BIO ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF EZETIMIBE AND PITAVASTATIN AND ITS APPLICATION TO PHARMACOKINETIC STUDIES USING UPLC

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Abstract

A simple, sensitive and rapid UPLC method was developed and validated for the simultaneous quantification of Ezetimibe and Pitavastatin. Separation was carried on C₁₈ column (50x2.1mm, 1.7μ) using a isocratic elution with a buffer containing 1mL of Formic acid in 1Lit of water and the mixture of two components like Buffer and Acetonitrile in the ratio of 40:60 as mobile phase with 1mL/min flow rate at ambient temperature. Analysis was carried out within 10 minutes. The calibration curve was linear in the concentration range from 5.0ng/mL to 100ng/mL ($r^2 = 0.999$) for Ezetimibe and 2.0ng/mL to 40ng/mL ($r^2 = 0.999$) for Pitavastatin. All the parameters of system suitability, specificity, linearity and accuracy are in good agreement with USFDA guidelines and applied effectively for the investigation of pharmacokinetic studies in rabbit. Simple and efficient method was developed and utilized in pharmacokinetic studies to see the investigated analyte in body fluids.

Key words: UPLC, Ezetimibe, Pitavastatin, Validation, Rabbit plasma.

1. Introduction

Ezetimibe is a medication used to treat high blood cholesterol ^[1-2] and certain other lipid abnormalities. Generally it is used together with dietary changes and a statin ^[3-4]. Alone, it is less preferred than a statin. It is taken by mouth. It is also available in the fixed combinations ezetimibe/simvastatin, ezetimibe/atorvastatin, and ezetimibe/rosuvastatin. The most commonly reported adverse events include upper respiratory tract infections ^[5-6], joint pain, diarrhea ^[7], and tiredness ^[8]. Serious side effects may include anaphylaxis ^[9-10], liver problems, depression, and muscle breakdown ^[11]. Use in pregnancy and breastfeeding ^[12] is of unclear safety. Ezetimibe works by decreasing cholesterol absorption in the intestines.

Pitavastatin (usually as a calcium salt) is a member of the blood cholesterol lowering medication class of statins ^[13]. Like other statins, it is an inhibitor of HMG-CoA

reductase^[14], the enzyme that catalyses the first step of cholesterol synthesis^[15]. Common statin-related side effects (headaches, stomach upset, abnormal liver function tests and muscle cramps^[16]) were similar to other statins. However, pitavastatin seems to lead to fewer muscle side effects than certain statins that are lipid-soluble, as a result of the fact that pitavastatin is water-soluble (as is pravastatin, for example). One study found that coenzyme Q₁₀ was not reduced as much as with certain other statins (though this is unlikely given the inherent chemistry of the HMG-CoA reductase pathway that all statin drugs inhibit)^[17-18]. As opposed to other statins, there is evidence that pitavastatin improves insulin^[19] resistance in humans, with insulin resistance assessed by the homeostatic model assessment (HOMA-IR) method^[20]. Hyperuricemia^[21] or increased levels of serum uric acid^[22] have been reported with pitavastatin^[23].

In drug discovery and production, bioanalysis is an integral component. Bioanalysis is related to the analysis of analytes in biological samples (drugs, metabolites, biomarkers) and requires several phases from sample collection to analysis of samples and reporting of results. The first phase is the selection of samples from clinical or preclinical trials, then sending the samples for analysis to the laboratory. Sample clean-up is the second step and it is a very critical step in bio-analysis. A robust and stable sample preparation system should be implemented in order to reach accurate results. The task of sample preparation is to remove interference from the matrix of the sample and improve the efficiency of the analytical method. Preparation of samples is often labor intensive and time consuming. The last step is the examination and detection of samples. The method of choice in bioanalytical laboratories is Ultra performance liquid chromatography (UPLC) for separation and detection. This is attributed to high selectivity and high sensitivity of the UPLC technique. In addition the information about the analyte chemical structure and chemical properties is important to be known before the start of bio-analytical work.

