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Development and validation by statistical treatment of stability indicating RP-HPLC method for quantification of Orlistat in Orlistat-loaded solid dispersion

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Abstract

Background: Most of the analytical methods reported for the estimation of Orlistat were complex, expensive, and deficient in reproducibility with no or very less informative regarding various statistical methods and equations used for the validation purpose. This study provides a fast, accurate, descriptive, and precise isocratic reversed phase high-performance liquid chromatographic (HPLC) method using Waters Spherisorb 5 μm Octadecyl-silica-2 (250 × 4.6 mm) column, for the estimation of Orlistat in bulk drug and pharmaceutical formulations with minimized drug extraction steps. The drug was detected in an analytical column with mobile phase comprising a mixture of methanol, acetonitrile, and 2% phosphoric acid in the ratio of 85:14:1 v/v/v at flow rate of 1 ml/min with elution monitoring at 215.0 nm.

Results: The retention time for Orlistat was found to be 5.9 min with sharp and proper peak. The linearity was covered over the concentration range of 1.00–10.00 μg/ml (r^2 = 0.9997) with a limit of detection and limit of quantitation 0.06 and 0.2 μg/ml, respectively. The developed analytical technique was found to be validated for all the parameters within the acceptance criteria of ICH guidelines. The mean ± standard deviation (SD) recoveries of Orlistat were 99.87 ± 0.45.

Conclusion: The optimized method was well precise, accurate, sensitive, stability indicating, and tested with all statistical parameters. Thus, the method can be conveniently used in quality control and routine analysis of Orlistat containing solid dispersions and other formulations. The main advantage of the developed method was its high specificity for the estimation of Orlistat in presence of various degradation products resulting from stress conditions and formulation excipients.

Keywords: Orlistat, RP-HPLC, Validation, Stability indicating, Solid dispersion

Background

Orlistat ((2S)-1-[(2S,3S)-3-hexyl-4-oxooxetan-2-yl]tridecan-2-yl (2S)-2-formamido-4-methylpentanoate) is reversible inhibitor of gastrointestinal lipases used for the management of obesity and weight loss [1]. It acts by inhibiting intestinal and pancreatic lipase, an enzyme that break down triglycerides present in the gastro intestinal tract into hydrolyzed form and thus reducing the absorption of fats into the human body and thereby decreasing the amount of calorie intake [2].

Orlistat is a white to off-white crystalline powder which is approximately insoluble in water but freely soluble in chloroform and extremely soluble in methyl alcohol and ethyl alcohol [3]. It is a highly lipophilic drug with high amount of lipophilic chains as shown in the Fig. 1. It has half-life of 1 to 2 h with more than 99% binding to plasma proteins (mostly lipoproteins and albumin were major binding proteins) and metabolized mostly in the gastrointestinal wall leading to the formation of relatively inactive metabolites. Metabolites M1 (4-member lactone ring hydrolyzed) and M3 (M1 with

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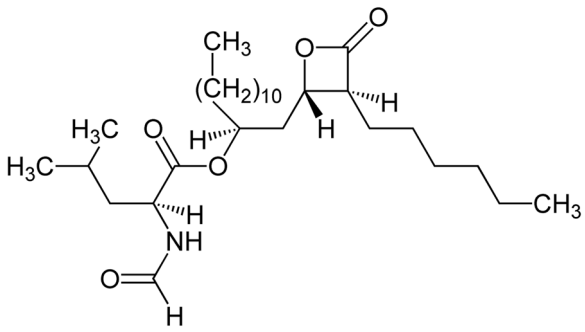

Fig. 1 Structural formula for Orlistat showing large hydrocarbon chains responsible for the lipophilicity of the drug

N-formyl leucine moiety cleaved) are open lactone ring and have very low activity [4].

Maximum analytical methods presented in the literature for the estimation of Orlistat were using tandem mass spectrometry in biological matrix where drug extraction was done and then chromatography was carried out using acetonitrile and ammonium acetate (90:10) as eluent with 2 mm i.d. × 50 mm Deltabond Phenyl column [5–7], which was complex, costly, difficult to perform, time consuming, and not useful for routine analysis of Orlistat formulations. Also, very few analytical methods are available or reported for the estimation of Orlistat in pharmaceutical formulations and bulk drug but all of them have limitations of lack of clear estimation of drug peak, deficient reproducibility, difficult test preparation procedure, and short of method applicability for the analysis of Orlistat in single and combination drug formulations, like differential derivative UV and visible spectroscopy with the help of hydroxylamine solution provides difficult estimation procedure with no sensitivity and specificity for the drug [8, 9]. This study provides a fast, accurate, and precise HPLC method for the assessment of Orlistat in pharmaceutical formulations for drug quality control purpose. The dissolution method was also developed and validated according to ICH and USP guidelines.

Methods

Materials

Orlistat was obtained as gift sample from CMG Biotech Pvt. Ltd. HPLC grade methanol, chloroform, acetonitrile, and water used were purchased from Merk Chemicals, India. Phosphoric acid, ethanol, Poloxamer 407, and mannitol were procured from Sigma Aldrich, India. Xenical capsules (Roche) were purchased from local pharmacy shop and used as reference formulation in the study. Syringe Filter [MILLEX-HV (0.45 μm)] and Millipore Filters [MILLIPORE GVWP04700, 0.45 μm] are used. The buffer system and solvents were filtered through 0.22 μ Millipore membrane filter (Merck, Darmstadt, Germany) and properly degassed in ultrasonic bath for 30 min.

