Bio analytical method development and validation for Ezetimibe and Pitavastain and its applications to pharmacokinetic studies in Rabbit plasma by using LCMS/MS

Potturi Ramadevi*, Kantipudi Rambabu

Department of Chemistry, RVR and JC College of Engineering, Chowdavaram, Guntur – 522 019, Andhra Pradesh, India

Article History:
Received on: 06 Nov 2020
Revised on: 11 Dec 2020
Accepted on: 14 Dec 2020

Keywords:
Ezetimibe, Pitavastain, LC-MS/MS, USFDA guidelines, validation, Rabbit plasma

ABSTRACT

For the gradation of Ezetimibe and Pitavastain in rabbit plasma, a highly sensitive and simple LC-MS/MS assay was developed and witnessed. The chromatographic conditions are isocratic with a waters symmetry C18 (150 x 4.6 mm, 3.5) column in isocratic mode. The detection was carried out using a mobile phase of 0.1 percent formic acid and 60:40 acetonitrile, and the detection was carried out using MS in a positive mode of electrospray ionisation. The valid approach was checked with a linear range of 10-200 ng/ml Ezetimibe and 2-40 ng/ml Pitavastain. The intraday and interday precision values were found to be within reasonable limits. The liquid extraction process is used to remove these drugs from rabbit plasma. And these drugs have been shown to be stable in freeze-thaw, autosampler, and benchtop tests in the future. The fluid chromatography coupled mass spectrometry strategy was approved by the US Food and Drug Administration for quantification of Ezetimibe and Pitavastain in rabbit plasma using D4–ezetimibe and D4–pitavastain as within norms using LC-MS consolidated with quadrupole spectrometer by electro shower ionisation process. The aim of this study is to evaluate the applicability of this approach to ezetimibe and pitavastain at different evaluation levels while taking into account various factors such as instrument stability, precision, and accuracy, sample preparation techniques, instrument calibration, recovery, and matrix effect by using Ezetimibe and Pitavastain, as well as their internal guidelines.

INTRODUCTION

Ezetimibe is a prescription used to treat high blood cholesterol (Alenghat and Davis, 2019) and certain other lipid irregularities. Ezetimibe works by diminishing cholesterol assimilation in the intestines (Lin and Zhang, 2017; Flannigan et al., 2015). Generally, it is utilized along with dietary changes and statin (Abd and Jacobson, 2011; Lamaida et al., 2013). Alone, it is less favored than a statin. It is taken by mouth. It is likewise accessible in the fixed blends ezetimibe/simvastatin, ezetimibe/atorvastatin, and ezetimibe/ rosuvastatin. Common results incorporate upper respiratory tract (Breeze and Turk, 1984) diseases, joint pain, diarrhea (Liang et al., 2019; Dupont and Vernisse, 2009) and tiredness (Zielinski et al., 2019). Genuine results may incorporate hypersensitivity (Black, 1999), liver issues, discouragement, and muscle breakdown (Chavez et al., 2016; War-
Use in pregnancy and breastfeeding is of hazy safety. Ezetimibe works by decreasing cholesterol absorption in the intestines. Pitavastain (for the most part, as a calcium salt) is an individual from the blood cholesterol bringing down the prescription class of statins. Like different statins, it is an inhibitor of HBG- CoA reductase (Istvan et al., 2000), the chemical that catalyzes the initial step of cholesterol synthesis. Common statin-related side effects migraines (Armstrong, 2013), stomach upset, anomalous liver function (Giboney, 2005), tests and muscle cramps (Garrison et al., 2020) were like other statins. However, Pitavastain appears to prompt less muscle side effects than specific statins that are lipid-solvent because of the way that Pitavastain is water dissolvable. Figure 1 represents the structural representation of Ezetimibe and Pitavastain.