This work provides an overview of bioanalytical method development and validation. The main principles of method validation will be discussed. Commonly used sample preparation techniques will be presented. In addition the role of LC-MS/MS in modern bioanalysis will be discussed. In the present review we have our focus on bioanalysis of small molecules. Till date, no method is available for bio-analysis of ezetimibe and pitavastatin in any type of biological matrix. This is the first time to report a bio-analytical method for these drugs.

2. Experimental Study

Chemicals

Acetonitrile, formic acid and water (HPLC grade), were purchased from Merck Ltd. Worli, Mumbai, India. APIs of Ezetimibe and Pitavastatin as reference standards were procured from Spectrum Pharma research solutions pvt. Ltd, Hyderabad.

Instrumentation

Agilent1290 Infinity II LC model of UPLC monitored with empower 2 data handling system and fitted with C₁₈ column (50x2.1mm, 1.7μ) and a detector of photo diode array was used for this study.

Preparation of Buffer

1ml of formic acid is dissolved in 1lt of HPLC grade water and filter through 0.45μ filter paper.

Preparation of Mobile phase

Add buffer and acetonitrile in 40:60 ratios, mixed thoroughly, sonicated for 5min and filtered through 0.45μ filter paper and used as mobile phase.

Diluent

Mobile phase was used as diluent.

Chromatographic conditions

Chromatographic separation, using C₁₈ column (50 x 2.1 mm, 1.7 micron), was administered in isocratic mode at room temperature. As a mobile phase, a mix of 0.1 percent formic acid and acetonitrile at 40:60 v/v with a flow of 1.0 ml/min was used. 5μl was the injection rate and the run time was 10 minutes.

Preparation of standard solution (Ezetimibe 50ng/ml and Pitavastatin 20ng/ml):**Preparation of Ezetimibe parent stock solution**

Accurately weigh and transfer 5mg of Ezetimibe working standard into 100ml volumetric flask and add app. 70ml of diluent, sonicate to dissolve it for 30min. and made up to the mark with diluent. Take 4 ml of the above solution and transferred into 10ml volumetric flask and made up to the mark with diluent.

Preparation of Pitavastatin parent stock solution

Accurately weigh and transfer 5mg of Pitavastatin working standard into 100ml volumetric flask and add approximately 70ml of diluent, sonicate to dissolve it for 30min. and made up to the mark with diluent. Take 1.6 ml of the above solution and transferred into 10ml volumetric flask and made up to the mark with diluent.

Preparation of Ezetimibe and Pitavastatin stock solution

Take 1ml of the Ezetimibe parent stock solution and 1 ml of Pitavastatin parent stock solution into 100ml volumetric flask and made up to the mark with diluent.

Preparation of Ezetimibe and Pitavastatin standard solution

For standard preparation, 200μl of plasma sample, 300μl of acetonitrile and 500μl of internal standard, 500μl of diluents and 500μl of standard stock to precipitate all the proteins and mix in the vortex cyclo mixture. Centrifuge at 500 rpm for 30 min. Collect the supernatant solution in HPLC vial and inject into the chromatogram.

Preparation of sample solution (Ezetimibe 25ng/ml and Pitavastatin 10ng/ml)

For sample preparation, 200µl of plasma sample, 300µl of acetonitrile and 500µl of internal standard, 500µl of diluents and 500µl of sample stock to precipitate all the proteins and mix in the vortex cyclo mixture. Centrifuge at 500 rpm for 30 min. Collect the supernatant solution in HPLC vial and inject into the chromatogram.

Preparation of Lovastatin (IS) stock solution

Accurately weigh and transfer 5mg of Lovastatin working standard into 100ml volumetric flask and add app 70ml of diluent, sonicate to dissolve it for 30min. and made up to the mark with diluent. Take 0.2ml of the above solution and transferred into 10ml volumetric flask and made up to the mark with diluent. Further transfer 0.8 ml of the above solution into 10 ml flask and made up to the mark with diluents.

