RESEARCH ARTICLE

Liquid Chromatography Tandem Mass Spectrometric Method Development and Validation for the Quantification of Orlistat in Biological Matrices

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ABSTRACT

A specific, linear and precise liquid chromatographic-tandem mass spectrometric method was established and validated for the quantitation of orlistat in sample plasma. Zorbax C_{18} (4.6 mm i.d.× 50.0 mm; 5.0 µm) stationary phase was utilized to achieve chromatography elution, through a flowing rate of 0.90 mL/min. Isocratic elution was done using methanol, acetonitrile and 0.10% v/v HCOOH in a fraction of 80:10: 10 v/v/v as the mobile phasic system. For drug and internal standard separation, the precipitation extraction technique used acetonitrile as solvent. A triple quadrupole mass detector was employed for the quantification of ions. Electrospray ionization in a positive ionizing method, which was executed in multiple reaction monitorings (MRM) with parent/product ion transitions of m/z 496.4→337.31 for orlistat and 506.23→57.07 for amprenavir internal standard. The calibration graph was executed between the concentrations of 4.75–190.0 ng/mL and the resulting equation was y = 0.0058x + 0.0022 with r^2 value of more than 0.99. Orlistat recovery values were found to be more than 93.65%, and its accuracy, measured in relative error, was in the range of -4.48 to 3.49%. Accuracy findings, sensitivity and recovery values of orlistat in the sample plasma for the established technique evidences its importance in pharmacokinetic and bioequivalence study.

Keywords: Accuracy, LC–MS/MS, Linearity, Obesity, Orlistat, Validation.

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INTRODUCTION

Orlistat chemically designated as (*S*)-((*S*)-1-((2*S*, 3*S*)-3-Hexyl-4-oxooxetan-2-yl) tridecan-2-yl) 2-formamido-4-methylpentanoate. Its molecular mass and chemical formula are 495.745 g/mol and $C_{29}H_{53}NO_5$, respectively (Figure 1). The number of obese people around the world is rising quickly. Obesity-related health problems are a big personal and financial burden because they lower the quality of life and raise healthcare cost.[1,2] Some people can't keep off the weight they've lost through diet and exercise alone, so they need help from drugs or surgery. Orlistat is a powerful and selective lipase enzyme inhibitor that breaks down fat. It works in the GI tract by covalently binding to the serine residues on the active site of both gastric and pancreatic lipase.[3,4] When orlistat is taken with fat-containing foods, it slows down the process by which triglycerides are broken down. This makes it harder for your body to absorb monoacylglycerides and free fatty acids, which help you keep or lose weight.

The amount of orlistat that gets into the body and stays there is low. However, systemic absorption of the drug is not necessary for orlistat to work. After taking 360 mg of radiolabeled orlistat by mouth, the level of radioactivity in the blood reached its peak in about 8 hours. Plasma levels of the parent drug that had not been changed were close to the lower limit of what could be found (5 ng/mL). Plasma samples from people taking orlistat sometimes showed unchanged drug at very low concentrations (10 ng/mL or 0.02 M), but there was no evidence that the drug was building up.[5]

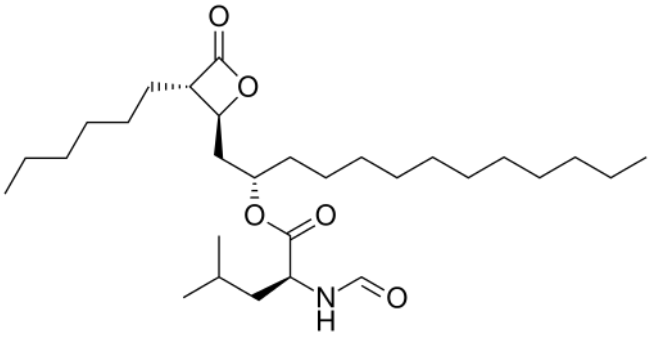

Figure 1: Orlistat chemical structure.

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Literature review on orlistat reveals that high performance liquid chromatography-mass spectrometry (HPLC/MS),[6] high performance thin layer chromatography (HPTLC),[7] reverse phase high performance liquid chromatography (RP-HPLC),[8] thin layer chromatographic (TLC)[9] and liquid chromatographic–tandem mass spectrometric (LC-MS/MS)[10] analytical approaches were reported for the assessment of orlistat in sample solutions. So, this work aimed to develop a specific, accurate, and reliable LC–MS/MS method for measuring orlistat in human plasma as a single drug.

