Stability Indicating Analytical Method Development and Validation for Estimation of Orlistat in Bulk and its Dosage form by HPTLC Technique and Finding Degradants by LC-MS.

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ABSTRACT

A new simple, accurate, precise and selective stability indicating high performance thin layer chromatographic method has been developed and validated for estimation of Orlistat Tablet. The mobile phase selected was Toluene: Methanol(8:2v/v) with UV detection at 211nm. The retention factor for Orlistat was found to be 0.60±0.02. The method was validated with respect to linearity, accuracy, precision and robustness as per the ICH guidelines. The drug were subjected to stress condition of hydrolysis (acid, base), oxidation, photolysis and thermal degradation. Results found to be linear in concentration range of 6000-36000 ng/band. The thermal method has been successfully applied for the analysis of drug in pharmaceutical formulation. The % assay (Mean ± S.D) was found to be 99.30±1.10. The developed method can be used for checking the stability of Orlistat in bulk drug and pharmaceutical dosage form.

Keywords: Forced degradation, High performance thin layer chromatography (HPTLC), Orlistat, stability-indicating method, Validation, High performance thin Liquid chromatography- Mass spectrometry(HPTLC-MS), Degraded products(DP).

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INTRODUCTION

Orlistat is a potent, specific and reversible long-acting inhibitor of gastrointestinal lipase. Orlistat is chemically designated as [(2S)-1-[(2S,3S)-3-hexyl-4-oxooxetan-2-yl]tridecan-2-yl] (2S)-2-formamido-4-methylpentanoate. It is a white amorphous powder with a molecular weight of 495.7g/mol. It is very soluble in methanol and chloroform and insoluble in water. The drug is formulated as a capsule form, and is used for the obesity management that acts by inhibiting the absorption of dietary fat. The literature survey revealed few methods like UV-spectroscopy, RP-HPLC and HPLC for the determination of Orlistat.

The above survey of literature shows no report of stability indicating HPTLC method for estimation of Orlistat. The aim of the study was to develop a simple, accurate, precise and specific stability indicating HPTLC method for the determination of Orlistat. The present work involve stress degradation as per ICH Q1A(R2) and Q1B. The proposed method was validated for linearity, accuracy, precision, robustness, LOD and LOQ according to ICH guidelines.

MATERIALS AND METHOD

Chemicals and Reagents

Orlistat was provided as a gift sample by Bills Biotech pvt ltd, Vadodra. Methanol and Toluene of AR grade purchased from Merck Pvt. Ltd. Mumbai, India. Hydrochloric acid, hydrogen peroxide and sodium hydroxide were purchased from LOBA CHEMIE PVT. LTD. Mumbai.

Selection of detection wavelength

The standard solution of Orlistat in methanol was scanned over the range of 200 – 400 nm by using UV-Visible spectrophotometer. Wavelength 211 nm was selected for analysis where Orlistat showed higher absorbance.

Instrumentations and Chromatographic conditions

Chromatographic separation of drug was performed on Aluminum plates pre-coated with silica gel 60 F\textsubscript{254}, (10 cm × 10 cm with 250 μm layer thickness). Samples were applied on the plate as a band of 4 mm width using Camag 100 μL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). The mobile phase was composed of Toluene:Methanol(8:2v/v).
CAMAG twin through glass chamber 10 cm × 10 cm was used for linear ascending development of TLC plate under 10 min saturation conditions and 10 mL of mobile phase was used per run, migration distance was 90 mm. Densitometric scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software, slit dimensions were 3.00 x 0.45 mm and Deuterium lamp was used as a radiation source.

**Preparation of Sample solution**

A standard stock solution of Orlistat was prepared by dissolving 60 mg of drug in 10 ml of methanol to get concentration of 6000μg/ml. The solution was filtered through 0.2 μ whatman filter paper. The above prepared solutions were analyzed by HPTLC for the content of Orlistat.