HPLC instrumentation

Orlistat HPLC method was developed using Waters HPLC system outfitted with binary pumps (515) photo-diode array (PDA) detector (Waters 2489 UV/Visible) and sample injection volume 20 μl. The column used was

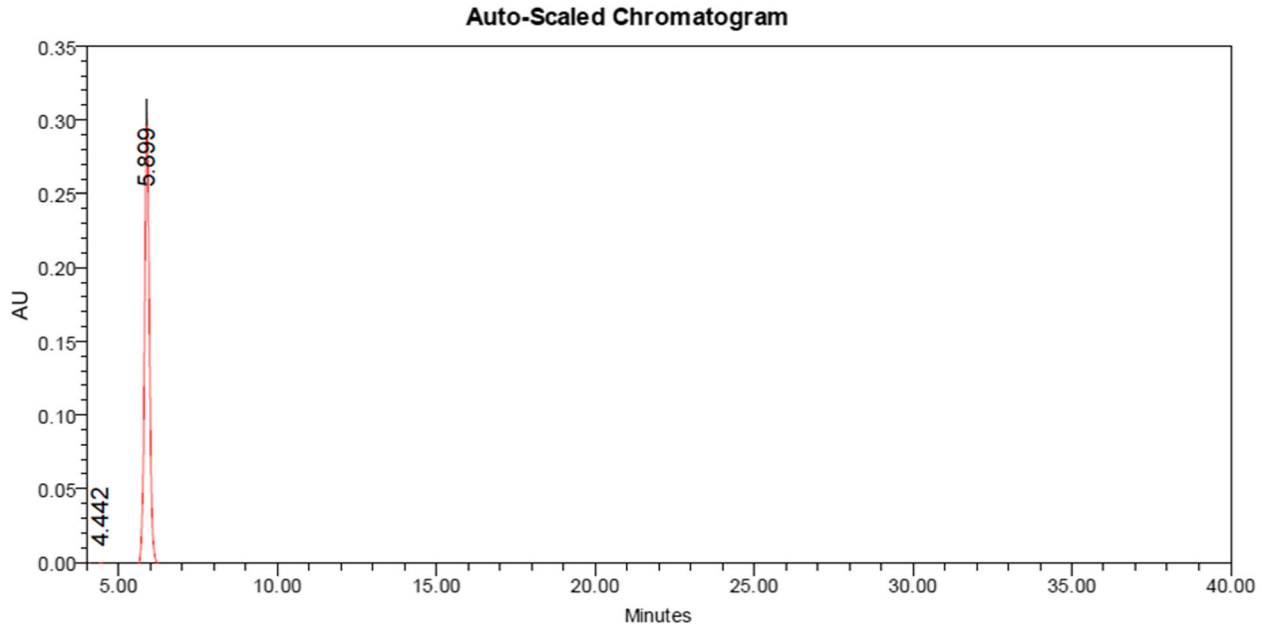

Fig. 2 HPLC chromatogram obtained by analysis of standard injection of Orlistat

Table 1 Mobile phase optimization using different solvents and their mixtures

Chromatographic parameters	Acceptance criteria	Results[a]					
Mobile phase		**A**	**B**	**C**	**D**	**E**	**F**
Methanol: buffer	–	65:44	70:29	75:24	80:19	85:14	90:9
Methanol: chloroform	–	65:44	70:29	75:24	80:19	85:14	90:9
Methanol : acetonitrile	–	65:44	70:29	75:24	80:19	85:14	90:9
2% phosphoric acid (%w/v)	–	1.0	1.0	1.0	1.0	1.0	1.0
Average retention time	–	7.8	7.2	6.7	6.2	5.9	5.5
Average tailing factor	0.8–1.5	1.6	1.36	1.24	1.13	1.02	1.23
Average number of theoretical plates	More than 2000	13,624	12,598	11,234	14,321	13,925	13,268

[a]Data represents mean ± S.D., $p < 0.001$ by two-way ANOVA

Spherisorb ODS2 column (250 × 4.6 mm) 5 μm and sample injection volume was 20 μl. The software control and data processing was made using LC solution version 1.2 [10].

Chromatographic conditions

The RP-HPLC method development for quantification of Orlistat was initiated with following columns, solvents, flow rate, and pH.

Effect of stationary phase The chromatogram was recorded using subsequent columns.

Hypersil ODS-2 columns (250 × 4.6 mm) 5 μm
Spherisorb ODS2 column (250 × 4.6 mm) 5 μm
Kromasil 100 C18 column (250 × 4.6 mm) 5 μm

Effect of solvent The three widely used organic modifiers for RP Chromatography methanol, chloroform, and acetonitrile were used.

Effect of pH The consequence of pH change on the chromatographic performance of the drug was studied by varying pH at 6.6, 6.4, 6.2, and 6 of respective mobile phase solvent system.

Effect of mobile phase ratio The chromatogram was recorded by using mobile phase containing various ratios of methanol, chloroform and acetonitrile.

Effect of 2 % phosphoric acid Varying amount of 2% phosphoric acid in small proportion was added to the mobile phase to improve the sharpness of the peak.

Effect of flow rate The flow rate 0.5, 0.7, 1.0, 1.2, 1.5 ml/min were used and chromatogram was recorded.