MATERIALS AND METHODS

Chemicals and reagents
Acetonitrile (LCMS grade) and formic acid (LCMS grade), water (HPLC grade) were purchased from Merck (India) Ltd, Worli, Mumbai, India. All APIs of Ezetimibe and Pitavastain as reference standards were procured from Glenmark, Mumbai.

Equipment
An HPLC system (waters alliance e-2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument (sciex) was used.

Chromatographic conditions
Chromatographic separation was administered in an isocratic mode at room temperature by using a symmetry C\textsubscript{18} column (150x4.6 mm, 3.5 microns). A mix of 0.1% Formic acid and acetonitrile in 40:60 v/v at a flow of 1.0 ml/min was used as a mobile phase. The injection volume was 10 µl, the run time was 6 min.

Preparation of standard and internal control samples
Preparation of standard stock A
Take 5mg of Pitavastatin and 25 mg of Ezetimibe working standard taken into 100ml volumetric flask and 70ml of diluent, sonicate for 15 min to dissolve the contents completely. After that, makeup to mark with diluent. Take 0.1ml of the above solution into a 10ml volumetric flask with diluent. Further, dilute 1.6ml of the above solution into a 10ml volumetric flask with diluent.

Preparation of standard stock B
Take 5mg of D\textsubscript{4}- Pitavastain and 25mg of D\textsubscript{4}- Ezetimibe working standard taken into 100ml volumetric flask and 70ml of diluent, sonicate for 15 min to dissolve the contents completely. After that, makeup to mark with diluent. Take 0.1ml of the above solution into a 10ml volumetric flask with diluent. Further, dilute 1.6ml of the above solution into a 10ml volumetric flask with diluent.

Methods validation
Selectivity
Selectivity was performed by analyzing the rabbit plasma tests from six unique rabbits to check for interference at the maintenance season of analytes.

Matrix effect
Matrix effect for Ezetimibe and Pitavastain was assessed by contrasting the tallness area proportion inside the post extracted plasma test from six diverse medication-free clear plasma tests and flawlessness reconstitution tests. Tests were performed at MQC levels three-fold with six diverse plasma lots with the appropriate exactness of \( \leq 15 \% \).

Precision and accuracy
It was determined by replicate analysis of inward control samples at a lower cutoff of measurement (LLOQ), low-quality control (LQC), medium quality control (MQC), top quality control (HQC) levels. The half of CV ought to be under 15 % and exactness should be inside 15% aside from LLOQ where 20%.

Recovery
The extraction productivity of Ezetimibe and Pitavastain is determined by analysis of six reproduce at each internal control concentration. The share recovery evaluated by comparing the height areas of extracted standards to the height areas of unextracted standards.

Carryover
During the grid with an analyte concentration above the ULOQC and diluting this sample with a transparent matrix, the analyte was retained by the chromatographic system.

integrity of dilution
Spiking the matrix with an analyte fixation over the ULOQC and diluting this sample with a simple structure should demonstrate dilution integrity.

Stability
The response of the analyte within the dependability test was compared to the world reaction of test arranged from new stock arrangement to determine stock arrangement power. Stability concentrates in plasma were performed using six replicates at the LQC and HQC fixation stages.

According to US FDA guidelines, an analyte was considered stable if the shift was less than 15%. The courage of spiked rabbit plasma kept at room temperature for 24 hours was checked. The integrity of spiked rabbit plasma stored at 2–8°C in an autosampler for 24 hours was checked. By comparing the concentrate plasma tests that were infused right away with the samples that were re-injected after being stored in the autosampler at 2-8°C for 24 hours, the autosampler steadiness was calculated.

The reproducibility of reinjection was determined by comparing extracted plasma samples that were rapidly injected with samples that were reinjected after being stored in the autosampler at 2-8°C for 24 hours. The freeze-thaw stability was tested by comparing freshly spiked internal control samples with steadiness samples that had been frozen at -30°C and thawed three times. For the freeze-thaw stability checks, six aliquots of each LOQ and HQC concentration level were used. The concentrations obtained after 24 hours were compared to the initial concentration for long-term stability evaluation.