**Stress degradation study of bulk drug**

Stress degradation studies were carried under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolytic. For each study, samples were prepared as follows

1. The blank subjected to stress in the same manner as the drug solution.
2. Working standard solution of Orlistat subjected to stress condition.

Dry heat was carried out in solid state. 3.0 μl of the resultant solution was then applied at TLC plate and densitogram was developed.

Stress test conditions like strength of reagent and exposure time were optimized to get 10-30% degradation. The optimized conditions are as follows:

**Degradation under alkali catalyzed hydrolytic condition**

To 60 mg of the Orlistat powder , 3mL of 0.05 M NaOH was added. The solution was kept for 30 min at room temperature. The volume was made upto 10 mL with methanol. After that the solution is sonicated and filtered through whatman filter paper and applied as 3 μl/band.

**Degradation under acid catalyzed hydrolytic condition**

To 60 mg of the Orlistat powder , 3mL of 0.05 N HCL was added. The solution was kept for 30 min at room temperature. The volume was made upto 10 mL with methanol. After that the solution is sonicated and filtered through whatman filter paper and applied as 3 μl/band.

**Degradation under neutral hydrolytic condition**

To 60 mg of the Orlistat powder , 3mL of Distilled water was added. The solution was kept for 2 hrs at room temperature. The volume was made upto 10 mL with methanol. After that the solution is sonicated and filtered through whatman filter paper and applied as 3 μl/band.

**Degradation under oxidative condition**
To 60 mg of the Orlistat powder, 3 mL of 3% v/v of H2O2 was added. The solution was kept for 1 hrs at room temperature. The volume was made up to 10 mL with methanol. After that the solution is sonicated and filtered through whatman filter paper and applied as 3 µl/band.

**Degradation under dry heat**

Dry heat studies were performed by keeping drug sample in sunlight for a period of 2 hrs. Sample was withdrawn, dissolved in methanol and diluted to get 6000 µg/ml.

**Method Validation**

The method was validated according to the ICH Q2 (R1) guidelines for the following parameters.

**Specificity**

The specificity of the method was ascertained by peak purity profiling studies in winCATS software. It involves comparison of UV spectra at peak start, middle and end. The peak purity values were found to be more than 0.998, indicating the noninterference of any other peak of degradation product or impurity.

**Linearity and Range**

From the sample solution (6000 µg/ml) of Orlistat, this solution was used for spotting. Six replicates per concentration were spotted. The linearity was determined by analyzing six concentrations over the concentration range of 6000-36000 ng/band for Orlistat. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. The LOD and LOQ were calculated based on the equation:

\[ \text{LOD} = 3.3 \times \frac{\sigma}{S} \quad \text{and} \quad \text{LOQ} = 10 \times \frac{\sigma}{S}. \]

Where, \( \sigma \) is standard deviation of the lowest response of linearity equation and \( S \) is slope of the calibration curve of the analyze.

**Accuracy**

To check accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 80%, 100% and 120%. Basic concentration of sample chosen was 6000 ng/band. % recovery was determined from linearity equation.

**Precision**

The precision of the method was demonstrated by Intra-day and Inter-day variation studies. In the Intra-day studies 3 replicates of 3 concentrations were analyzed on the same day, for the inter day variation studies, 3 replicates of 3 concentrations were analyzed on 3 consecutive days and % RSD was calculated.

**Robustness**
Robustness of the method was determined by introducing small deliberate changes in mobile phase ratio, chamber saturation time and Total mobile phase. For all changes in conditions, the samples were analyzed in triplicate and the effects on the peak area and $R_f$ value was noted. It was found that results did not vary by more than 2%.

**High performance thin layer chromatography and Tandem mass spectroscopy for isolation and identification of degradation product in Orlistat.**

**Preparation of standard stock solution:**

Accurately weighed quantity of 60.0 mg Orlistat, was transferred to 10.0 ml volumetric flask, added 5 ml of methanol and ultrasonicated for 10 minutes, volume was then made up to the mark with methanol. (Concentration obtained 6000 µg /ml).