Preparation of standard solutions

A standard stock solution of Orlistat (1.00 mg/ml) was prepared by dissolving precisely weighing 100.00 mg of Orlistat in 100 ml of methyl alcohol. The stock solution was protected from light by covering the flask with aluminium foil and stored for about one week at 4 °C and was found to be stable throughout this period. The working standard solutions of 100.00 μg/ml and 10.00 μg/ml were prepared by further diluting the stock standard solution using methanol:acetonitrile:2% phosphoric acid (85:14:1 v/v). The secondary standard solutions were prepared from working standards (100.00 μg/ml) by diluting an aliquot of 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 ml in 5 ml volumetric flask respectively using the mobile phase to get 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00 μg/ml standard solutions. Further, 10.00 μg/ml was used to prepare 160.00, 320.00, 485.00, 640.00, and 800.00 ng/ml by properly diluting aliquot using methanol:acetonitrile:2% phosphoric acid (85:14:1 v/v) to find limit of detection (LOD) and limit of quantitation (LOQ) [11].

Preparation of Orlistat solid dispersion

Orlistat is a BCS class II drug with dissolution limited activity due to its low water solubility. Solid dispersion of Orlistat was prepared with poloxamer and mannitol to improve its dissolution profile and stability in the aqueous medium. The prepared dispersion was also tested by developed method to check the sensitivity of the HPLC method to detect the drug peak in presence of various excipients as solid dispersions were the formulations with generally the highest amount of excipients or carriers.

A mixture of Poloxamer and Orlistat (1:1 by weight) was wetted with a small amount of ethanol:water (1:1) and kneaded carefully for 30 min in a glass mortar pestle [12]. The paste produced was desiccated under vacuum for about 24 h. The obtained dried mass was passed through sieve no. 120 and then mixed with mannitol thoroughly to get a freely flowing powder mixer. Every formulation equivalent to 120 mg of Orlistat was packed into size '3' hard gelatin capsule [13] and stored in dessicator till further evaluation.

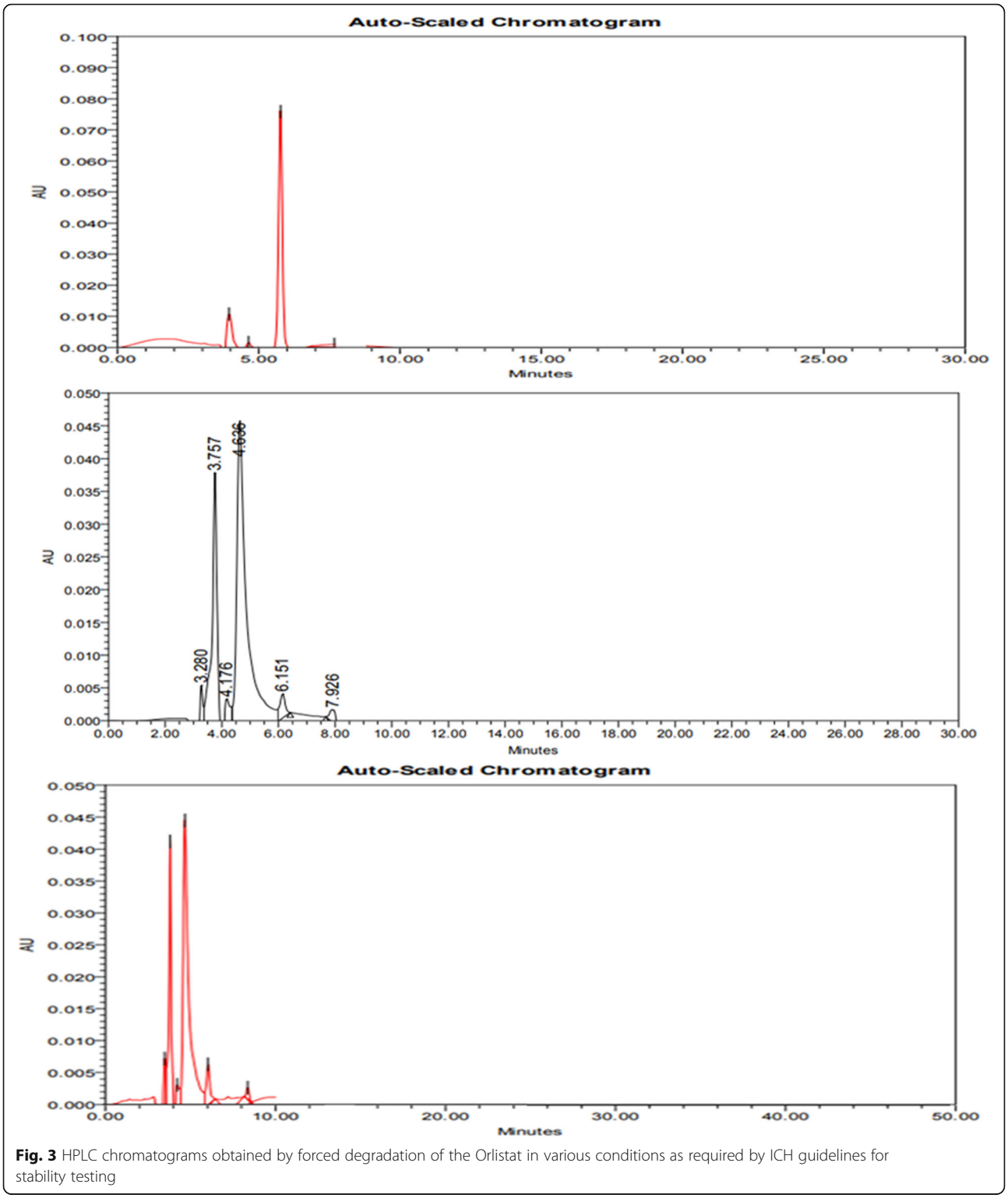

Fig. 3 HPLC chromatograms obtained by forced degradation of the Orlistat in various conditions as required by ICH guidelines for stability testing

