Stability Indicating Method Development and Validation of the Trandolapril in Human Plasma by LCMS/MS in Bulk and Tablet Dosage Form

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Abstract: *A selective, sensitive and rapid HPLC-MS/MS method has been developed and validated for simultaneous quantification of trandolapril in human plasma using meloxicam as internal standard (IS). The analytes and IS were extracted from 200 µL of plasma by liquid-liquid extraction technique. Chromatographic separation was achieved in 5.3 min and run time was 6.5min, on a Zorbax Eclipse XDB-Phenyl 3.5µ, 7.5 x 4.6 mm using a 0.1% formic acid and acetonitrile (30/70 V/V) as mobile phase. The quantification of target compounds was performed in a positive electrospray ionization mode and multiple reaction monitoring (MRM). The proposed method was validated over the concentration ranges of 5.061-500.682 pg/ml for each analyte. The intra- and inter-day precision and accuracy results were acceptable as per FDA guidelines. Stability of compounds were established in a battery of stability studies, i.e. bench top, auto sampler, dry extract and long term storage stability as well as freeze-thaw cycles. The validated method has been successfully used to analyze human plasma samples for stability studies under various storage conditions.*

Keywords: Trandolapril, meloxicam, HPLC-MS/MS, development, validation

1. Introduction

Trandolapril is a non-sulfhydryl prodrug that belongs to the angiotensin-converting enzyme (ACE) inhibitor class of medications. It is metabolized to its biologically active diacid form, Trandolaprilat, in the liver. Trandolaprilat inhibits ACE, the enzyme responsible for the conversion of angiotensin I (ATI) to angiotensin II (ATII). ATII regulates blood pressure and is a key component of the renin angiotensin aldosterone system (RAAS). Trandolapril may be used to treat mild to moderate hypertension, to improve survival following myocardial infarction in clinically stable patients with left ventricular dysfunction, as an adjunct treatment for congestive heart failure, and to slow the rate of progression of renal disease in hypertensive individuals with diabetes mellitus and microalbuminuria or over nephropathy.

Literature survey reveals that several methods have been reported for the quantitative determination of trandolapril individually in biological fluids. However, till date no LC-MS/MS method has been reported in literature for the determination of trandolapril in human plasma. Hence, authors attempted to develop a simple and reproducible LC-MS/MS method for simultaneous quantification of these three analytes which helps the researchers for therapeutic drug monitoring and pharmacokinetics. The aim of this work is, to develop a simple, selective and sensitive method, which employs liquid-liquid extraction technique for sample preparation and liquid chromatography with electrospray ionisation-tandem mass spectrometry for simultaneous quantitation of trandolapril in human plasma. The proposed method has significant advantages over earlier reported methods like Shorter run time, Wider linearity range with a more sensitivity and Simple reproducible extraction.
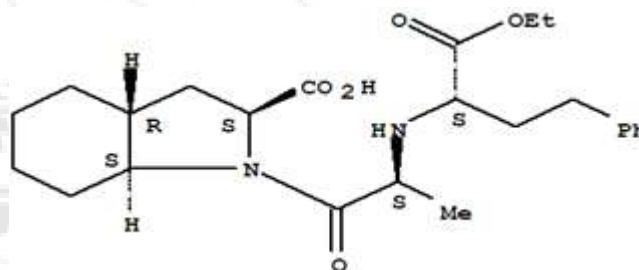

Figure 1: Chemical structures of trandolapril

2. Materials and Method

2.1 Materials and reagents

Reference standards of trandolapril (Figure. 1), internal standard (meloxicam) were purchased from Sigma-Aldrich (Hyderabad, India). HPLC grade methanol was procured from J.T Baker (Phillipsburg, USA). Milli-Q water (18.2 mΩ and TOC≤50 ppb) from Milli-Q purification system, Millipore (Bangalore, India) was used throughout the study. Drug free human plasma was procured from King George Hospital (Visakhapatnam, India).

