. The samples withdrawn were diluted to 20 µ g/mL with the mobile phase and injected into the HPLC system.[12]

2.8 *Stability Studies*

A stability study is designed to provide substantiation of how the quality of drug products affects time under the influence of different environmental conditions such as temperature, humidity, and light. The standard stock solution of orlistat (1000 µ g/mL) was prepared in acetonitrile: water was dissolved into two volumetric flasks. This volumetric flask was kept at room temperature and at 4°C inside the refrigerator. The stability of orlistat at room temperature was evaluated for 24 hrs, whereas the stability at refrigerated conditions was evaluated for both the short term (72 h) and long term (1 month). The solution that was exposed under the stability stress was further diluted to 20 µ g/mL by using the mobile phase and injected into the HPLC system.[13]

2.9 *Robustness*

To determine the robustness of the developed method, intentional changes were made to the chromatographic system, and the system suitability parameters were verified. The changes to the parameters include the change in flow rate by ±0.05 mL/min, change in pH of the buffer by ±0.1 unit and change in the ratio of mobile phase (±2% absolute).[17]

2.10 *Ruggedness*

The impact of the bioanalytical method should remain unchanged with minor variations. The ruggedness of the developed method was evaluated by one precision and accuracy run using different columns and instruments.[18]

3. RESULTS AND DISCUSSION

To develop and validate an HPLC method, primarily optimization of the mobile phase has to be done after an appropriate selection of columns. Orlistat is practically insoluble in water, freely soluble in chloroform, and very soluble in methanol and ethanol. The literature review identified that acetonitrile was a good solubilizer of orlistat, forming a clear solution on addition. So, it is distinct that a mixture of acetonitrile and water in different proportions can be used to separate orlistat through the C18 column. Caffeine is an internal standard; it can be eluted with acetonitrile and water mixture without any buffer.

3.1 System Suitability

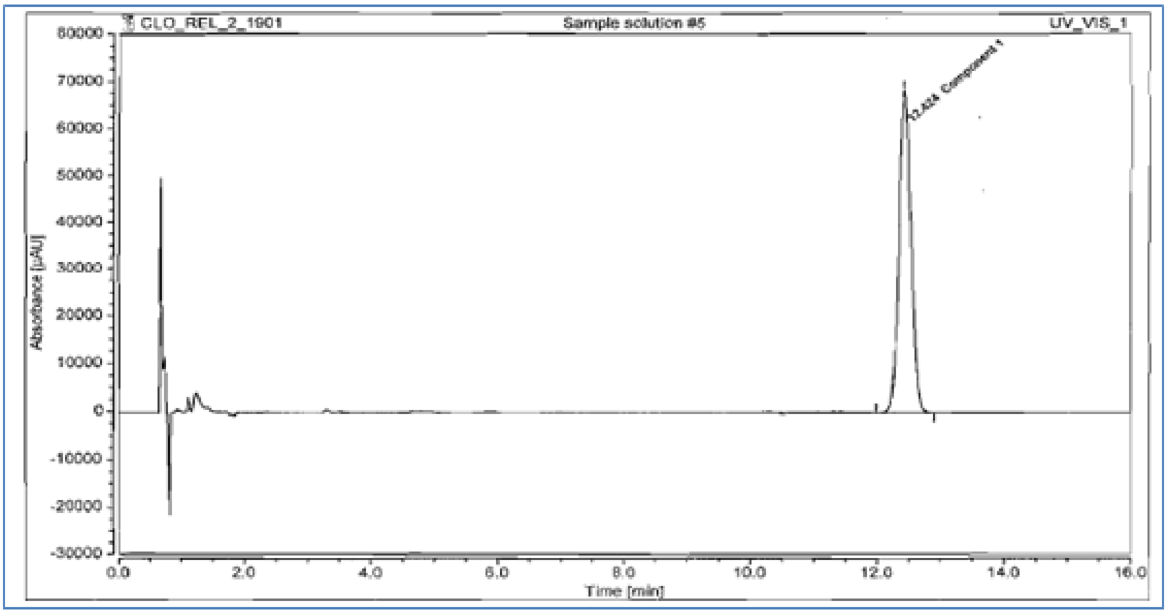

Fig 2: Representative chromatograms obtained under the optimum chromatographic conditions for Orlistat standard sample and Blank

Table 1: Relative standard deviation and tailing by area count determination of standard orlistat.