Preparation of sample solution

Twenty capsules each of prepared formulation and Xenical (marketed formulation) were weighed and opened then all contents are mixed. An aliquot of powder equivalent to 120 mg of Orlistat was precisely weighed and transferred to 100 ml volumetric flask and volume was make up with HPLC grade methyl alcohol. The volumetric flasks were sonicated for about 30 min for complete extraction of drug. Suitable aliquots of the solution were

Table 2 Statistical data of calibration curves of Orlistat

Parameters	Orlistat
Linearity	1.00 to 10.00 μg/ml
Regression equation	98056x-19122
Standard deviation of slope	0.023
Relative standard deviation of slope (%)	0.045
Standard deviation of intercept	0.136
Correlation coefficient (*r*)	0.9997

filtered using 0.45 μm nylon filter. Appropriate amount of filtered solution were transferred to the tubes and centrifuged at 2500 rpm for 15 min. Initial supernatant volume of 1 ml was discarded, and then about 1 ml was properly diluted with methyl alcohol and then samples were analyzed by HPLC [14].

Method validation

The analytical method validation for the estimation of Orlistat in the formulation and bulk drug was done as per ICH Q2 (R1) guidelines with respect to specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, robustness, and system suitability [15].

Specificity

Specificity was the capability of the method to quantify the analyte in the presence of components which may be accepted to be present. Normally, these consist of impurities, degradants, matrix, etc. [16]. In order to determine specificity, the Orlistat capsules (prepared formulation and marketed formulation) and Orlistat powder were stressed under various conditions to perform forced degradation studies. For all the series, API 1 mg/ml and capsule contents equivalent to 120 mg of Orlistat were taken and prepared for analytical validation.

- Preparation of acid and base induced degradation product

To 10 ml of prepared stock solution, 10 ml each of 0.1 N HCl and 0.1 N NaOH were added separately. These mixtures were refluxed for 8 h in the dark in order to keep out the probable degradative effect of light.

- Preparation of hydrogen peroxide induced degradation product
  To 10 ml of prepared stock solution, 10 ml of 3.0% hydrogen peroxide was added. The solution was heated in a boiling water bath for 10 min to completely eliminate the surplus of hydrogen peroxide and then refluxed for 8 h in the dark in order to keep out the possible degradative effect of light.
- Preparation of wet heat induced degradation product
  The stock solution was refluxed with water for 8 h for wet heat induced degradation. Refluxing was done in the dark in order to keep out the possible degradative effect of light.
- Preparation of photochemical degradation product
  The photochemical stability of the drug was studied by exposing the Orlistat formulation (prepared and marketed) powder and API powder to direct sunlight for 48 h in the open air.
  A paired two-tailed *t* test was done to evaluate the concentration signals obtained for the six standards at t = 0 h with those obtained at t = 8 h. To evaluate the stability of formulations containing Orlistat were analyzed instantaneously after preparation and after 8 h storage at stressed conditions and concentrations were estimated by comparing the relative error (Er), formula as Eq. (1), with injector repeatability relative standards deviation.

$$\mathrm{Er} = (C_8 - C_0)/C_0 \times 100 \quad (1)$$

Where C_8 was the concentration after 8 h storage and C_0 was the concentration before storage [17].

Linearity and range

The linearity of a method was its capability to get the results which are straightway proportional to the concentration of analyte in the sample. For the estimation of linearity and range, solutions of different concentration of Orlistat containing 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, and 10.00 μg/ml were injected six times per concentration. Area under the curve for each concentration was observed and a graph of Orlistat concentration versus area under the curve was prepared [18].

Method of least squares

Procedure of developing a calibration graph preferably requires a linear relationship between the various responses and the least square method is ideally used for fitting the obtained data into a linear model.

The relationship between the concentration of drug (x, independent variable) and the absorbance of UV (y, dependent variable) can be represented by least square regression analysis as:

$$Y = f(x, a, b_{\mathrm{i}}.....b_{\mathrm{n}})$$

Where a, b_{i}.....b_{n} are the parameters of the function.

The data of unknown parameters must be evaluated in a way the linear regression model fit the experimental value points (X_i & Y_i) as nearly as possible.

If $Y_i = \alpha + \beta X_i + e_i$

The e_i represents residuals and a,b are the values for intercept and the slope of the graph given by the Eqs. (2) and (3).

$$b = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - \left[\sum_{i=1}^{n} x_i\right]^2} \tag{2}$$

$$a = \frac{\sum_{i=1}^{n} y_i \sum_{i=1}^{n} x_i^2 - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i y_i}{\sum_{i=1}^{n} x_i^2 - \left[\sum_{i=1}^{n} x_i\right]^2} \tag{3}$$

Evaluation of standard error (S_e)

It is the estimation of the difference between the obtained values and the calculated values of the dependent variable given by Eq. (4).

$$Se = \sqrt{\sum_{i=1}^{n} \left(y_i - y_p\right)^2 \Big/ (n-2)} \tag{4}$$

y_i and y_p are observed values and predicted values.