2.2 Chromatographic and mass spectrometric conditions

Equipment used is API 4000 LC-MS/MS coupled with UFLC model separation module. Mobile phase is 0.1% formic acid and acetonitrile (30/70 V/V). The analytes and IS were separated by using Zorbax Eclipse XDB-Phenyl 3.5µ, 7.5 x 4.6 mm column maintained at 40 ± 20C with flow rate of 0.6 mL/min. The sample manager was maintained at 5°C and injection volume was 5 µL. The total chromatographic run time was 6.5 min. The analytes and IS were detected using a Waters XEVO TQ mass spectrometer

(Waters corporation, Milford, USA) equipped with Z spray source. The quantification of analytes and IS were achieved by operating the mass spectrometer in positive ion ESI with multiple reaction monitoring (MRM) mode. Nitrogen gas was used as both cone gas and desolvation gas with a flow rate of 50 L/Hr and 800 L/Hr respectively. The source dependent parameters capillary voltage, extractor voltage, source temperature and desolvation temperature were set at 3.50 KV, 3 V, 150°C and 400°C respectively.

2.3 Sample pretreatment

A simple and rapid protein precipitation method was used for the preparation of plasma samples. Required number of plasma samples from the deep freezer was retreived and thawed them at room temperature or in a water bath maintained at room temperature and vortexed the tubes to mix. Transfer 200 µL of plasma was transfered into pre-labeled tubes.100.0 µL of extraction buffer was added to all the vials and vortex for about 5 seconds. 2 mL of tertiary butyl methyl ether (TBME) was added to the all vials and vortex for a period of 10 min, interrupting the Vortexer for every 1 minute. All the vials were centrifuged at 4500 rpm, at 4°C for 5 min. About 1.8 mL of supernatant was transferred into pre-labeled tubes and the supernatant solutions were evaporated to dryness under nitrogen at 40 ± 2°C. 500.0 µL of reconstitution solution was added to all the tubes and vortexed for about 2 min.Appropriate volumes of the reconstituted solution were transferred into pre-labeled autosampler vials and 5 µL was injected into LC-MS/MS.

2.4 Preparation of calibration standards and quality control (QC) samples

Stock solutions of trandolapril and IS were prepared in methanol at 1.0 mg/ml and 100 µg/ml respectively. The standard stock solution was prepared in a concentration range of 263.95-83400.00 ng/ml, for trandolapril. The IS solution was similarly diluted to 2 µg/ml with methanol. Calibration standard samples of trandolapril were obtained by spiking 10 µl of the appropriate working solutions to 100 µl blank plasma. Quality control (QC) samples at low, medium and high concentrations (713.37, 45036 and 83400 ng/ml) were prepared separately in the same fashion. All stock solutions, working solutions and quality control samples were stored at −70 °C until used.
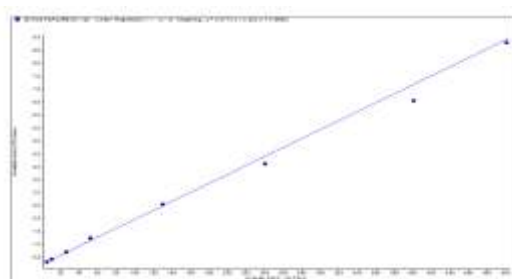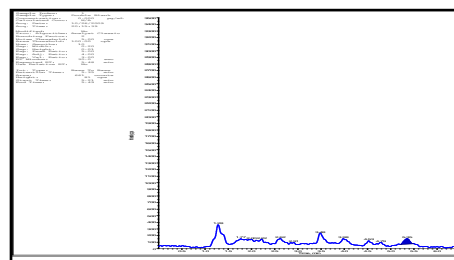

Figure 2: A Representative CalibrationCurve

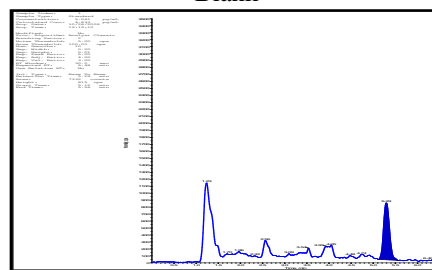**Figure 3:** A Representative Chromatogram of Standard Blank
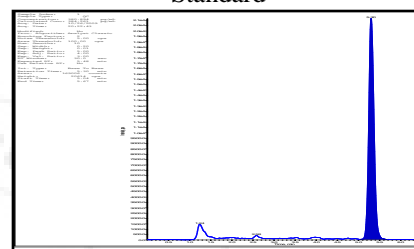