Pharmacokinetic Study**Animals' parameters**

In this study six healthy white rabbits (body weight app. 2500grams) were obtained from Bioneds India pvt ltd, Bengaluru. The protocol of animal study was approved by institute of animal ethics committee (Reg.No:1074/PO/Re/S/05/CPCSEA).

Bio analytical Method validation

The method was validated in selective, sensitive, linearity, accuracy and precise, matrix condition, recovery study, re-injection reproducibility and stability.

Selectivity

By analyzing the six different rabbit's plasma samples and to check interference at the retention time selectivity was conducted.

Matrix effect

By comparing the height area ratio from the six various drug free plasma samples for Ezetimibe and Pitavastatin to get matrix effect. Experiments were performed at MQC levels in triplicate with six different plasma lots with the suitable precision of $\leq 15\%$.

Precision and accuracy

It was determined by replicate analysis of internal control samples at a lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) levels. The half of CV should be less than 15 % and accuracy should be within 15% except LLOQ where 20%.

Recovery

The analysis of six samples reproduce at each internal control concentration is by extracting the Ezetimibe and Pitavastatin. By comparing the height areas of extracted standards to the height areas of un-extracted standards, recovery is evaluated.

Carry over

Carry over deals with the analyte retained by the chromatographic system during the matrix with an analyte concentration ULOQC and above the diluting this sample with blank matrix.

Dilution integrity

By spiking the matrix with an analyte concentration above the ULOQC and diluting this sample with blank matrix, the dilution integrity should be explained.

Stability

By comparing the act of stock solution stability under the stability sample with the sample from the fresh stock sample preparation. Sample Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is smaller amount than 15 % as per US FDA guidelines. The perfectness of spiked rabbit plasma stored at room temperature was evaluated for twenty four hrs. The stability of spiked rabbit plasma stored at RT in auto sampler was evaluated for twenty four hrs. The auto sampler stability(LQC,MQC and HQC) was evaluated by comparing the extract plasma samples that were injected immediately, with the samples that were re-injected after storing with wet extract stability at room temperature after 12 h and 18 h at 2-8°C . the reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately, with the samples that were re injected after storing in the dry extract stability at room temperature after 12 h and 18 h at $-20^{\circ}\pm 3^{\circ}\text{C}$. The freeze thaw stability was conducted by comparing the steadiness samples that had been frozen at -31°C and thawed 3 times, with freshly spiked internal control samples. The short term stability was conducted 7 days at 7°C . For long term stability evaluation the concentrations obtained after 24 h were compared with initial concentration.

Pharmacokinetic Study

Before experimentation all animals are starved overnight and had water ad-libitum. Topical anesthetic procedure was used. Pharmacokinetic evaluation was performed for Ezetimibe and Pitavastatin formulations. The samples were administered to each rabbit under fasting conditions. After oral administration of Ezetimibe and Pitavastatin, blood samples were collected from rabbit marginal ear vein using a 25-gauge, 5/8 inch needle by clipping the marginal ear vein with a paper clip shown in figure 2 with volume of 0.5 ml to 1.0 ml at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24hrs. The blood was collected in Eppendorf containing 10% EDTA solution. Blood was centrifuged at 5000 rpm for 30 mins at 2-8°C temperature. The clear supernatant plasma were collected & stored at -30°C till its analysis. The plasma samples were treated for liquid-liquid phase extraction and analyzed for drug content with developed analytical method. After the study the animals were returned to animal house for rehabilitation.

The pharmacokinetic parameters for Ezetimibe and Pitavastatin oral administration were determined from plasma concentration data. Pharmacokinetic parameters like AUC, $C_{\max}$, $T_{\max}$ the time at which $C_{\max}$ occurred, K_{el} , $t_{1/2}$, K_a and MRT were calculated using the data. Data was measured by the trapezoidal rule method from time zero to infinity of concentration-time curve. $C_{\max}$ and $T_{\max}$ were obtained from the graph. All values are expressed in mean $\pm$ SD.