Set No.	System Precision (Area Count for standard)	Acceptance criteria
1	1366587.016	The system suitability parameters such as % Relative Standard deviation, tailing factor, and Theoretical plate count was evaluated for method suitability.
2	1360448.375	
3	1357808.355	
4	1358409.069	
5	1362485.866	
Mean	1361148	
% RSD*	0.26	Not more than 2.0
Tailing	1.21	Not more than 2.0

RSD = Relative standard deviation

System suitability test was applied to the chromatograms to ensure the parameters such as column efficiency (Plates), peak tailing, capacity factor, and resolution. Suitable area counts for the standards were obtained concerning the total analysis time. The system suitability precision was given in Table 1. The % RSD for the five consecutive injections of 25 µL working standard solution at 2 ml/min into HPLC chromatography using UV detector at 205 nm was found to be 0.26 and tailing 1.21, respectively. All these results confirm the adequacy and reproducibility of the proposed HPLC method for routine analysis of orlistat.

3.2 Method Validation

The proposed method was validated to select the linearity range, sensitivity, precision, accuracy robustness, and ruggedness as per the ICH guideline (ICH 2005).

3.3 Forced Degradation

The ICH guideline entitled stability testing of drug substance and product requires the stress testing to be done to illustrate the inherent stability characteristics of the active substances and provide a fine identification of differences that would result from change in the manufacturing process or source sample (ICH 2005). To proceed with the testing, various stress conditions were applied, such as pure drug, 0.1 N HCl stressed, 0.1 N NaOH stressed, 3.0%w/v H_20_2 and stressed under high temperature of 80 °C for 2 hours. Under all these conditions, the degradation products were observed. This forced degradation testing shows that the product is pure and stable over provided stress conditions. The forced degradation and purity results were given (Table 2).