RESULTS AND DISCUSSION

Electro shower ionization having the greatest reaction over air pressure chemical ionization mode selected in this strategy. The advancement of the instrument to offer affectability and signal stability during in fusen of the analyte inside the constant progression of the mobile phase to electro-spray ion source operated at both polarities at a flow of 1 ml/min Ezetimibe and Pitavastain give more reaction in positive ion mode in examination with negative ion mode. Figure 2 shows the blank chromatogram and Figure 3 shows the standard chromatogram.

Specificity
Interfering peaks weren’t observed at Ezetimibe, Pitavastain, Ezetimibe-D4, Pitavastain-D4 retention times within the chromatogram of blank rabbit plasma. Thus proved the specificity of the tactic to research Ezetimibe and Pitavasatin simultaneously.

Matrix effect
Percent RSD for within the signal, ion suppres-
### Table 1: Results of Linearity

| Linearity | Ezetimibe | Pitavastain |
|-----------|-----------|-------------|
|           | Conc.(ng/ml) | Area response ratio | Conc.(ng/ml) | Area response ratio |
| 1         | 10         | 0.401        | 2           | 0.082             |
| 2         | 25         | 0.926        | 5           | 0.221             |
| 3         | 50         | 1.815        | 10          | 0.424             |
| 4         | 75         | 2.724        | 15          | 0.604             |
| 5         | 100        | 3.615        | 20          | 0.826             |
| 6         | 125        | 4.515        | 25          | 1.027             |
| 7         | 150        | 5.418        | 30          | 1.278             |
| 8         | 200        | 7.131        | 40          | 1.604             |
| Slope     | 0.0095     |              | 0.0489      |                   |
| Intercept | 0.01184    |              | 0.00021     |                   |
| CC        | 0.9999     |              | 0.9992      |                   |

### Table 2: Precision and Accuracy of Ezetimibe

| QC Name | LLQC | LQC | MQC | HQC |
|---------|------|-----|-----|-----|
| Conc.(ng/ml) | 10   | 50  | 100 | 150 |
| QC sample -1 | 10.211 | 50.2 | 100.1 | 150.1 |
| QC sample -2 | 10.328 | 50.1 | 100.09 | 149.08 |
| QC sample -3 | 10.1 | 50.09 | 100.08 | 149.09 |
| QC sample -4 | 10 | 49.09 | 100.12 | 149.08 |
| QC sample -5 | 10.241 | 49.08 | 100.2 | 150.2 |
| QC sample -6 | 10.437 | 50.01 | 100.3 | 150 |
| Mean | 10.171 | 49.795 | 100.127 | 149.591 |
| SD | 0.149 | 0.489 | 0.096 | 0.560 |
| %CV | 1.147 | 0.984 | 0.096 | 0.374 |
| Accuracy | 98 | 98.62 | 99.16 | 100 |

### Table 3: Precision and Accuracy of Pitavastain

| QC Name | LLQC | LQC | MQC | HQC |
|---------|------|-----|-----|-----|
| Conc.(ng/ml) | 2 | 10 | 20 | 30 |
| QC sample -1 | 2.05 | 10 | 20.1 | 30.1 |
| QC sample -2 | 2 | 10.1 | 20 | 30.06 |
| QC sample -3 | 2.04 | 10.09 | 20.04 | 30.08 |
| QC sample -4 | 2.1 | 10.08 | 20.05 | 30 |
| QC sample -5 | 2.745 | 10.1 | 20.1 | 30.09 |
| QC sample -6 | 2.142 | 10.164 | 20.06 | 30.2 |
| Mean | 2.031 | 10.095 | 20.058 | 30.088 |
| Std.dev | 0.040 | 0.063 | 0.038 | 0.065 |
| %CV | 1.979 | 0.631 | 0.190 | 0.216 |
| Accuracy | 99 | 98 | 99 | 100 |
Table 4: Stability results of Ezetimibe