Standard deviation of slope (S_b) can be estimated by the formula as given by Eq. (5):

$$Sb = \sqrt{\frac{\sum_{i=1}^{n} \left(y_i - y_p\right)^2}{(n-2)}} * \sqrt{\frac{1}{\sum_{i=1}^{n} \left(x_i - x_p\right)^2}} \tag{5}$$

where x_p and y_b are the arithmetic mean value of x_i and y_i

Standard deviation of intercept (S_a) can be given Eq. (6)

$$Sa = \sqrt{\frac{\sum_{i=1}^{n} \left(y_i - y_p\right)^2}{(n-2)}} * \sqrt{\frac{1}{\sum_{i=1}^{n} \left(x_i - x_p\right)^2}} * \sqrt{\frac{\sum_{i=1}^{n} x_i^2}{n}} \tag{6}$$

Correlation coefficient (r)

It is used to confirm the linear association between the absorbance and the concentration of the Orlistat. The value of '*r*' can be obtained by applying the Eq. (7)

$$r = \frac{\left[\sum_{i=1}^{n} (x_i - x_p)(y_i - y_b)\right] \Big/ (n-1)}{\left[\sum_{i=1}^{n} (x_i - x_p)^2 (y_i - y_b)^2\right] \Big/ (n-1)^2} \tag{7}$$

Ordinary least square (OLS) can give prompt factually mistaken outcomes for heteroscedastic information; both OLS and weighted least square (WLS) were tried for heteroscedasticity by F test. In light of the connection acquired among F_{critical} and $F_{\text{statistic}}$ ($F_{\text{critical}} > F_{\text{statistic}}$), WLS relapse investigation was performed on various weights (w_i). Percent relative error (% Er) and complete percent relative error (Σ%Er) were resolved for each model of various weights and model with least Σ%Er was chosen [18]. Scope of created diagnostic technique was resolved dependent on plot got for top zone against concentration and reaction factor against focus for each alignment standard. Further, percent RSD of got reaction factor was resolved to set up proper range [19].

Accuracy

Analytical method accuracy represents the nearness of the values between the standard value and the obtained value. As confirmed by ICH, accuracy gives information regarding the distinction between the mean value and the true value. For the validation of analytical method, there are two practicable methods the estimation of the accuracy, first one was absolute method and the second one was comparative method [20].

Accuracy of the method was evaluated by carrying out the recovery studies at three different levels. The already estimated samples were spiked with additional 50, 100, and 150% of the standard Orlistat and the samples were reanalyzed by the developed method. The practical was performed in triplicate for finding the percent recovery of the Orlistat at different levels in the formulations [21].

Precision

The analytical procedure precision represent the proximity of agreement involving in a series of estimation obtained from manifold sampling of the identical sample under the approved conditions. It represents the 'reproducibility' of the method and the most familiar statistical

terms used for the precision was standard deviation (SD) [21]. The equation for SD was given by Eq. (8):

$$\sigma=\sqrt{\frac{\sum(x_i-\mu)2}{N}} \tag{8}$$

σ =	Sample standard deviation
N =	The size of the sample
x =	Each value from the sample
μ =	The sample mean

The square of the standard deviation is known as Variance. RSD stands for relative standard deviation and also called coefficient of variance. Percent relative standard deviation is given by Eq. (9):

$$\%\mathrm{RSD} = (\sigma/\mu)*100 \tag{9}$$

Precision was considered at two level, i.e., intraday and interday precision. Method repeatability was estimated by six replicate injections and six fold estimation of 5.0 μg/ml concentration. The intra- and inter-day variation for the estimation of Orlistat was performed at three different concentration levels of 2.5, 5.0, and 7.5 μg/ml, respectively [22].

Estimation of precision and accuracy

Student t test

Student *t* test was usually used to evaluate the means of two correlated samples estimated by standard and test methods. It also gives response to the rightness of the null hypothesis within a limit of confidence from 95 % to 99%.

$$t = \frac{d_r}{S_D \Big/ \sqrt{n}} \tag{10}$$

Where,
d_r = X_r (standard method) − X_t. (Test method)
S_D was standard deviation

F test

F test is usually used to test the importance of the difference in variances of standard and test methods. If one preformed n_1 replicate observations by test methods and *n*2 replicate observations by means of standard method, then S_T^2 and S_R^2 will not differ extremely if null hypothesis is correct. The *F* ratio can obtain by Eq. (11) as:

$$F = S_T^2/S_R^2 \tag{11}$$

where S_T^2 = variance of the test method
S_R^2 = variance of reference method
If the value of *F* is less than unity, then procedures used are not significantly different in precision in a given confidence interval [23].

Limit of detection and limit of quantitation

The detection limit of an analytical procedure was the minimum quantity of drug in a sample which can be identified and the quantitation limit of an analytical methodology was the minimum quantity of drug present in a sample which can be quantitatively estimated with reasonable precision and accuracy [17].

The limit of detection (DL) can be calculated by Eq. (12) as:

$$\mathrm{LOD} = 3.3\ \sigma/S \tag{12}$$

where σ = standard deviation
S = slope of the standard curve
The limit of quantitation (LOQ) can be calculated by Eq. (13) as:

$$\mathrm{LOQ} = 10\ \sigma/S \tag{13}$$

The slope *S* was determined from the equation of the standard curve of the drug (Mohammadi et al. 2006).

Robustness

For any analytical experimentation, the robustness represents the proportion of its ability to stay unaffected by little changes in the system; however, conscious variations in technique parameters gives a sign of its unwavering quality during typical utilization. To evaluate the HPLC method robustness, a few parameters were deliberately varied. Thus, pH of the mobile phase, column temperature, and flow rate were varied. By introducing small changes in the established parameters, the effects on the results were examined [24].

System suitability

So as to confirm the appropriateness of chromatographic framework for planned investigation, system suitability test was performed by six duplicate injections of standard solutions of Orlistat [25]. The various parameters used for testing system suitability were as follows.