3.4 Specificity Study Data

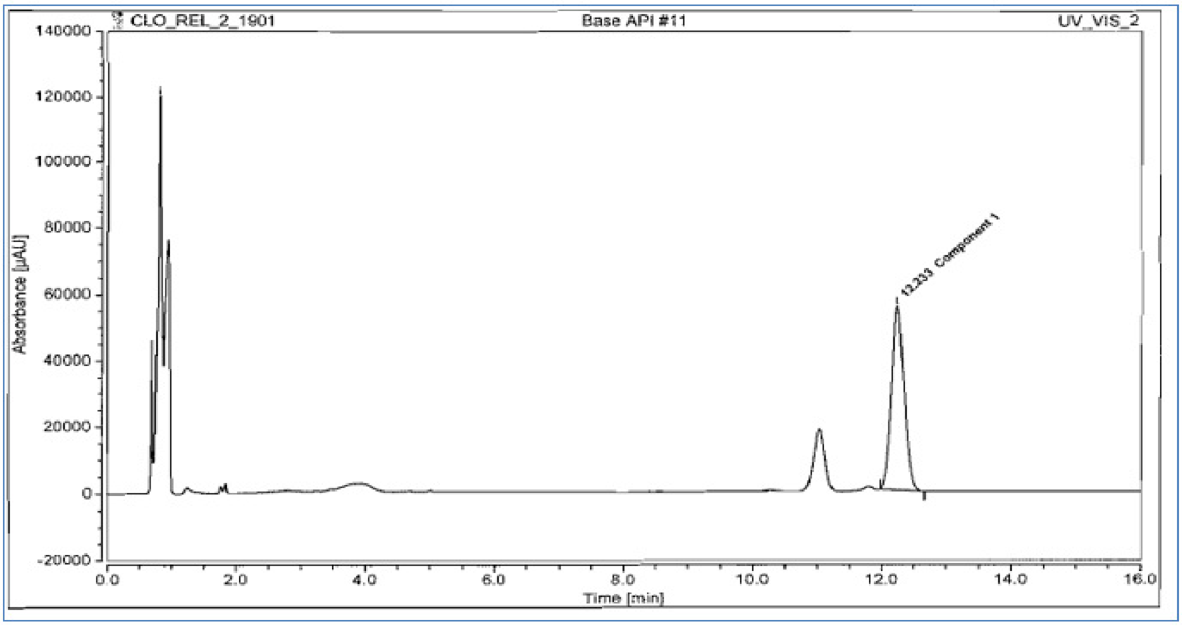

Fig 3: Representative chromatograms obtained under the optimum chromatographic conditions for Orlistat standard undergone stress conditions.

Specificity was achieved by injecting blank solution, working standard solution into the chromatography system & verified the exhibition blank and Orlistat peak from WS.

Table 2: Purity match (%) of orlistat after the exposition on various conditions of temperature and pH

Specificity in Stress Condition's	Peak Purity**	Purity Match
Sample as Such	P	994
0.1 N HCl Stressed	P	998
0.1 N NaOH Stressed	P	998
3.0%w/v H202 Stressed	P	997
High temperature of 80 °C for 2 hours	P	998

*****P= Pure***

3.5 Linearity Data of Orlistat

Table 3: Linearity data of orlistat analyses by the developed method

Set. No.	Concentration in ppm*	Area count
1	150	463074
2	245	639444
3	370	957856
4	500	1279717
5	600	1527782
Regression Coefficient		0.9991

****PPM = Parts per mole***

Linearity was established by generating a sequence of dilutions in different concentrations (in ppm) ranging from 150-600 ppm of Orlistat. The linearity of an analytical procedure, which are directly proportional to the concentration of analyte in the sample, indicates the capacity to acquire test results Calibration curve of orlistat was constructed by plotting the concentration in parts per mole against area count. The graph confirmed that the method was linear upto 600 ppm. (Table 3) Five different standard solutions within the linear range containing 150, 245, 370,500, and 600 ppm of orlistat were prepared and injected into the HPLC system. The linearity of the standards was evaluated by linear regression analysis. The standard deviations of the slope and the calibration curve intercept were calculated using a regression equation. (Figure 4)

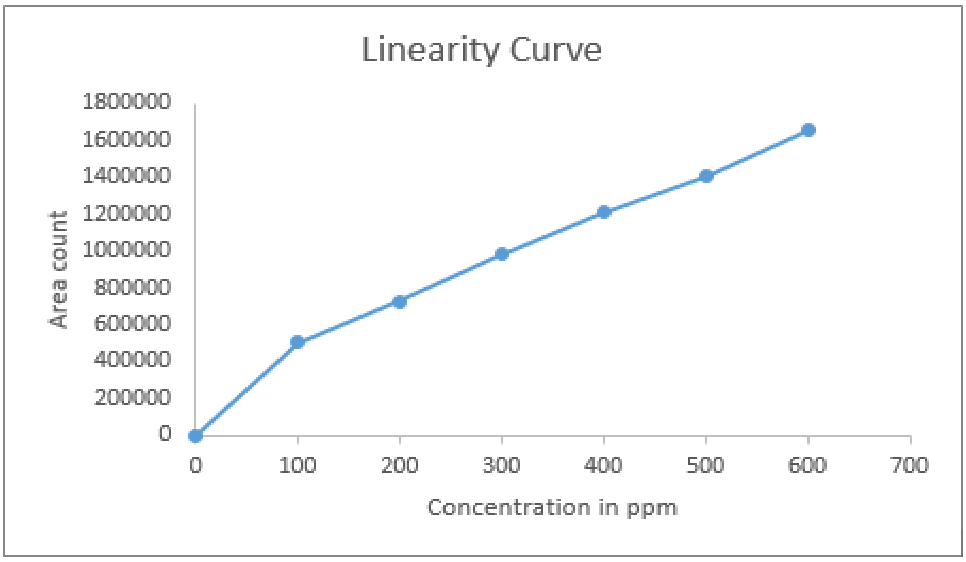

Fig 4: Linearity curve of orlistat plotted in concentration(ppm) against time

3.6 *Limit of Detection (LOD) And Limits of Quantification (LOQ)*

The LOD and LOQ of the analyte were determined using the y-intercepts of regression lines in the linearity curve. The detection limit is defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantitation limit is defined as the lowest concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (RSD%) and accuracy (Bias%) within ±10%.[36] The area counts of the LOD and LOQ were found to be 39 and 21 μ g /mL, respectively. Hence this is the variability limit that a user can expect to encounter in the field of analysis of orlistat in HPLC system.