3. Results and Discussion

To acquire the best chromatographic conditions, we used different buffers with acetonitrile as mobile phase in different ratios for isocratic and gradient mode was tested. The mobile phase composition was modified at each trial to enhance the resolution and also to achieve acceptable retention times. Finally, 0.1% formic acid and ACN in isocratic mode at 40:60 v/v ratios was selected as mobile phase because it gives a maximum response of the selected drugs. In the optimization method we used different stationary phases like C₁₈, C₈ and CN-propyl was used. From the different trials we get good peak shapes of Ezetimibe and Pitavastatin by using C₁₈ column of dimensions 50mmx2.1mm, 1.7 μ connected to a PDA detector. Mobile phase flow rates were performed at 1 mL/min. By applying the above conditions, we get the retention times of the two drugs Ezetimibe and Pitavastatin were 7.963 min and 6.838 min respectively. We get 0.40% Ezetimibe and 0.10% of Pitavastatin as % CV of six replicate injections; from the % CV results we conclude that the suggested strategy is very specific. The method under progress has been validated pursuant to USFDA guidelines.

Figure 1. PDA spectra of Ezetimibe and Pitavastatin

Table 1. Optimized conditions of chromatography

Parameter	Suitable condition
Stationary Phase	C ₁₈ (50x2.1mm, 1.7 μ)
Mobile Phase	Acetonitrile : 0.1% formic acid (60:40)
Injection volume	5 μ l
Flow rate	1.0 ml/min
Column temperature	25°C
Wave length	236 nm
Run time	10 min.
Retention time of Ezetimibe	7.963 min.
Retention time of Pitavastatin	6.838 min.
Retention time of Lovastatin (IS)	2.946 min.

Specificity

The specificity of the method to research Ezetimibe and Pitavastatin simultaneously is proved. The chromatograms of blank, standard and internal standard as shown in figure 2, 3 and 4. The chromatograms of blank rabbit plasma and standard having no interference peaks were observed.

Figure 2. Chromatogram of Standard

Figure 3. Chromatogram of blank

Figure 4. Chromatogram of blank plasma spiked with internal standard

Matrix Effect

The ion suppression/enhancement %CV in the signal was found to be 1.0% at MQC level for Ezetimibe and Pitavastatin. It indicates that the matrix effect on the ionization of analyte is within the acceptable limit. In matrix effect LQC and HQC of Ezetimibe were 99.89 and 100.24 and Pitavastatin were 100.32, 100.15%. %CV of the both drugs at LQC level were 1.59, 1.78 and HQC level is 0.89, 0.52 respectively. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

Linearity

From the calibration curve, it was clear that the peak area ratios were proportional to the concentration. The concentration range of Ezetimibe is 5.0-100ng/ml and 2-40ng/ml of Pitavastatin. The calibration curves were appeared linear and correlation coefficient was found to be 0.999 for Ezetimibe and Pitavastatin. Linearity results of Ezetimibe and Pitavastatin are shown in table 2.

Table 2. Linearity results of Ezetimibe and Pitavastatin

Linearity	Ezetimibe		Pitavastatin	
	Conc. ng/ml	Peak response	Conc. ng/ml	Peak response
1	5.00	6259	2.00	1522.000
2	12.50	16584	5.00	3925.000
3	25.00	31287	10.00	7551.000
4	37.50	47569	15.00	10765.000
5	50.00	63254	20.00	14229.000
6	62.50	79635	25.00	17564.000
7	75.00	93521	30.00	21264.000
8	100.00	122659	40.00	27947.000
Slope	0.0231		0.0131	
Intercept	0.00087		0.00242	
CC	0.99967		0.99964

Figure 5. Calibration plot of Ezetimibe

Figure 6. Calibration plot of Pitavastatin

Precision and Accuracy

The inter-run and accuracy were determined by pooling all individual assay results of replicate quality control over five separate batch runs analyzed on four different days. The %CV of inter-run precision was <5% and the value of inter-run accuracy were in between 85 and 115 for Ezetimibe and Pitavastatin. From the data given in the following table 3, 4. It was clear that the method is precise and accurate.