MATERIALS AND METHODS

Reagents and Chemicals

The Orlistat (98.96% pure) standard and amprenavir (99.84% pure) were acquired from Dr. Reddys, Bollaram, Telangana, India. Methyl alcohol and acetonitrile of HPLC level grade were attained from Merck, Vikhroli, Maharashtra, India. The present research produced water of LC-grade purity from the Milli-Q instrument, USA.

LC–MS/MS Instrument and Parameters

The LC–MS/MS instrument consists of an Agilent3200 liquid chromatography system with two pumps (dual-SL) and Agilent/6164 mass triple quadrupoles spectrometric detector with the source of electrospray ionization (CA, America). Chromatography statistics were executed thru MassHunter software. Zorbax C_{18} (4.6 mm i.d.×50.0 mm; 5.0 µm) stationary phase was utilized to achieve chromatography elution through a flowing rate of 0.90 mL/min. Isocratic elution was done using methanol, acetonitrile and 0.10% v/v HCOOH in a fraction of 80:10: 10 v/v/v as the mobile phasic system. A triple quadrupole mass detector was employed for the quantification of ions. Electrospray ionization in a positive ionizing method, which was executed in multiple reaction monitorings (MRM) with parent/product ion transitions of m/z 496.4→337.31 for orlistat and 506.23→57.07 for amprenavir internal standard. The MS/MS parameters were optimized as: capillary voltage at 4.50 kV, source temperature at 300ºC; dryer gas (N_2) flow at 10 L/min and nebulization gas at 50 psi. The autosampler temperature and infusion volumes were kept at 8.0ºC and 10 µL, respectively. In 20 eV of collisional energy was employed in the chromatography elution.

Standard Quality Controls

1000 µg/mL orlistat and amprenavir stock solutions were individually employed in mobile phase (as diluent). The resulting orlistat solution was processed for serial dilutions with mobile phase to make working standard controls. Amprenavir internal standard working standard at 250 ng/mL was processed accordingly to get in all the orlistat quality. The prepared quality controls were monitored at -20ºC till the sample analysis.

Linearity quality controls of orlistat (4.75, 9.5, 21.0, 45.0, 81.0, 118.0, 155.0 and 190.0 ng/mL) were achieved by the method of spiking to plasma blanks. Quality control solutions at low, medium and high concentrations (13.3, 95.0 and 142.5 ng/mL), were employed individually in the same manner.

Sample Preparation Method

A 250 µL blank plasma solution was transferred into a 10 mL tube for processing. Drug and 100.0 µL of internal standard solutions were added to tubes to get required concentration in the final dilution to be infused. The mixture was added to 5 mL of acetonitrile for the protein precipitation method and employed for the centrifugation (15 minutes). The upper organic solvent system was transferred to another clean tube and n-hexane of 5 mL was mixed and subjected for centrifugation for the formation of 2 layers. The upper n-hexane layer was isolated and dried by the vaporization. The resulting dried product was subjected for reconstitution with movable phase and 10 µL was infused to LC–MS/MS instrument for analysis.

Method Validation

The developed analytical method was subjected for validation according to the rules of the USFDA for variable validation parameters to fulfill the requirements.[11,12]

RESULTS AND DISCUSSION

Mass System Optimization

During the development stage, fresh orlistat solution was injected to make sure that the product and parent ions were working at their best. The positive ionization method found a precursor ion with a value of 496.4 m/z. When the precursor ion broke apart, pieces with masses of 466.38, 337.31, 155.10, 142.08, and 100.11 were found. At 337.31 m/z, the most intense value was found for the daughter ion of orlistat. Amprenavir has similar physical and chemical properties to orlistat, which makes it a good choice as an internal standard for this bioanalytical method development and for good recovery during the sample preparation and validation process. MRM scan was used to find both drugs' product and parent ions. The final transitions for orlistat were m/z 496.4→337.31 and for amprenavir internal standard, they were m/z 506.23→57.07.