3.7 *Precision Study Data of Orlistat*

Table 3: ***Precision of the developed method by assay of orlistat***

Set No.	Method Precision (% Assay)
1	99.0
2	99.2
3	100.1
4	100.8
5	100.6
6	99.9
Mean	99.9
Standard deviation	0.726
% RSD*	0.73
Acceptance criteria	NMT 2.0%
% RSD* = (Standard deviation *100)/Mean	

RSD – Relative standard deviation

Method precision was performed by preparing 6 set of samples from a single batch.

3.8 *Accuracy Study Data of Orlistat*

Accuracy of the developed assay method was determined using the six times analysis of the quality control samples of orlistat. Six sets of samples from a single batch were analyzed for its accuracy within the same day. The obtained values for the percentage relative standard deviation were found to be 0.73 which is under the acceptance range of NMT 2.0%, which indicated the precision and accuracy of the method. The results were summarized in the (Table 3).

Table 4: ***Accuracy of the developed method by standard addition method***

Set. No	Level (%)	Area count	Amount added (mg)	Amount recovered (mg)	Recovery (%)	Mean (%)
1	75%	1005215	37.56	37.26	99.2	99.3
2		994512	36.89	36.86	99.9	
3		988258	37.12	36.63	98.7	
1	100%	1345648	50.12	49.88	99.5	99.2
2		1350121	50.48	50.04	99.1	
3		1335648	49.99	49.51	99.0	

1	125%	1594878	60.24	59.11	98.1	98.1
2		1582154	60.05	58.64	97.7	
3		1608512	60.52	59.62	98.5	
Over all Mean Recovery						98.9%
Over all % RSD*						0.67
Acceptance Criteria						RSD-NMT 2.0%

RSD- Relative standard deviation

An accuracy study was performed to measure the closeness of the experimental values to the actual number of samples. The recovery was established at three different levels as per the target concentrations' ICH (80%, 100% and 120%). Inorder to identify whether the excipients of the developed formulation interfere with the analysis, the recovery test was performed using by standard addition technique. Three concentrations at 75%,100%, and 125% were selected by adding a known amount of Orlistat standard solutions into the formulation. The final concentration was within the linear range. These solutions were prepared three times and analyzed through the method developed. The amount of the drug added was compared with the amount of drug recovered. Comparison of the intercepts of calibration curve with standard addition technique indicated that the formulation with this excipient was identical (overall mean recovery – 98.9%), and there was no interference from matrix components. (Table 5)

3.9 Dissolution Study

Table 5: Orlistat release profile from Branded drug and Formulated drug samples at pH 6.0

Time (minutes)	Brand average % release ±SD	Sample 1 average % release ±SD	Sample 2 average % release ±SD
0	0	0	0
5	53.65±1.58	59.81±1.79	69.37±3.68
15	95.87±2.31	93.45±1.22	95.50±1.20
25	104.50±1.69	101.32±0.63	99.35±1.68
35	107.68±1.76	101.83±1.42	101.12±1.37
45	103.75±0.72	100.20±0.45	100.72±1.53