Table 3. Precision and accuracy of Ezetimibe

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	5 ng/ml	25 ng/ml	50 ng/ml	75 ng/ml
QC sample -1	6235	31142	63529	93456
QC sample -2	6198	31578	63124	93740
QC sample -3	6207	31659	63857	93851
QC sample -4	6224	31027	63765	93376

QC sample -5	6189	31246	63329	93451
QC sample -6	6199	31285	63058	93652
Mean	6209	31323	63444	93588
SD	17.466	247.253	330.289	188.417
%CV	0.28	0.79	0.52	0.20
Accuracy (%)	97.37	98.24	99.49	97.84

Table 4. Precision and accuracy of Pitavastatin

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	2 ng/ml	10 ng/ml	20 ng/ml	30 ng/ml
QC sample -1	1574	7536	14775	21643
QC sample -2	1569	7531	14096	21098
QC sample -3	1493	7581	14052	21457
QC sample -4	1482	7493	14875	21536
QC sample -5	1528	7500	14653	21857
QC sample -6	1553	7569	14356	21265
Mean	1533	7535	14468	21476
SD	38.994	35.434	351.598	269.969
%CV	2.54	0.47	2.43	1.26
Accuracy	104.19	102.43	98.33	97.31

Recovery

For recovery determination low, medium and high quality control concentration levels for Ezetimibe and Pitavastatin were prepared and the obtained areas for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery of Ezetimibe and Pitavastatin were 100.54%, 101.57% and %CV were 1.74%, 2.56%.

Ruggedness

The percent recoveries and percent CV of Ezetimibe and Pitavastatin determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The results proved method is ruggedness. The percent recoveries ranged from 97.89 – 102.83% for Ezetimibe and 98.35% -103.88% for Pitavastatin. The results proved method is ruggedness.

Auto sampler carryover

Peak area response of Ezetimibe and Pitavastatin, wasn't observed within the blank rabbit plasma samples after successive injections of LLQC and ULQC at the retention times of Ezetimibe and Pitavastatin.

Stability

In solution stability analysis, Ezetimibe and Pitavastatin solutions were prepared with diluents and put in storage at 2-8°C in a refrigerator. Fresh stock solutions were related to age stock

solutions prepared earlier 24 hrs. The stock solutions were stable up to 24h when stored in 2-8°C. Bench top and auto sampler stabilities were observed at LQC and HQC levels.

At room temperature Ezetimibe and Pitavastatin were stable in plasma for 24h, and 24h in auto sampler at 20°C. From this it was confirmed that, at LQC and HQC levels repeated freezing and thawing of plasma samples spiked with Ezetimibe and Pitavastatin did not affect their stability. From long-term stability it was clear that Ezetimibe and Pitavastatin were stable up to 24h at a storage temperature of -30°C. The overall stability results of Ezetimibe and Pitavastatin were tabulated in table 5 and 6.

Table 5. Stability results of Ezetimibe

Stability experiment spiked plasma		Spiked plasma Mean conc. (n=6, ng/ml)	Std Dev (n=6, ng/ml)	%CV
Bench top stability	LQC	25.452	0.066	0.28
	MQC	50.021	0.057	0.25
	HQC	75.518	0.039	0.23
Auto sampler stability	LQC	25.509	0.095	0.54
	MQC	50.114	0.038	0.45
	HQC	75.523	0.074	0.52
Long term stability (Day 28)	LQC	25.537	0.066	0.75
	MQC	50.312	0.037	0.34
	HQC	75.541	0.043	0.16
Freeze thaw stability	LQC	25.532	0.025	0.58
	MQC	50.129	0.064	0.3
	HQC	75.498	0.072	0.29
Wet extract stability	LQC	25.496	0.038	0.48
	MQC	50.958	0.049	0.29
	HQC	75.481	0.073	0.31
Dry extract stability	LQC	25.501	0.059	0.75
	MQC	50.009	0.061	0.33
	HQC	75.518	0.027	0.25
Short term stability	LQC	25.529	0.038	0.34
	MQC	50.463	0.060	0.26
	HQC	75.518	0.057	0.07