- Number of theoretical plates (*N*)
  According to sigma or tangential method column, efficiency can be evaluated by degree of peak dispersion as column characteristics. The formula for calculation *N* can be given by Eq. (14) as:

Table 3 Test for homogeneity of variance, *F* test

Concentration (ng/ml)	Responses	Variance (s^2)	$F = S_2^2/S_1^2$
1000	87,674	25,032,694.67	71.69
	91,545		
	99,686		
	97,468		
	98,564		
	90,237		
10,000	24,63,652	1,794,832,656	
	24,56,843		
	25,46,753		
	24,73,896		
	24,39,569		
	25,26,547		

$F_{critical}$ value is greater than $F_{statistic}$ value, therefore responses are heteroscedastic

$$N = 16\,(\mathrm{V}/\mathrm{T})^2 \tag{14}$$

where N = no. of theoretical plates
V = retention time
T = width of the peak

- Height equivalent to theoretical plate (HETP) was estimated by column length and no. of theoretical plates in the column in which each plate was considered to be of a fixed height, given by Eq. (15) as:

$$\mathrm{HETP} = L/N \tag{15}$$

where L = length of column
N = plate number

- Resolution
  Resolution was used to check the ability of the column to differentiate two drugs in separate individual peaks. Resolution can be estimated using the Eq. (16) as:

$$Rs = (t_{R2} - t_{R1})/0.5\,(t_{w1} + t_{w2}) \tag{16}$$

where t_{R1} and t_{R2} were the retention times of two peaks
t_{w1} and t_{w2} were the baselines lying between the tangents drawn to the sides of the peaks [21].

- Tailing factor (A_s)
  Tailing factor represents the information regarding peak symmetry and it was calculated using the formula (17):

$$A_S = B/A \tag{17}$$

where B = distance from the midpoint to the trailing edge
A = distance from the leading edge to the midpoint

- Relative standard deviation (RSD) also known as coefficient of variance [22] used for measuring the precision of the average of obtained values, can calculated using the Eq. (18).

$$\mathrm{RSD} = (\mathrm{SD}{*}100)/X \tag{18}$$

Where SD = standard deviation
X = mean of the obtained data

Method applicability

The dissolution of Orlistat capsules was performed out by eight-station USP type II dissolution rate test apparatus with dissolution media of 3% sodium lauryl sulfate and 0.5% sodium chloride in water to which added 1–2 drops of *n*-octanol, and adjusted with phosphoric acid to a pH of 6.0 at 37 ± 0.5 °C with paddle rotation speed of

Table 4 Regression equation, correlation coefficient, and sum of the relative errors for each weighted and unweighted factor

Model no.	W_i	WLS/LS regression equation	Correlation coefficient (*r*)	Σ% RE
1	Unweighted	$y = 96732x - 16734$	0.9997	65.45
2	$1/\sqrt{x}$	$y = 98963x + 32786$	0.9994	51.32
3	$1/x$	$y = 96289x - 18764$	0.9993	44.57
4	$1/x^2$	$y = 99726x + 9978$	0.9996	34.65
5	$1/\sqrt{y}$	$y = 98786x + 16783$	0.9993	57.45
6	$1/y$	$y = 95638x - 18976$	0.9998	56.32
7	$1/y^2$	$y = 97863x - 26134$	0.9995	39.67

Table 5 Intra and inter assay precision data (n = 6)

Actual concentration (μg/ml)	Measured concentration (μg/ml), ± RSD (%)	
	Intra day	Inter day
2.5	2.48, 2.02	2.61, 2.86
5	5.1, 1.64	5.77, 2.82
7.5	7.48, 1.78	7.73, 2.69

75 rpm for 45 min [26]. Aliquots of 5 ml were removed at different time intervals (5, 10, 20, 30, and 45 min) and replaced with corresponding amount of fresh dissolution media. The obtained samples were then diluted further with phosphate buffer of pH 6 and were centrifuged at 2500 rpm for 15 min and filtered through 0.45 μm nylon filter. The obtained samples were appropriately analyzed by validated HPLC method [27].

Statistical data analysis

Statistical analysis of the obtained values was performed using student t test and all the observations were expresses as mean ± standard deviation recovered from three different experiments (n = 6).

Results

Optimized conditions

The RP-HPLC method developed for quantification of Orlistat on Spherisorb ODS2 column (250 × 4.6 mm) 5 μm giving good peak symmetry for the basic analyte. All samples were tested with separate solvent systems in isocratic mode at room temperature.

- Mobile phase optimization: First, phosphate buffer (pH 6) with organic methanol tested as an aqueous step, and then chloroform methanol was used in several ratios, but none of them had the right peak of Orlistat eluted. Afterwards, mobile phases consisting of different ratios of methanol and acetonitrile were used in the study of Orlistat and analyzed for multiple chromatographic parameters as seen in Table 1.
- Retention time optimization: The retention time found to be decreased, as the ratio of acetonitrile in the mobile phase increased but this tends to increase the peak tail factor. Thus, the mobile phase with ratio of 85:15 (methanol: acetonitrile) was selected to give appropriate peak.
- Peak optimization: Improvement in the peak sharpness was obtained by adding 2 per cent phosphoric acid in limited quantities with a 5.9 min retention period and spectrophotometric identification at 215.0 nm. The final moving step has been methanol, acetonitrile, and 2% phosphoric acid at ratio 85:14:1 bv/v. Before the study of Orlistat samples, the column was saturated for 2 h with mobile level.

Method validation

The developed method was validated for a range of parameters as per the guideline of ICH Q2R(1) [11].