**Isolation of degradation product by using HPTLC method.**

Accurately weighed quantity of 60.0 mg of Orlistat was transferred to 10.0 ml of volumetric flask than add 3.0 ml of 0.05 M NaOH in flask no 1 and 3.0 ml of distilled water. The forced degradation study was carried out by exposing samples to the stress condition as 0.05 M NaOH & 0.05 N HCl for degradation, contents of the flask were reflux in a water bath at 80°C for about 30 min .After the respective time intervals all the flasks were removed and allowed to cool. Than the samples were applied on the TLC plate with the sample volume of about 3µl/ band, 5 band of degraded sample and 1 band of std. were applied. Than allow the TLC plate to developed under optimized chromatographic conditions for Orlistat.

After development of the plate this plates was kept under the UV chamber on the basis of RF value of the std. and degradation product they are marked and that portion of TLC plate was cut and allow it to extract in to methanol. Than MS-MS spectra was recorded for interpretation of probable structure of the degradation product .

**Isolation and identification of degradation product of Orlistat. (Alkaline Degradation).**

Isolation of Alkalyzed product was carried out by using procedure which is mentioned above. This Alk-1 was identified by using MS-MS.

**Identification of Alk-1 structure by Tandem Mass spectroscopy.**

Chemically Orlistat is (2S)-1-[(2S,3S)-3-hexyl-4-oxooxetan-2-yl]tridecan-2-yl] (2S)-2-formamido-4-methylpentanoate) which has empirical formulaC$_{29}$H$_{53}$NO$_5$, and molecular weight is of about 495.745 g/mol. when this drug is allowed to degrade under the Alkali condition Table.6.

**Isolation and identification of degradation product of Orlistat, Acid-1 (Acid Degradation).**
Isolation of Acid degraded product (Acid-1) was carried out by using procedure which is mentioned above. This Photo-1 was identified by using MS-MS.

**Identification of Acid-1 structure by Tandem Mass spectroscopy.**

Chemically Orlistat is (2S)-1-[(2S,3S)-3-hexyl-4-oxooxetan-2-yl]tridecan-2-yl] (2S)-2-formamido-4-methylpentanoate) which has empirical formula C\textsubscript{29}H\textsubscript{53}NO\textsubscript{5}, and molecular weight is of about 495.745 g/mol. when this drug is allowed to degrade under the Acid condition on Table 8.

**RESULTS AND DISCUSSION**

**Optimization of mobile phase**

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 211 nm. The stationary phase used for study of Orlistat was Aluminum plates pre-coated with silica gel 60 F\textsubscript{254}, (10 cm × 10 cm) with 250 μm layer thickness. Different ratios of mobile phase constituents were studied, mobile phase with Toluene: Methanol in the ratio of 8:2 v/v was chosen due to good symmetrical peak. Retention Factor was 0.60 ± 0.02. The analytical method was found linear over the range of 6000-36000 ng/band.

**Forced degradation studies**

Forced degradation studies were conducted to evaluate the stability and specificity of the method. Very less Degradation products of Orlistat was observed when the drug was subjected to acidic, basic and neutral treatment, exposure to heat. The drug peaks obtained from all the stressed samples were found to be homogenous and pure. Hence the method is found to be specific. The results are given in (Table 1).