Table 6. Stability results of Pitavastatin

Stability experiment spiked plasma		Spiked plasma Mean conc. (n=6, ng/ml)	Std Dev (n=6, ng/ml)	%CV
Bench top stability	LQC	10.021	0.014	0.38
	MQC	20.214	0.059	1.19
	HQC	30.229	0.011	1.08
Auto sampler stability	LQC	10.127	0.075	0.79
	MQC	20.174	0.038	0.88
	HQC	30.368	0.074	1.02
Long term stability	LQC	10.218	0.065	0.47

	MQC	20.316	0.029	1.47
	HQC	30.004	0.014	0.55
Freeze thaw stability	LQC	10.429	0.077	0.31
	MQC	20.316	0.032	0.61
	HQC	30.279	0.065	1.18
Wet extract stability	LQC	10.041	0.051	0.22
	MQC	20.462	0.047	0.79
	HQC	30.318	0.043	1.16
Dry extract stability	LQC	10.218	0.087	0.38
	MQC	20.164	0.026	1.01
	HQC	30.084	0.022	0.78
Short term stability	LQC	10.032	0.015	0.34
	MQC	20.246	0.048	0.34
	HQC	30.328	0.033	1.18

Pharmacokinetic Study

The method has been validated successfully to quantify the concentration of Ezetimibe and Pitavastatin in six different rabbits, after administration of Ezetimibe and Pitavastatin sample as an oral dose, under fasting condition. After injecting the drug samples into a rabbit body, collect the samples at different time intervals like 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24hrs from the rabbit body. After that as per test method sample is prepared and injected into the chromatographic system and record the values. The evaluated pharmacokinetic parameters were C_{max} (maximum observed drug concentration-time measured 6hrs, using the trapezoidal rule), t_{max} (time to observed maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and $t_{1/2}$ (terminal half-life as determined by the quotient $0.693/K_{el}$). The ratio of test/reference for C_{mx} , AUC_{0-12} and AUC were 89.24, 93.17 respectively and found to be within the acceptable limit of 80%-125%. Table 7 shows the pharmacokinetic parameters of Ezetimibe and Pitavastatin.

Table 7. Mean pharmacokinetic parameters of Ezetimibe and Pitavastatin

Pharmacokinetic parameters	Ezetimibe	Pitavastatin
AUC_{0-t} (ng h/ml)	985 ng h/mL	248 ng h/mL
C_{max}	47 ng/mL	19 ng/mL
$AUC_{0-\infty}$ (ng h/ml)	985 ng h/mL	248 ng h/mL
$T_{1/2}$	22 hrs	12 hrs
T_{max} (h)	4 hrs	1 hr

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity

AUC_{0-t} : Area under the curve up to the last sampling time

C_{max} : The maximum plasma concentration

T_{max} : The time to reach peak concentration

$T_{1/2}$: Time the drug concentration

Figure 7. Recovery plot of Ezetimibe

Figure 8. Recovery plot of Pitavastatin

4. Conclusion

For the first time higher sensitive UPLC method was developed and validated for the determination of Ezetimibe and Pitavastatin in rabbit plasma. Here the described method is fast, rugged, reproducible bio analytical method. Simple and efficient method was developed and can be used in pharmacokinetic studies and to check the investigated analyte in body fluids.

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Conflicts Of Interest

Authors declare that there were no conflicts of interest.

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