Specificity

The optimized method provided the Orlistat peak without the presence of any interference from any other peaks relating to the formulation excipients, dissolution media ingredients, or any other contributing factor as shown in Fig. 2. In testing the specificity, the dissolution media alone and a solution containing only the excipients and phosphate buffer (pH 6) were tested on the HPLC and it was found that these solutions do not present any peak with the same retention time of Orlistat. The appropriate resolution between the Orlistat and disso media peak was found, which established the specificity of the developed method.

Similarly, no interference of various degradants was found through the stressed degradation studies. It was found that Orlistat was stable under alkaline conditions with retention time of 5.87 ± 0.05 min and 39% decomposition on the other hand it was found unstable under the acidic conditions resulting in 78% decomposition. The analyte was found relatively more stable to oxidation with only 28% decomposition but showed extensive hydrolytic degradation with various degradation product like 5-(2-Formylamino-4-methyl-pentanoyloxy)-2-hexyl-3-hydroxyhexadecanoic acid or 3-Hexyl-4-(2-hydroxy-tridecyl)oxetan-2-one. Further, Orlistat showed better stability upon light exposure. Thus the RP-HPLC method developed was observed to be specific for the evaluation and quantification of Orlistat. Percentage recovery and number of degradants peaks during forced degradation was shown in Fig. 3.

Table 6 Robustness results at different conditions

Condition	Recovery % (±SD)		
	2.5 μg/ml	5 μg/ml	7.5 μg/ml
HPLC 2	97 ± 2.45	98 ± 2.76	101 ± 1.67
Mobile phase with composition 84:15:1 (methanol, acetonitrile and 2% phosphoric acid)	99 ± 3.51	101 ± 2.12	102 ± 2.73
Flow rate 1.2 ml/min	98 ± 4.78	99 ± 3.85	104 ± 3.76

Table 7 System suitability parameters

Parameter	Result ± RSD (%)
Retention time (min)	5.9 ± 0.005
HETP	25.7 ± 0.67
N	13672 ± 128
Tailing factor	0.98 ± 0.003

Linearity and range

The calibration curve was constructed for Orlistat and linearity was evaluated by least square linear regression analysis for all the six calibration curves (as shown in Table 2). The curves were found to be linear in the concentration range of 1.00 to 10.00 μg/ml. The linear regression equation for the calibration curve was found to be Eq. (19) as:

$$Y = 98056x - 19122 \tag{19}$$

where y represents the area under the curve and x represents the concentration (μg/ml) of the Orlistat.

The RSD value provides information about the smaller or larger deviation of the data compared to the mean value. For example, the RSD value between 10 and 20 was considered to be good as data was slightly spread out and the RSD value of 10 or less than 10 indicates the tight clustering of data around the mean, which was considered as very good and the responses were considered to be linear. As the percent RSD was found to be 4.5%, then it shows the linearity of the developed method, but the OLS regression analysis was quiet appropriate for homoscedastic data, for that reason obtained responses were tested for heteroscedasticity by F test (shown in Table 3). Since the developed RP-HPLC method had to be utilized for assessment of Orlistat from different solid dispersions and formulations as well as dissolution medium, a broad calibration range was advantageous. That is why, WLS regression equation, regression coefficient (r^2), and Σ% RE for every weighted factor were obtained and shown in Table 4.

Accuracy and precision

The analytical method accuracy represents the proximity of test results to true value. On the whole percentage recovery for three diverse concentrations levels 2.5, 5.0, and 7.5 μg/ml, ranged from 99.76 to 100.98 with confidence interval ranging from ± 0.218 to ± 0.653 presenting that any minute change in the drug concentration can be precisely estimated with high accuracy.

Precision signifies the effects of random errors on the repeatability and reproducibility of the optimized method on the estimation of the drug. Precision was investigated by injecting six replicate samples of 2.5, 5.0, and 7.5 μg/ml in the same day and for interday study the same three concentrations were injected six consecutive days with % RSD as shown in Table 5. The optimized method ruggedness was estimated by evaluation of intra and inter-day results for Orlistat obtained by two analysts.

LOD and LOQ

Under the experimental conditions employed, LOD and LOQ were calculated from standard deviation and slope of the standard curve according to the formula (xii) and (xiii). The LOD was found to be0.06 μg/ml and LOQ was found to be 0.2 μg/ml with a resultant % RSD of 0.3% (n = 6).

Robustness

The evaluation of robustness was studied by changing the analytical instrument from HPLC Waters to HPLC Shimadzu. This change led to shift in the retention time of peak to 5.2 ± 0.06 min for Orlistat with correlation coefficient of 0.999 with less than 2% RSD value. By slight change in various chromatographic parameters (as shown in Table 6) like mobile phase composition, flow rate (1 to 1.2) and instrument the percent recovery of Orlistat was found to be 97 ± 2.45% to 104 ± 3.76%.

System suitability

System suitability for an analytical method can be tested by checking of a system, before or during analysis of the drug samples, to ensure system performance. A data from six replicate injections of standard solution of Orlistat were utilized for estimating suitability parameter like %RSD, tailing factor, and theoretical plates. The % RSD value for all was found to be less than 2% (Table 7).