| Stress degradation conditions          | Percent recovery (%) | Percent degraded (%) | Peak purity data r(s,m) | r(m,e) |
|---------------------------------------|----------------------|----------------------|-------------------------|--------|
| Initial                               | 100                  | -                    | 0.999                   | 0.999  |
| Base (0.05 N NaOH, kept for 30 min at RT.) | 83.89                | 16.11                | 0.998                   | 0.997  |
| Acid (0.05 N HCl, Kept for 30 min at RT.) | 86.28                | 13.71                | 0.999                   | 0.997  |
| H\textsubscript{2}O\textsubscript{2} 3% v/v (kept for 1 hrs at RT.) | 82.55                | 17.45                | 0.997                   | 0.998  |
| Neutral (kept for 2 hrs)              | 88.67                | 11.32                | 0.998                   | 0.998  |
| Thermal (in sunlight for 2 hour at about 50°C) | 80.46                | 19.54                | 0.997                   | 0.998  |
| Photolytic(at 254nm for 72 hour)      | 99.47                | 0.53                 | 0.998                   | 0.999  |

**Linearity and Range**

The linear calibration range was found to be 6000 to 36000 ng/band. The calibration curve obtained by the least square regression analysis between average peak area and concentration...
showed linear relationship with a correlation coefficient of 0.999, the equation of the calibration curve found for Orlistat was \( y = 0.195x - 72.09 \) (Fig. 2 & 3).

**Accuracy and Precision**

Recovery of standard drug was found to be 100.75-98.70 % with less than 2% of RSD values, indicating that the proposed method was accurate (Table 2). (Table 3) shows the precision study results. The RSD values for intraday and interday-precision were not more than 2 %, indicating the repeatability and reproducibility of the method.

**Limit of detection and quantification (LOD and LOQ)**

The LOD (Limit of Detection) and LOQ (Limit of quantitation) were estimated from the standard deviation of the lowest response and the slope of the calibration curve. LOD and LOQ were found to be 671.9112 ng/ band and 2036.095ng/band respectively.

**Robustness**

The %RSD values of all robustness parameters were examined and found to be within the limit of 2%, showed that the proposed method was robust (Table 4).

**Solution Stability**

Freshly prepared solution was kept in a freeze (cool condition) after use. UV absorbance of freeze solution was compared with absorbance of fresh solution. It was observed that drug solution have stability of 3 days.

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**Figure 2: Densitogram of linearity of Orlistat (6000-36000 ng/band).**
**Figure 3: Calibration curve for Orlistat.**

**Table 2: Accuracy of the proposed method**

| Conc’n Level (%) | Theoretical Conc. (mg/band) | Avg. Area of Recovered Conc. (mg/band) | Recovered Area | % Recovery |
|------------------|----------------------------|--------------------------------------|----------------|------------|
| 80               | 48                         | 6535.8                               | 6559.3         | 100.75     |
| 100              | 60                         | 7262                                 | 7215.7         | 98.70      |
| 120              | 72                         | 7988.2                               | 7958           | 99.30      |

**Table 3: Precision study**

| Concentration (ng/band) | Mean area* | SD  | % RSD |
|-------------------------|------------|-----|-------|
| **Inter day**           |            |     |       |
| 6000                    | 1237.47    | 0.115| 0.115 |
| 12000                   | 2340.24    | 0.838| 0.842 |
| 18000                   | 3591.5     | 1.163| 1.176 |
| **Intra day**           |            |     |       |
| 6000                    | 1290.66    | 1.4709|1.4772 |
| 12000                   | 2395.77    | 1.5421|1.5447 |
| 18000                   | 3609.29    | 0.5394|0.5399 |

**Table 4: Results of robustness study**

| Sr. No. | Parameters | Robust condition | % RSD |
|---------|------------|------------------|-------|
| 1.      | Saturation time (10min) ± 5 min | 5min | 5min | 0.0113 |
| 2.      | Mobile phase composition | Toluene: Methanol (7.9:2.1 v/v) ±0.1 toluene | Toluene: Methanol (8.1:1.9 v/v) | 0.0072 |
| 3.      | Total mobile phase (Toluene 10ml + Methanol 10m=Total 20 mL) | 11mL | 9mL | 0.011 | 0.018 |

y = 0.1955x + 72.095

R² = 0.9992
**Table 5: Summary of validation study**

| Sr. No. | Validation parameters | Orlistat |
|---------|-----------------------|----------|
| 1.      | Linearity Range       | \( y = 0.195x + 72.09 \) R² = 0.999 6000-36000 ng/band |
| 2.      | Precision (% RSD)     | Interday 0.709 Intraday 1.1872 |
| 3.      | Accuracy              | % Recovery 80 100.75 100 98.70 120 99.30 |
| 4.      | Limit of Detection    | 671.911ng/band |
| 5.      | Limit of Quantitation | Specific |
| 6.      | Specificity           | Specific |
| 7.      | Robustness            | Robust |