Specificity

Blank plasma and plasma spiked at LLoQ level (4.75 ng/mL), of orlistat and amprenavir were infused into an LC-MS/MS instrument and the resulting chromatograms were given in Figure 2. Due to interference, the sample plasmas of orlistat and amprenavir did not show any peaks. Orlistat and amprenavir were eluted from the system in a 4 minutes time. Orlistat and

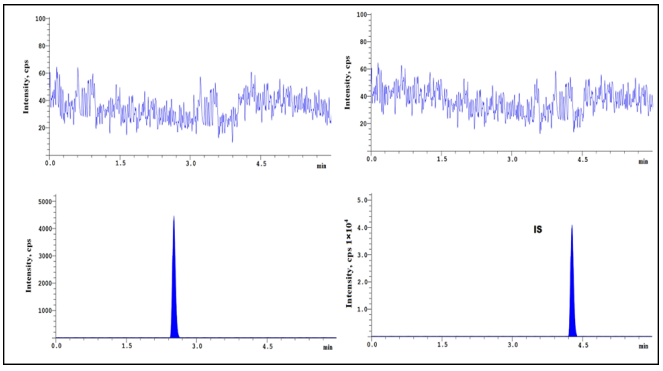

Figure 2: Orlistat A) Plasma blank chromatogram and B) LLOQQC chromatogram.

Table 1: Orlistat calibration quality controls

St-ID	*Conc (ng.mL^{-1})*	*Drug Area*	*IS Area*	*Area ratio (drug/IS)*
St -1	4.75	1578	55921	0.028218
St -2	9.5	3294	56034	0.058786
St -3	21	7039	55219	0.127474
St -4	45	13947	56135	0.248455
St -5	81	26421	55627	0.474967
St -6	118	38418	55926	0.686943
St -7	155	50126	55265	0.907012
St -8	190	60920	56234	1.08333

amprenavir resided in the system for 2.03 and 4.25 minutes, respectively.[13]

Linearity and Sensitivity

The signal/noise results were >10.0 at this concentration (4.75 ng/mL) level, and the accuracy and precision findings were 4.25% RSD, hence the LLoQQC of the orlistat was set at 4.75 ng/mL. Every set of orlistat plasma concentrations between 4.75 and 190.0 ng/mL was analyzed using rectilinear plots (Table 1). Calculated from the average values of six replica calibration standards,[14,15] the equation of regression plot for orlistat was determined to be: y = 0.0058x + 0.0022, where 'x' stands for plasma concentration and 'y' for peaks ratio, or analytes/IS.

Accuracy, precision and recovery

Inter day, and intra day precision and accurateness outcomes were shown in Figure 3 and Table 2. Precision findings in a day were present between %RSD of 2.89 to 4.68% for orlistat,[16] where the accuracy outcomes were present between the relative error of −4.48 to 3.50%. Similarly, between different experimental days, precision values varied in the limits of 2.77 to 4.25% (RSD) for orlistat, whereas the accuracy was present between the relative error of -3.56 to 3.49%.

Orlistat average recoveries were exist in between the limits of 93.65 to 103.84% at 3 quality controls (Table 3). The processed extraction technique for sample solution evidenced that orlistat[17] and amprenavir(98.86%) were improved with high percentage outcomes from blank plasma.

Matrix effects

The peak response ratios of orlistat/amprenavir in blank plasma extract to those with diluent was present in between 94.24 to 102.75% for orlistat (Table 4) at low-QC level and 94.26 to 103.54% at high QC level.[14,17]

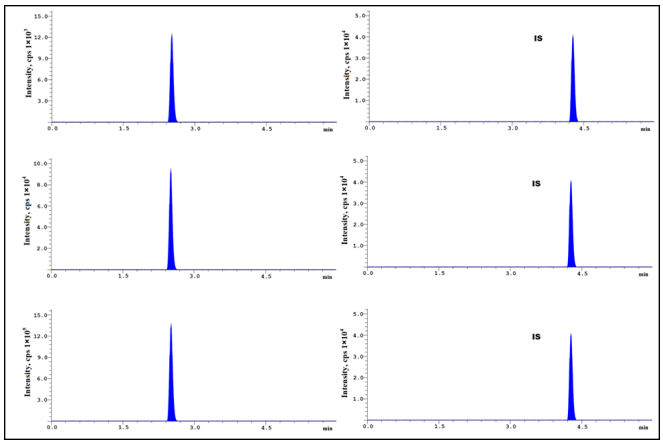

Figure 3: Orlistat outcomes at A) Low-QC B) Median-QC and C) High-QC level.