| Stability experiment spiked plasma | Spiked plasma conc. (n=6, ng/ml) | Conc. measured (n=6, ng/ml) | % CV |
|------------------------------------|----------------------------------|-----------------------------|------|
| Benchtop stability                 | LQC 50                           | 50.150                      | 1.2  |
|                                   | MQC 100                          | 100.02                      | 0.233|
|                                   | HQC 150                          | 150.211                     | 0.236|
| Autosampler stability              | LQC 50                           | 50.104                      | 0.336|
|                                   | MQC 100                          | 100.052                     | 0.325|
|                                   | HQC 150                          | 150.022                     | 0.258|
| Long term (Day 28) stability      | LQC 50                           | 50.111                      | 0.485|
|                                   | MQC 100                          | 100.356                     | 0.462|
|                                   | HQC 150                          | 150.457                     | 0.218|
| Wet extract stability             | LQC 50                           | 50.267                      | 0.269|
|                                   | MQC 100                          | 100.426                     | 0.214|
|                                   | HQC 150                          | 150.438                     | 0.482|
| Dry extract stability             | LQC 50                           | 50.108                      | 0.432|
|                                   | MQC 100                          | 100.129                     | 0.326|
|                                   | HQC 150                          | 150.254                     | 0.821|
| Freeze-thaw stability             | LQC 50                           | 50.321                      | 0.621|
|                                   | MQC 100                          | 100.265                     | 0.653|
|                                   | HQC 150                          | 150.253                     | 0.821|
| Short term stability              | LQC 50                           | 50.08                       | 0.901|
|                                   | MQC 100                          | 100.241                     | 1.230|
|                                   | HQC 150                          | 150.243                     | 1.326|

Table 5: Stability results of Pitavastain

| Stability experiment spiked plasma | Spiked plasma conc. (n=6, ng/ml) | Conc. measured (n=6, ng/ml) | % CV |
|------------------------------------|----------------------------------|-----------------------------|------|
| Benchtop stability                 | LQC 10                           | 10.562                      | 1.021|
|                                   | MQC 20                           | 20.365                      | 2.562|
|                                   | HQC 30                           | 30.451                      | 0.248|
| Autosampler stability              | LQC 10                           | 10.623                      | 0.261|
|                                   | MQC 20                           | 20.152                      | 0.328|
|                                   | HQC 30                           | 30.256                      | 3.014|
| Long term (Day 28) stability      | LQC 10                           | 10.1056                     | 2.125|
|                                   | MQC 20                           | 20.325                      | 1.026|
|                                   | HQC 30                           | 30.254                      | 0.625|
| Wet extract stability             | LQC 10                           | 10.325                      | 1.022|
|                                   | MQC 20                           | 20.425                      | 0.389|
|                                   | HQC 30                           | 30.250                      | 0.521|
| Dry extract stability             | LQC 10                           | 10.362                      | 0.326|
|                                   | MQC 20                           | 20.254                      | 0.127|
|                                   | HQC 30                           | 30.256                      | 1.238|
| Freeze-thaw stability             | LQC 10                           | 10.233                      | 2.014|
|                                   | MQC 20                           | 20.856                      | 1.023|
|                                   | HQC 30                           | 30.623                      | 0.223|
| Short term stability              | LQC 10                           | 10.241                      | 0.410|
|                                   | MQC 20                           | 20.526                      | 0.566|
|                                   | HQC 30                           | 30.286                      | 0.860|
Table 6: Pharmacokinetic parameters of Ezetimibe and Pitavastain

| Pharmacokinetic parameters | Ezetimibe          | Pitavastain        |
|----------------------------|--------------------|--------------------|
| $AUC_{0-t}$                | 1048 ng-hr/ml      | 237 ng-hr/ml       |
| $C_{max}$                  | 86.2 ng/ml         | 17.8 ng/ml         |
| $AUC_{0-\infty}$           | 1048 ng-hr/ml      | 237 ng-hr/ml       |
| $T_{max}$                  | 22 hr              | 12 hr              |
Recovery enhancement was observed as 1.0 per cent for Ezetimibe and Pitavastatin in LC-MS/MS, suggesting that under these circumstances, the matrix effect on analyte ionization is within an acceptable range of ionization.