S.D n=3

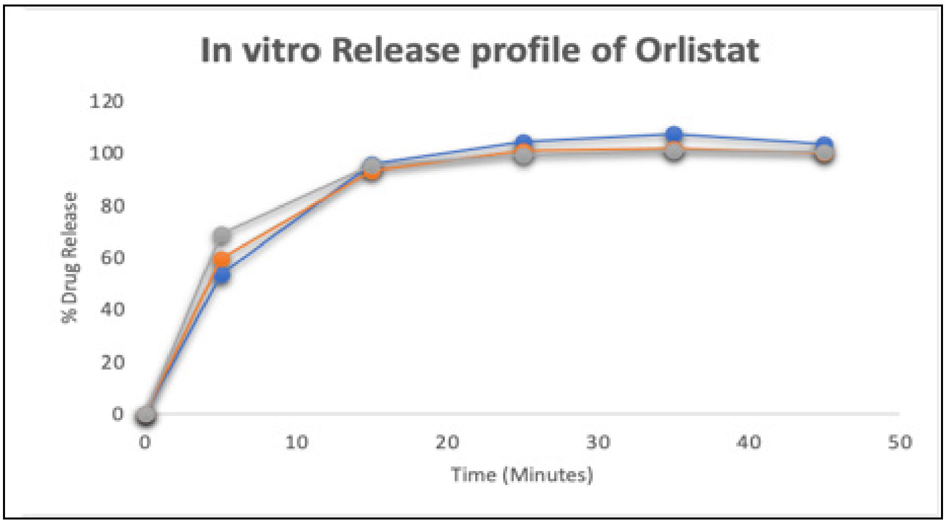

Fig 5: Orlistat drug release profile from Branded drug and Formulated drug samples at pH 6.0

An *in vitro* dissolution study for orlistat microparticles and marketed formulations was conducted in accordance with the reported FDA dissolution method. In association with this dissolution testing both samples 1 and 2 were tested at the recommended pH media parameters (FDA conditions). In both the samples, the products release more than 90% of their active ingredients within 15 minutes as shown in (Table 5 and Figure 5). Hence the calculations of similarity and dissimilarity factors vanished according to ICH guidelines. However, the dissolution of orlistat from all selected test products was below 6%, and near to 1% when tested at pH 6.8, 4.5, and acidic pH (0.1 N HCl solution).

Table 6: *Assay data of marketed formulation*

Sample No.	% Assay	Average Assay (%)
Orlistat Microparticles	99.5%	99.8%
Orlistat Marketed formulation	100.1%	