**Degradation Products**

Table 6 Molecular ion peak of different m/z ratio recorded in positive mode of MS.

| Degradation peak name | m/z ratio |
|-----------------------|-----------|
| DP-1                  | 86.0601   |
| DP-2                  | 356.353   |
| DP-3                  | 218.212   |
| DP-4                  | 373.332   |

Proposed mass fragmentation pattern of the hypothetical mass fragmentation pattern Alkali -1 for Orlistat drugs on the basis of MRM transitions has been incorporated and that helps in confirmation of Structure on the basis of diagnostic ions (Fig 4, 5, 6, 7).

![Figure 4 MS/MS spectrum of m/z-86.0601 in the positive mode(DP-1).](image-url)
Figure 5  MS/MS spectrum of m/z-356.353 in the positive mode(DP-2).

Figure 6  MS/MS spectrum of m/z-218.212 in the positive mode(DP-3).

Figure 7  MS/MS spectrum of m/z-373.332 in the positive mode(DP-4)
Table 7: Characteristic fragment ion of Alk-1 obtained in ESI positive mode of MS-MS.

| Analyte/functional group attached | Structure of analyte | m/z (observed) |
|----------------------------------|----------------------|---------------|
| C₄H₇NO                           | ![Structure of C₄H₇NO](image) | 86.0601       |
| C₂₂H₅NO₂                         | ![Structure of C₂₂H₅NO₂](image) | 356.353       |
| C₁₂H₂₇NO₂                         | ![Structure of C₁₂H₂₇NO₂](image) | 218.212       |
| C₂₂H₄₄O₄                         | ![Structure of C₂₂H₄₄O₄](image) | 373.332       |

Table 8 Molecular ion peak of different m/z ratio recorded in positive mode of MS.

| Degradation peak name | m/z ratio |
|-----------------------|-----------|
| DP-5                  | 86.0966   |
| DP-6                  | 230.248   |
| DP-7                  | 294.207   |
| DP-8                  | 440.441   |

Proposed mass fragmentation pattern of the hypothetical mass fragmentation pattern Alkali -1 for Orlistat drugs on the basis of MRM transitions has been incorporated and that helps in confirmation of Structure on the basis of diagnostic ions (Fig 8, 9, 10, 11).
Figure 8 MS/MS spectrum of m/z-86.0966 in the positive mode (DP-5).

Figure 9 MS/MS spectrum of m/z-230.248 in the positive mode (DP-6).

Figure 10 MS/MS spectrum of m/z-294.207 in the positive mode (DP-7).
Figure 11 MS/MS spectrum of m/z-440.411 in the positive mode (DP-8).

Table 9 Characteristic fragment ion of Acid -1 obtained in ESI positive mode of MS-MS.

| Analyte/functional group attached | Structure of analyte | m/z (observed) |
|----------------------------------|----------------------|----------------|
| C₅H₁₁N                          | ![Structure](image1) | 86.0966        |
| C₁₄H₃₁NO                         | ![Structure](image2) | 230.248        |
| C₁₇H₂₇NO₃                       | ![Structure](image3) | 294.207        |
CONCLUSION

The developed method was found to be simple, sensitive, selective, cost-effective and time saving for analysis of Orlistat in Capsule form without any interference from the excipients. The results indicated the suitability of the method to study stability of Orlistat under various forced degradation conditions as prescribed by ICH Q1A(R2) guidelines.

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