Table 3: Orlistat and amprenavir recovery studies

Concentration level	*Y*	*Z*	*% Recoveries*	*% Mean recoveries*	*% RSD*
LQC	4264	4146	97.24	98.24	4.29
MQC	30460	28525	93.65		
HQC	45690	47444	103.84		
Amprenavir	55234	54604	98.86		

Y, mean recoveries of un-extracted samples; Z, mean recoveries of extract samples.

Table 4: Orlistat matrix effect at low-QC and high-QC level.

	LQC			*HQC*		
S.No	*Peak area without matrix*	*Peak area with of matrix*	*Matrix factor*	*Peak area without matrix*	*Peak area with of matrix*	*Matrix factor*
1	4286	4403	102.75	45705	44704	97.81
2	4308	4151	96.36	45647	47262	103.54
3	4326	4210	97.32	45716	43571	95.31
4	4274	4143	96.95	45637	44190	96.83
5	4302	4054	94.24	45720	47023	102.85
6	4294	4095	95.38	45677	43055	94.26
Mean			97.17			98.43
± SD			2.95			3.89
%RSD			3.04			3.95

SD:standard deviation; RSD: Relative standards deviation.

Table 2: Intra-day and inter day precision and accuracy of orlistat.

Spiked conc. (ng/mL)	*Intra day(n= 6)*			*Inter day(n = 6×3)*		
	Measured conc(mean± SD;ng/mL)	*Precision (RSD%)*	*Accuracy (RE %)*	*Measured conc(mean± SD;ng/mL)*	*Precision (RSD%)*	*Accuracy (RE%)*
4.75	4.92 ± 0.19	4.01	3.51	4.58 ± 0.184	4.02	-3.57
13.3	13.61 ± 0.43	3.11	2.33	13.77 ± 0.447	3.25	3.49
95	92.07 ± 4.31	4.68	-3.08	92.41 ± 3.93	4.25	-2.73
142.5	136.11 ± 3.94	2.89	-4.48	146.12 ± 4.06	2.78	2.54

RSD: Relative standard deviation; RE: Relative error.

Table 5: Orlistat stability studies at variable environmental conditions(n=3).

Storage condition	*LQC-35.0 ng.mL^{-1}*		*MQC-250.0 ng.mL^{-1}*		*HQC-375.0 ng.mL^{-1}*	
	Accuracy (Mean%)	*Precision (RSD%)*	*Accuracy (Mean%)*	*Precision (RSD%)*	*Accuracy (Mean%)*	*Precision (RSD%)*
Room temp., 8 hours	93.64	2.86	96.34	3.62	94.68	1.76
30 days at −20.0°C	95.38	1.09	101.98	2.94	103.77	3.64
3 freeze-thawed cycles	103.84	3.85	94.39	2.86	97.45	3.29
Extracts, 24.0 hours at 4.0°C	96.49	4.2	95.23	4.37	95.39	2.88

RSD: relative standard deviation.

Stability study

Orlistat stability was proven by executing the control samples to variable storage environments.[12,15] The exposed environments comprise long-time stabilities and subsequent storage of samples at −20°C for 30 days, short-time stabilities at room temperature upto 8 hours, and three completely freeze-thawed cycles (frozen at −20.0°C for 12.0 hours) and processed (extracts) samples stabilities after 24 hours at 4.0°C. Table 5 shows the results for stability for control sample solutions in the plasmas. According to regulatory requirements, the orlistat drug's evaluated accuracy levels ranged from 93.64 to 103.84% were acceptable.

CONCLUSION

A specific, linear and precise liquid chromatographic - tandem mass spectrometric method was established and validated for the quantitation of USFDA approved orlistat in sample plasma. Zorbax C_{18} (4.6 mm i.d. × 50.0 mm; 5.0 µm) stationary phase was utilized to achieve chromatography elution, through a flowing rate of 0.90 mL/min. Electrospray ionization in a positive ionizing method, which was executed in MRM with parent/product ion transitions of m/z 496.4→337.31 for orlistat and 506.23→57.07 for amprenavir internal standard. The calibration graph was executed between the concentrations of 4.75 to 190.0 ng/mL and the resulting equation was y = 0.0058x + 0.0022 with r^2 value more than 0.99. Orlistat recovery values were more than 93.65%, and its accuracy, measured in relative error, was in the range of -4.48 to 3.49%. Lastly, the method made was within the guidelines for bioanalytical method validation and can be used to measure the amount of orlistat in different biological samples.

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