**Linearity**

The peak area ratio of calibration standards was proportional to the concentration. The concentration range of Ezetimibe is 10-200 ng/ml and Pitavastain is 2-40 ng/ml. The calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Ezetimibe and Pitavastain. Linearity results of Ezetimibe and Pitavastain were shown in the following Table 1, and the calibration plots were represented in Figure 4.

**Precision and accuracy**

The precision and accuracy were determined by pooling all individual assay results of various internal control samples. The accuracy results of Ezetimibe in quality control samples 98.7–99.9 and Pitavastatin in quality control samples 99.1–99.9. The half of CV of Ezetimibe and Pitavastain is 5% altogether internal control samples. Basing on the given data, it had been clear that the method is precise and accurate. Precision and accuracy results were shown in Table 2 and Table 3.

**Recovery**

The recoveries for Ezetimibe (98.78%-100.70%) and Pitavastain (99.58%-100.42%) at LQC, MQC and HQC levels and % CV ranged from 0.19-0.61 for Ezetimibe and 0.82-1.23 for Pitavastain. The results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery wasn’t hooked into concentration.

**Ruggedness**

The percent recoveries and percent CV of Ezetimibe and Pitavastain determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The percent recoveries ranged from 99.82–100.78% for Ezetimibe and 99.32%-99.96% for Pitavastain. The %CV values ranged from 0.12-0.37 for Ezetimibe and 0.57-1.52 for Pitavastain. The results proved method is ruggedness.

**Autosampler carryover**

Peak area response of Ezetimibe and Pitavastain, Ezetimibe –D₄, Pitavastain –D₄ wasn't observed within the blank rabbit plasma samples after successive injections of LLQC and ULQC at the retention times of Ezetimibe and Pitavastain, Ezetimibe –D₄, Pitavastain –D₄. Therefore, this method doesn’t
exhibit autosampler carry over.

**Stability**

Ezetimibe and Pitavastain solutions were prepared with diluents and processed at 2-8°C in a refrigerator for solution stability checking. Stock solutions prepared in the previous 24 hours were compared to new stock solutions. The stock solutions were stable for up to 24 hours when stored at 2-8 degrees Celsius. At 20°C, the benchtop and autosampler stabilities were stable in plasma for twenty-four hours and in the autosampler for twenty-four hours. This confirmed that repeated freezing and thawing of plasma samples spiked with Ezetimibe and Pitavastain did not affect their stability at LQC and HQC levels. From future stability, it had been clear that Ezetimibe and Pitavastain were stable up to 24 hrs at a storage temperature of -30°C. The general stability results of Ezetimibe and pitavastain were tabulated in Table 4 and Table 5.

**Pharmacokinetic study**

After the oral organisation of the Ezetimibe and Pitavastain sample as an oral dose under fasting conditions, the method was successfully accepted to test the convergence of Ezetimibe and Pitavastain in six separate rabbits. After injecting drug samples into a rabbit body, take samples at various time intervals, such as 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 hours. The sample is then prepared and inserted into the chromatographic system according to the test process, and the results are registered. $C_{max}$ (most observed drug concentration-time measured using trapezoidal rule), $t_{max}$ (time to observed maximum drug concentration), $K_{el}$ (apparent first-order terminal rate constant determined from the semi-log plot of the plasma concentration versus time curve using the smallest amount regression tactic), and $t_{1/2}$ (terminal half-life as deduced from the semi-log plot of the plasma concentration versus time curve using the tactic of the smallest amount regression) were the pharmacokinetic boundaries tested. The proportion of test/reference for $C_{max}$, AUC$_{0–12}$ and AUC were 89.31 and 93.17 respectively and situated to be inside the reasonable limit of 80%-125%. The subsequent table shows the pharmacokinetic boundaries of Ezetimibe and Pitavastain. Pharmacokinetic parameters of Ezetimibe and Pitavastatin were tabulated in Table 6 and the recovery plots were shown in Figure 5.

