Quantification of Orlistat by a Validated, Simple and Sensitive High Performance Thin Layer Chromatographic-Densitometric Assay Method

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Abstract: *The Objective of the current study was quantification of orlistat by a validated, simple and sensitive high performance thin layer chromatographic-densitometric assay method. Separation of Orlistat was carried out on silica gel 60F254, 10×10 cm^2 thin layer chromatography (TLC) plates with 6 mm band length and 10 µl injection volume. Development chamber was saturated for 30 min. prior. Ascending chromatography TLC plate developed using mobile phase containing n-hexane, ethyl acetate, glacial acetic acid (7:3:0.1 v/v/v) respectively in glass twin-trough development chamber. The plates were developed up to a distance of 80 mm at temperature of 25°C and dried with hair drier at 50°C. Quantification was carried out with CAMAG TLC Scanner 3 operated by WinCats software using a deuterium lamp. The linear regression equation for Orlistat was $y = 847.15x + 3557.3$ with correlation co-efficient 0.9984. The LOD value for Orlistat was found to be 1.14 µg/ml, and the LOQ value 3.81 µg/ml. The mean recovery for Orlistat was 99.13-99.92%. The %RSD values for intermediate and method precision study was 0.017% and mean % assay was 99.79% as well as absolute difference between mean % assay values of method precision and intermediate precision found 0.04 % for Orlitat. The effects of such deliberate changes on peak area and % assay were calculated, which found 99.60 % to 100.12 %. as well as % RSD for retardation factor found 0.67%. There was no indication of compound instability in the sample solution was found through the study.*

Keywords: *orlistat; high performance thin layer chromatographic; densitometric assay method; LOD value; Method validation; Assay Method*

1. INTRODUCTION

Obesity is medical condition of human in which execs body fat store in body to extant that it may pose bad effect on human health [1]. Person with Body mass index which is the ratio of person's weight to the square of his/her height, is over 30 kg/m^2 is considered obese generally people with body mass index between 25–30 kg/m^2 defined as overweight [1]. Obesity in human increases the tendency of various diseases especially heart decease, diabetes type 2, obstructive sleep apnea, certain class of cancer, and osteoarthritis.[2]

Obesity is combination of excessive food in regular intake, no any physical activity and genetic susceptibility [1][3].some cases observed due to the mental illness, genes, endocrine disorders [4]. Obese people require more energy to maintain increased body mass as compare to other peoples. [5][6]

Obesity must be preventable through social change and personal choice. [1] Change in regular diet and exercise is main treatment for obesity. [2] Reducing high sugar and fat containing food, and increasing intake of fiber in regular diet, quality of diet can increases.[1]combination of medicinal treatment and suitable diet is also taken to reduce appetite or decrease fat absorption.[7]

2. ORLISTAT

Orlistat is drug which is used for the treatment of obesity and it sold in market as a prescription drug with tread name Xenical by in most of nations, also sold in UK with name alli by GlaxoSmithKline. It act as lipase inhibitor and prevents reducing of fate from human diet thus calorie intake is reduces [8].
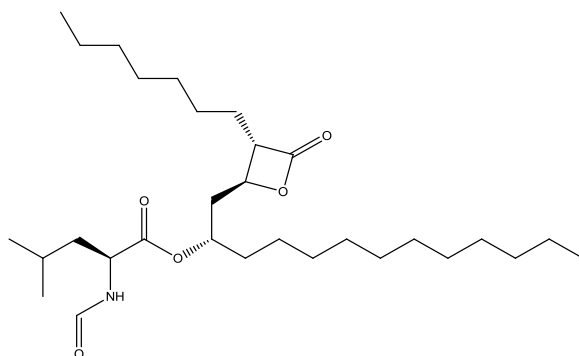
Figure1. *Chemical structure of Orlistat*

Orlistat is derivative of lipstatin with high potency of pancreatic lipases which is isolated from bacterium Streptomycestoxytricini [9]. Due to its simplicity and stability from others lipstatin, it was chosen as an anti obesity drug. [10]

Orlistat is useful for the various treatments such as weight loss [11] and as a secondary effect it reduces the blood pressure. Its preventive effect for the type 2 diabetes is also reported [12].

Each drug has positive and negative effects too. Orlistat is notorious due to its gastrointestinal side effect which can include steatorrhea, which are decreases with time and reported as adverse effect of drug. [12]

3. LITERATURE REVIEW

The literature reviews regarding Orlistat suggested that various analytical methods are reported for stability of Orlistat in pharmaceutical formulations and in various biological fluids by using various analytical techniques as UV, HPLC, UPLC, and TLC method. The literature reviews regarding these methods are as under.