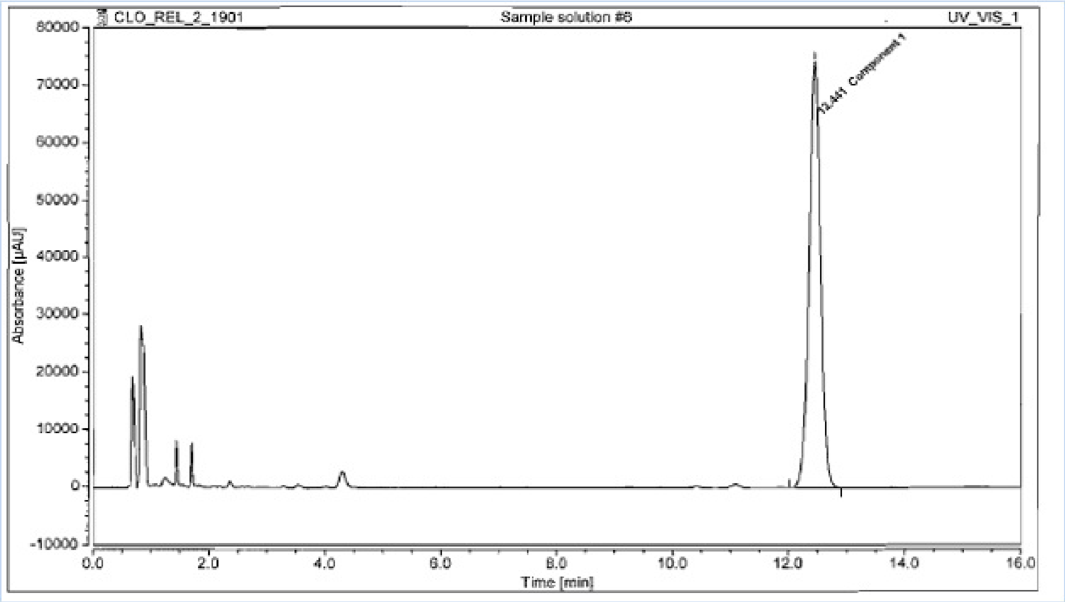

Fig 5: Assay of marketed formulation

3.10 *Robustness*

To demonstrate the system's suitability for the developed method, several statistical values such as retention time, tailing factor, resolution, range, slope, Intercept, LOD, and LOQ were calculated by making deliberate changes to the chromatographic conditions. The values of the parameters were listed (Table 7), and it was evident that the method developed was robust over an acceptable working condition of its HPLC operational parameters.

Table 7: ***System suitability parameters***

S.No	Parameters	Orlistat system suitability
1.	Retention time (min)	1.2
2.	Tailing factor	0.21
3.	Range (ppm)	150-600
4.	Slope	0.622
5.	Intercept	1934
6.	LOD (µ g mL- 1)	39
7.	LOQ (µ g mL- 1)	21
8.	Assay	99.3%

To identify the method's reproducibility, a system suitability test was employed to establish the tailing factor. The peak asymmetric factor was observed to be 0.21, less than 1.6. The result indicated the absence of peak tailing as the asymmetric peak factor falls within the acceptable range.

3.11 *Matrix Factor*

Table 8: ***IS normalized matrix factor for orlistat at low concentration***

Parameter	Analyte Peak Area	IS Peak Area	IS Normalized MF
Mean	611.0000	64533.6667	0.9857
S.D	18.43253	357.65495	0.01432
% CV	2.94	0.56	1.55

Acceptance criteria: Overall CV% for IS normalized matrix factor ≤ 15%

Table 8: ***IS normalized matrix factor for orlistat at high concentration***

Parameter	Analyte Peak Area	IS Peak Area	IS Normalized MF
Mean	43875.0000	53283.6667	1.0654
S.D	238.43256	188.54387	0.00564
% CV	0.55	0.36	0.57

Acceptance criteria: Overall CV% for IS normalized matrix factor ≤ 15% Internal standard normalized matrix factors for orlistat at low concentration had mean 0.9867, SD 0.01528 and % Co-efficient of variation 1.55 and for the highest concentration, mean 1.0167, SD 0.00577 and % Co-efficient of variation 0.57. The acceptance criteria with overall CV% for IS normalized Matrix factor ≤ 15%.

3.12 Ruggedness

Ruggedness was achieved by analyzing different batches in analytical columns and instruments and it was identified that the method was unaffected by variations in different of columns and temperatures.

3.13 Application of The Developed Method

The validated method developed was applied to determine Orlistat in commercially available formulations. The figure demonstrated a typical type of HPLC chromatogram of tablet solution of orlistat with no interference of excipients present in floating microparticles. The analytical results are given in (Table 5). This result shows that the method selected for the analysis provides results without the interference of the excipients.

4. CONCLUSION

In this study, an attempt was strived to develop an easy, simple, precise, selective, and delicate RP-HPLC method for the pharmaceutical analysis of orlistat. This method acts as a surrogate for determining orlistat in dosage forms. This method was validated for its suitability, accuracy, precision, selectivity, robustness, and ruggedness per the ICH guidelines. The forced degradation testing results include isolation of degradation products and determination of the amount of orlistat after exposure to stress conditions. This data revealed that the method is stability indicating and has the ability to determine orlistat in the presence of its degradation products. Moreover, this RP-HPLC method is acceptable for concurrently determining the best possible condition. Its robustness and effectiveness strongly indicate a powerful strategy that can be used to estimate the drug in different pharmaceutical dosage forms. Hence the HPLC analytical method developed was suitable for the quantitative and qualitative analysis of orlistat.

5. ACKNOWLEDGEMENTS

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6. AUTHORS CONTRIBUTION STATEMENT

V. Vivekanandhan conceptualized and gathered the data about this work. Dr. D Kumudha organized these data, and all necessary inputs were given towards the design of the manuscript. Finally, both authors discussed the methodology, and results and contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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