**CONCLUSION**

For the first time, a more sensitive HPLC-ESI-LCMS/MS technique for detecting Ezetimibe and Pitavastain in rabbit plasma was developed and validated. The strategy described here is a tough, simple, and repeatable bioanalytical tool. The method was designed to be simple and effective, and it can be used in pharmacokinetic studies and to see the investigated analyte in body fluids.

**ACKNOWLEDGEMENT**

I am grateful to my guide for his support and assistance in completing this research project.

**Conflict of Interest**

The authors declares that they have no conflict of interest for this study.

**Funding Support**

The authors declare that they have no funding support for this study.

**REFERENCES**

Abd, T. T., Jacobson, T. A. 2011. Statin-induced myopathy: a review and update. *Expert Opinion on Drug Safety*, 10(3):373–387.

Alenghat, F. J., Davis, A. M. 2019. Management of Blood Cholesterol. *Jama*, 321(8):800–801.

Armstrong, C. 2013. AAN/AHS update recommendations for migraine prevention in adults. *American Family Physician*, 87(8):584–585.

Black, C. A. 1999. Delayed type hypersensitivity: Current theories with a historic perspective. *Dermatology Online Journal*, 5(1):7.

Breeze, R., Turk, M. 1984. Cellular structure, function and organization in the lower respiratory tract. *Environmental Health Perspectives*, 55:3–24.

Chavez, L. O., Leon, M., Einav, S., Varon, J. 2016. Beyond muscle destruction: a systematic review of rhabdomyolysis for clinical practice. *Critical Care*, 20(1):135–135.

Dupont, C., Vernisse, B. 2009. Anti-Diarrheal Effects of Diosmectite in the Treatment of Acute Diarrhea in Children. *Pediatric Drugs*, 11(2):89–99.

Flannigan, K. L., Geem, Duke, Harusato, Akihito, Denning, T. L. 2015. Intestinal Antigen-Presenting Cells: Key Regulators of Immune Homeostasis and Inflammation. *The American Journal of Pathology*, 185(7):1809–1819.

Garrison, S. R., Koroynyk, C. S., Kolber, M. R., Allan, G., Michael, Musini, Vijaya, M., Sekhon, Ravneet, K., Dugré, N. 2020. Magnesium for skeletal muscle cramps. *The Cochrane Database of Systematic Reviews*, (9):CD009402.

Giboney, P. T. 2005. Mildly Elevated Liver Transami-
nase Levels in the Asymptomatic Patient. *Am Fam Physician, 71*(6):1105–1110.

Istvan, E. S., Palnitkar, M., Buchanan, S. K., Deisenhofer, J. 2000. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. *The EMBO Journal, 19*(5):819–830.

Lamaida, N., Capuano, E., Pinto, L., Capuano, E., Capuano, R., Capuano, V. 2013. The safety of statins in children. *Acta Paediatrica, 102*(9):857–862.

Liang, Y., Zhang, L., Zeng, L., Gordon, M., Wen, J. 2019. Racecadotril for acute diarrhoea in children. *Cochrane Database of Systematic Reviews, 12*(12):CD009359.

Lin, L., Zhang, J. 2017. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunology, 18*(1):2.

Warren, J. D., Blumbergs, P. C., Thompson, P. D. 2002. Rhabdomyolysis: A review. *Muscle & Nerve, 25*(3):332–347.

Zielinski, M. R., Systrom, D. M., Rose, N. R. 2019. Fatigue, Sleep, and Autoimmune and Related Disorders. *Frontiers in Immunology, 10*:1827–1827.