4. EXPERIMENTAL

4.1. Chemicals and Reagents

An active pharmaceutical ingredient (API) working standard of Orlistat was gifted by Dr. Y. T. Naliapara, Assistant Professor, Department of Chemistry, Saurashtra University, Rajkot.

HPLC grade Dichloro methane (MDC), ethyl acetate, glacial acetic acid and n-hexane were procured from Merck India Limited, Mumbai, India. High purity deionised water was prepared using Milli-Q (Millipore, Milford, MA, USA) water purification system. Nylon syringe filters 0.22 μm were from Millex-Hn (Mumbai, India). TLC plates used were obtained from Merck India Limited, Mumbai, India.

4.2. Instrumentation

CAMAG HPTLC system used for quantitative analysis consisted of 100 μl sample syringe (Hamilton, Switzerland), CAMAG Linomat V applicator (Camag, Switzerland), CAMAG glass twin-trough chambers (20 × 10 × 4 cm^3); TLC plate visualizing chamber and CAMAG TLC scanner III. Chromatographic data Equalization and instrument control were done using WinCATS software (V 1.4.2, CAMAG). The source of radiation was a deuterium lamp emitting a continuous UV spectrum in the range 190–400 nm.

4.3. Mobile Phase Preparation

The mobile phase was made of, n-hexane, ethyl acetate, glacial acetic acid (7:3:0.1 v/v/v)

4.4. Diluents Preparation

Dichloro methane used asdiluents.

4.5. Standard Preparation

Standard stock solution containing Orlistat 2000μg/mlwas prepared by taking 20.0mg Orlistat in 10 ml volumetric flask, 5ml of diluents (methylene dichloride) was added, sonicated and cooled to room

temperature. The solution was diluted to the mark with diluents. Standard solution containing Orlistat (200 µg/ml)   was prepared by pipetting 1 ml stock solution into a 10 ml volumetric flask and diluted up to the mark with diluents.

4.6. Chromatographic Conditions

Separation of Orlistat was carried out on silica gel 60F254, 10×10 cm^2 thin layer chromatography (TLC) plates with 6 mm band length and 10 µl injection volume. Development chamber was saturated for 30 min. prior. Ascending chromatography TLC plate developed using mobile phase containing n-hexane, ethyl acetate, glacial acetic acid (7:3:0.1 v/v/v) respectively in glass twin-trough development chamber. The plates were developed up to a distance of 80 mm at temperature of 25°C and dried with hair drier at 50°C. Quantification was carried out with CAMAG TLC Scanner 3 operated by WinCats software using a deuterium lamp.

5. RESULTS AND DISCUSSION

5.1. Development and Optimization of the HPTLC Method

An analytical method based on TLC using densitometric detection was developed and validated for assay determination of Orlistat API. The analytical conditions were selected, keeping in mind the different chemical nature of Orlistat. The chemical and physical parameters e.g. solubility, pH, polar or nonpolar nature of the compound and peak wavelength. According to the literature review, the λ max values of were reported 190-220 nm. For the multi-component analysis of the drugs, most suitable wavelength was chosen; considering the maximum response were observed at 208 nm.

The mobile phase selection has been done on the basis of resolution, Symmetry of peak, spot definition sensitivity and day-to-day reproducibility of the retardation factor and intensity or peak height and peak area Orlistat peaks. After evaluating all these factors, Nonpolar and polar solvent mixture was found to be giving satisfactory results. Initial trial was taken using methanol and chloroform as a mobile phase but we didn't get good peak shape (Trial 1, fig.2).
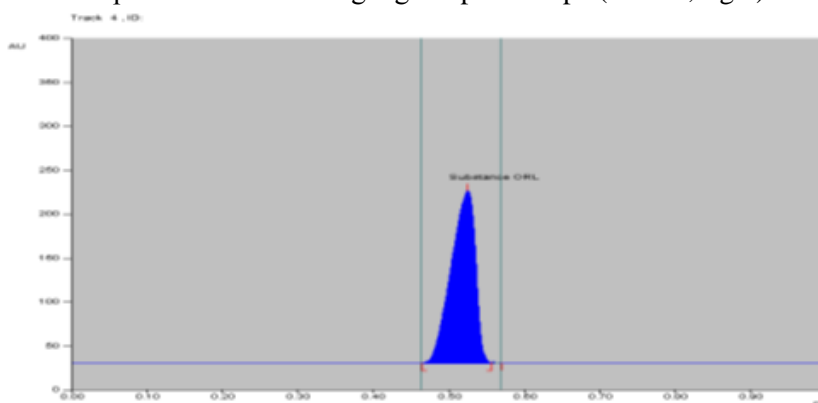