Method applicability

In vitro release profile of Orlistat from solid dispersion and from marketed formulation was carried out in dissolution media (as per USP) of 3% sodium lauryl sulfate and 0.5% sodium chloride in with 1–2 drops of n-octanol, and adjusted with phosphoric acid to a pH of 6. The rotation speed of paddle was 75 rpm and aliquots of 5 ml were removed at different time intervals (5, 10, 20, 30, and 45 min) and diluted further with phosphate buffer of pH 6. Around 80% Orlistat was released from solid dispersion formulation and 65% Orlistat

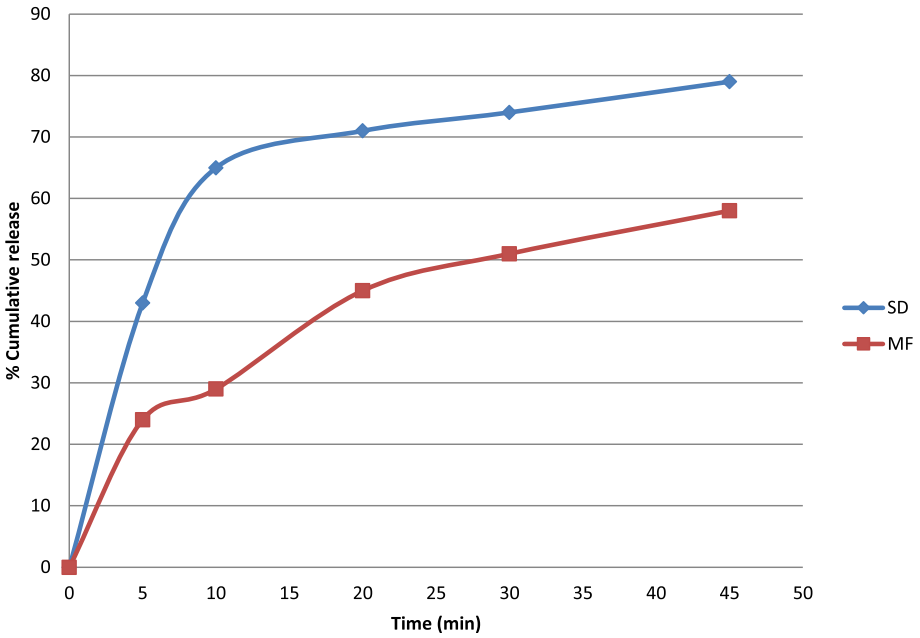

Fig. 4 Cumulative in vitro release of Orlistat form prepared solid dispersion. *SD represents prepared Orlistat solid dispersion and MF represents marketed formulation

was released from marketed formulation with no interference from dissolution media and formulation components (as shown in Fig. 4). Thus, it can be stated that the developed RP-HPLC method could be efficiently utilized to assess the in-vitro release of Orlistat.

Discussion

This research work focused on the development of an appropriate stability indicating RP-HPLC method with its succeeding validation for the estimation of Orlistat in any formulation with various excipients using Spherisorb ODS2 column (250 × 4.6 mm) 5 μm and PDA detector [28]. All the samples were analyzed in isocratic mode at room temperature with various solvent systems. Firstly, phosphate buffer (pH 6) as aqueous phase with methanol as organic phase then methanol with chloroform was used in several ratios but none of them eluted Orlistat with proper peak. Thereafter, mobile phase comprising of methanol and acetonitrile of various ratios were used for the analysis of Orlistat and evaluated for several chromatographic parameters like retention factor, theoretical plates, retention time, tailing factor, etc. As the ratio of acetonitrile in the mobile phase is increased, the retention time was found to decrease but the peak tailing factor seemed to increase which was not acceptable. So the mobile phase with ratio of 85:15 (methanol:acetonitrile) provided better peak with adjusted flow rate of 1 ml/min. Improvement in peak sharpness was made by adding 2% phosphoric acid in small amount (the final mobile phase became methanol, acetonitrile, and 2% phosphoric acid in the ratio of 85:14:1 v/v/v) with retention time of 5.9 min and spectrophotometric detection at 215.0 nm. The column was saturated for 2 h with mobile phase before the analysis of Orlistat samples.

Most analytical method reported for the analysis of Orlistat are aligned with mass spectrophotometer with difficult sample analysis and time consuming making it tedious during routine analysis [29]. Rest of the other methods has limitations like improper peak resolution and very less reproducibility; moreover, none of the method emphasis over statistical interference of the obtained data [6]. Our method tested for the all the possible experimental and statistical tests to prove its certainty and relationship between different variables. While examining each variable and its related equations, we developed an effective method with good sensitivity, accuracy, and reproducibility for rapid analysis of the samples.

The RP-HPLC method could be suitably utilized for the analysis of drug content and in vitro release from solid dispersions or marketed formulations. The retention time of 5.9 min allowed analysis of large samples in shorter period of time with sharp peak estimation without any interference from the formulation excipients and release media.

Conclusion

A validated stability indicating RP-HPLC analytical method was developed [as per ICH guideline Q2(R1)] for the estimation of Orlistat in bulk powder and different pharmaceutical dosage forms by testing various validation parameters. The selected method is simple, accurate, precise, and specific and can estimate the Orlistat in presence of its degradation products without any interference. The results undoubtedly demonstrate the ruggedness of the optimized method and its system suitability. The method developed eliminates extraction steps thus reduce analytical time, cost and minimize the extraction errors. Thus the described procedure is suitable for the routine analysis of Orlistat in various formulations and API.

Abbreviations

ICH: International Conference on Harmonization; TFA: Trifluoroacetic acid; ODS: Octadecyl-silica; USP: United States Pharmacopoeia; Er: Relative error

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Authors' contributions

KS done the HPLC analysis with interpretation of the data and was the major contributor in writing the manuscript. All authors have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission. We confirm that the order of authors listed in the manuscript has been approved by all named authors.

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Availability of data and materials

The research work has been carried out by us, and we assure you that it can be provided to you whenever required.

Ethics approval and consent to participate

Not applicable because study does not involve any human or animal data.

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts or competing of interest.

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