Figure2. *Chromatogram of method development (Trial 1)*

When chloroform was replaced by ethyl acetate the peak shape and little separationof drug was improved**.** (Trial 2, fig.3)
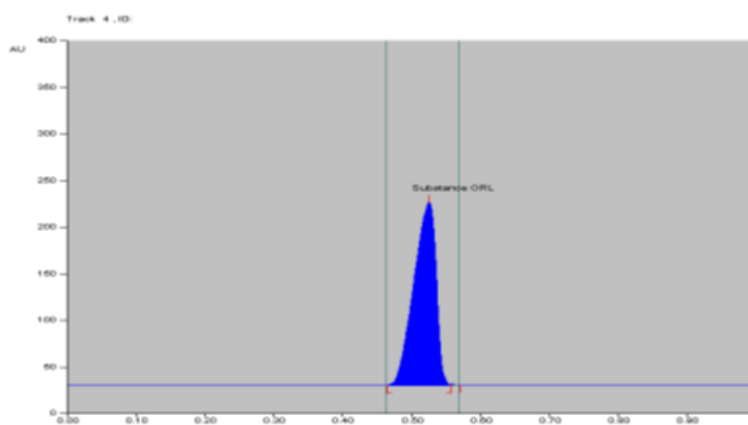

Figure3. *Chromatogram of method development (Trial 2)*

After so many trial of method optimization, Orlistat was found suitable for faster development, resolution and peak shape of both components. Finally, the mobile phase composition consisted of a mixture of Hexane: Ethyl acetate: Glacial acetic acid (7.0:3.0:0.1 v/v/v). Optimized mobile phase proportion was providing good resolution between Orlistat. For the selection of polar organic constituent of mobile phase, Ethyl acetate was chosen to increase the retardation factor and to attain good peak shape with resolution. Figure (4 and 5) represents the chromatograms of standard and test preparation, respectively. The final optimized chromatographic conditions are given in the Table 1.
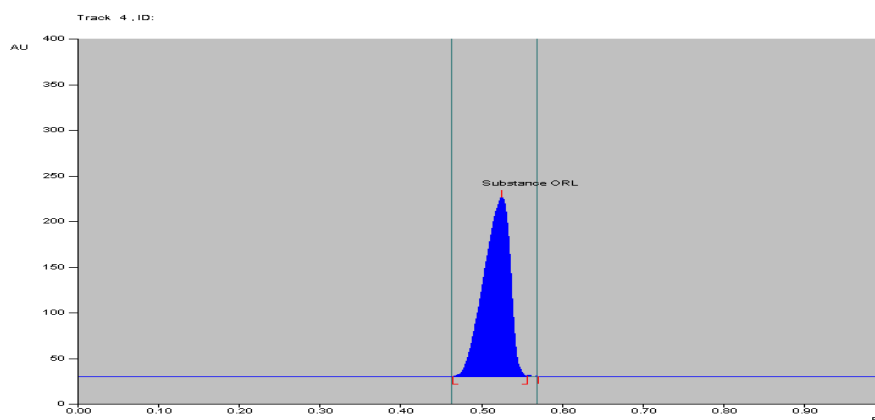

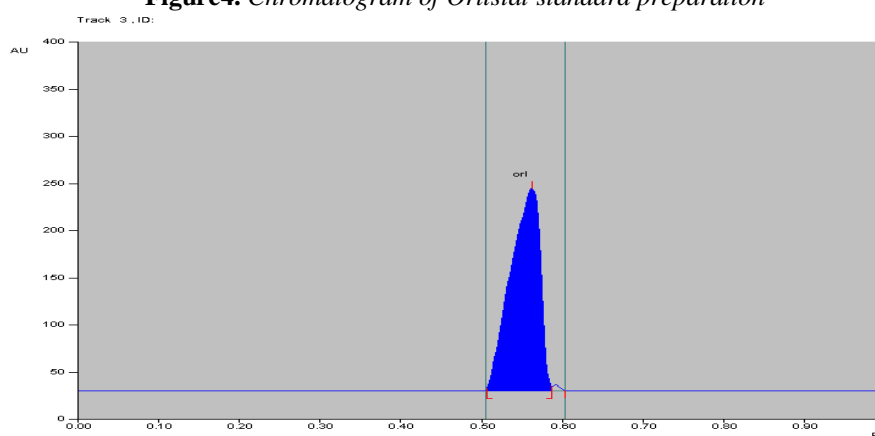**Figure4.** *Chromatogram of Orlistat standard preparation*

Figure5. *Chromatogram of Orlistat test preparation*

Table1. *Optimized chromatographic conditions for HPTLC*

Parameters	Chromatographic Conditions
Stationary phase	Silica gel GF254 pre-coated (aluminum sheet)
Diluents	Dichloro methane
Sample con.(ng/band)	2000 (ng/band) Orlistat
Sample applicator	CAMAG Linomate V
Sample application speed	100 nl/sec
Volume	10µl
Band	6mm
Development chamber	CAMAG Twin Trough Chamber
Method development	Hexane: Ethyl acetate: Glacial acetic acid (7:3:0.1v/v/v)
Chamber saturation	30 minutes
Development distance	80 mm
Drying of plate	Hair Drier
Visualization	CAMAG UV Visualizing Chamber
Densitometric scanner	CAMAG TLC Scanner III
Scanning speed	20mm/sec
Detector	Deuterium Lamp
Wavelength	208 nm
Retardation Factor	0.54 min

5.2. Method Validation

5.2.1. Solution Stability

Solution stability test was carried out by using sample and standard preparation stored at room temperature for 48 h. Sample and standard were stored without protection of light. The responses for the old solution were evaluated by comparison with freshly prepared standard solutions at the interval of 0 h, 12 h, 36 h, and 48 h. For the duration of the study of the stability of stored solutions assay was determined. At each interval solutions were applied on the plates; after development of plates, the chromatographs were evaluated for extra spots. There was no indication of compound instability in the sample solution was found through the study. Assay values acquired after 24 h and 36 h were statistically alike with the first value without measurable loss for Orlistat, respectively. Table 2 shows the summary of solution stability study.

Table2. *Summary of solution stability study of Orlistat*

Duration	Orlistat		
	Mean area of Standard	Mean area of test	%Assay
Initial	20481.8	20461.3	99.70
12 h	20479.3	20456.5	100.18
24 h	20477.5	20426.3	99.95
36 h	20482.1	20139.9	98.13
48 h	20475.4	19936.9	97.17

5.2.2. Specificity

Specificity is the measurement of the degree of interference from Impurity that may be expected to be present in the sample. Typically these might include impurities, degradation products, and diluents. The peak purity of bands for Orlistat in the sample was confirmed by comparing the R_f values and spectra of the bands with standards. **(Figure 6)** It was assessed by comparing spectra at different levels, e. g. peak- start (S), peak- max (M) and peak-end (E) position of spots. There was no interference of any peakfromthese extraneous materials.
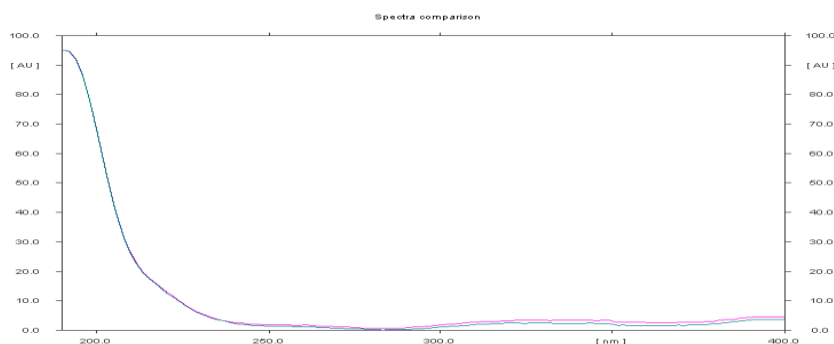

Figure6. *Overlay UV spectra of Orlistat, standard and sample*

5.2.3. Linearity

In a chromatographic method, linearity study was carried out preparing seven point calibration curve of concentration range from 80-320 µg/band (800-3200 ng/band) for Orlistat. The linearity assessed by test results which are directly proportional to the concentration of analyte which are present in the sample. (Table 3) The linear regression equation for Orlistat was y = 847.15x+3557.3 with correlation co-efficient 0.9984 Where x is the concentration in µg/ml and y is the peak area in absorbance unit. The overlay chromatogram of all the levels of linearity is given in the figure 7

Table3. *Summary of concentration and linearity evaluation for Orlistat*

Linearity		Orlistat		
Level	%of Level	Con. (µg/ml)	Con. (ng/band)	Mean Area
1	40	80	800	10083.50
2	60	120	1200	14066.70
3	80	160	1600	17358.30

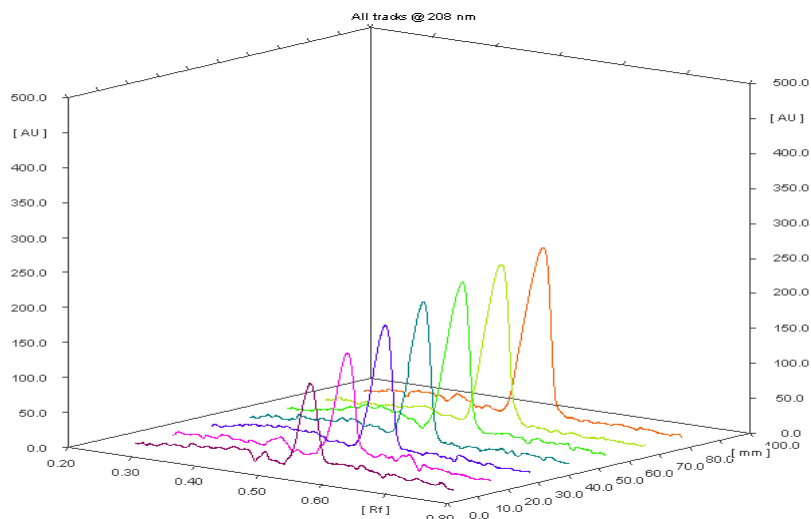| 4	100	200	2000	20481.80
5	120	240	2400	24363.00
6	140	280	2800	27797.80
7	160	320	3200	30537.90
Correlation Co-efficient			0.9984	
Slope			847.15	
Intercept			3557.3	

Figure7. *Overlay chromatograph of seven level calibration curve*

5.2.4. LOD and LOQ

The limit of detection and limit of quantification were calculated using 3σ/S and 10σ/S equation for the developed method for LOD and LOQ, respectively. Where σ is the standard deviation of the y-intercept and S is the slope of the calibration curve. For the LOD and LOQ study, concentrations were selected in the range of 800-3200 ng/band for Orlistat, respectively. The LOD value for Orlistat was found to be 1.14 μg/ml, and the LOQ value 3.81 μg/ml. The result shows the sensitivity of the methods

5.2.5. Accuracy

The accuracy study of an analytical procedure states the degree of closeness of agreement between the conventional true value or accepted reference value and the practically found value. Recovery of an ingredients was determined at three different concentration levels. The mean recovery for Orlistat was 99.13-99.92% .The satisfactory results indicating that the method was accurate and % recovery found within acceptance criteria.

5.2.6. Precision

The precision of the method, as repeatability was evaluated by performing six independent assays of the test sample preparation and calculating the SD, % RSD and mean % assay. The intermediate (inter-day) precision of the method was assessed by performing same procedure on different days by other analyst under the same experimental conditions with different make chemicals. The results obtain from the repeatability study was consider for the intra-day precision study also. The %RSD values for intermediate and method precision study was 0.017% and mean % assay was 99.79% as well as absolute difference between mean % assay values of method precision and intermediate precision found 0.04 % for Orlitat.

5.2.7. Robustness

The robustness study was evaluated with respect to the minute but a deliberate alteration in the chromatographic conditions, the result of the study delivers the reliability of the analysis. The change in Chromatographic parameters, e.g. composition of mobile phase, volume of mobile phase, chamber saturation time, detection wavelength. The effects of such deliberate changes on peak area and % assay were calculated, which found 99.60 % to 100.12 %. as well as % RSD for retardation factor found 0.67%.

5.2.8. System Suitability Study

Before measurement of validation parameter a system suitability test was performed by measurement of very important characteristics such as % RSD of peak area, % RSD of retardation factor observed and calculated for standard solutions. (Table 4)

Table4. *Summary of system suitability data of Orlistat*

System Suitability Parameter In-house Limits	%RSD[a] of Area NMT[b] 2.0	%RSD[a] of Rf[c] NMT[b] 2.0
Validation Parameter	Orlistat	
Solution Stability	1.160	0.83
Specificity	0.057	0.62
Linearity	0.094	1.24
Method Precision	0.023	0.78
Intermediate Precision	0.150	1.16
Accuracy	0.008	1.15
Robustness	0.160	0.67

The experimental results obtained and calculated are suitable for the chromatographic method with in-house limits.

6. CONCLUSION

A new analytical method was developed to be routinely applied to quantification of orlistat by a validated, simple and sensitive high performance thin layer chromatographic-densitometric assay method. Orlistat act as lipase inhibitor and prevents reducing of fate from human diet thus calorie intake is reduces. In this study, an analytical method based on TLC using densitometric detection was developed and validated for assay determination of Orlistat API. The developed procedure was evaluated for System suitability study, linearity, accuracy, precision, Robustness, LOD and LOQ. Hence, the method is recommended for routine quality control analysis of assay determination of Orlistat API.

ACKNOWLEDGEMENTS

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