Figure 4: A Representative Chromatogram of LLOQ Standard

Figure 5: A Representative Chromatogram of HQC sample
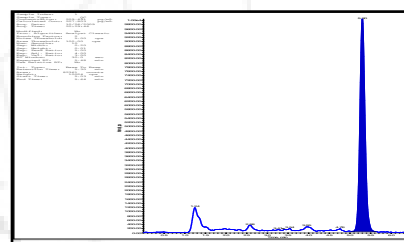
Figure 6: A Representative Chromatogram of MQC sample

Figure 7: A Representative Chromatogram of LQC sample
Figure 3-7 – MS/MS spectra of trandolapril and meloxicam (IS)

3. Stability of Analytes

3.1 Stock Solution Stability

3.1.1 Short Term Stock Solution Stability for Analyte and Internal Standard
Short term stock solution stability of the analyte was determined by storing SSHQC for07hrs 15mins at room temperature. The % mean atability was found to be 99.97%.

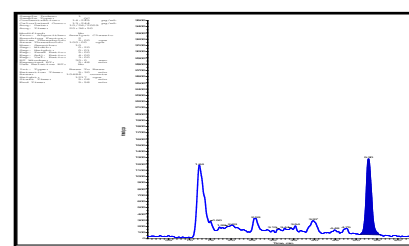Short term stock solution stability of the IS was determined by storing SSHQC for 06hrs 20mins at room temperature. The %mean stability was found to be 99.92%. The Stability was assessed by comparing with the freshly prepared solutions of both standard solution and internal standard. The acceptance limit was 90.00% - 110.00 %.

Short Term Spiking Solution Stability for Analyte and Working Solution Stability for Internal Standard:

Short term stock solution stability of the analyte was determined by storing SSHQC and SSLQC for 07hrs 10 mins at room temperature. The % mean atability was found to be 100.20% and 99.49%. Short term stock solution stability of the IS was determined by storing for 06hrs 30mins at room temperature. The %mean stability was found to be 100.78%.The Stability was assessed by comparing with the freshly prepared solutions of both standard solution and internal standard .The acceptance limit was 90.00% - 110.00 %.

3.1.2 Long Term Stock Solution Stability for Analyte and Internal Standard:
Long term stock solution stability of the analyte was determined by storing SSHQC for 11days 16hrs 20 mins at 5±3º c. The % mean atability was found to be 99.79%. Long term stock solution stability of the IS was determined by storing for 11 days 16hrs 20mins at 5±3ºc. The %mean stability was found to be 99.98%. The Stability was assessed by comparing with the freshly prepared solutions of both standard solution and internal standard .The acceptance limit was 90.00% - 110.00 %.

Long Term Spiking Solution Stability for Analyte and Working Solution Stability for Internal Standard:
Long term stock solution stability of the analyte was determined by storing SSHQC and SSLQC for11days 1hr at 5±3º c. The % mean atability was found to be 100.18% and 101.52%. For IS it was determined by storing for 11 days 1hrs 45mins at 5±3ºc. The %mean stability was found to be 99.86%. The Stability was assessed by comparing with the freshly prepared solutions of both standard solution and internal standard. The acceptance limit was 90.00% - 110.00 %.

3.2 Stability of Analyte in Plasma:

Stability studies in plasma were conducted in the various conditions using six replicates of LQC and HQC samples, as described below:

3.2.1 Freeze Thaw Stability:
Freeze thaw stability of the spiked quality control samples was determined after four freeze thaw cycles stored at -28 °C ± 5 °C. Stability was assessed by comparing them against the freshly spiked calibration standards and quality control samples. The % mean stability for HQC and LQC was found to be 99.48% and 101.31% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

3.2.2 Bench Top Stability:
Bench top stability of the spiked quality control samples was determined for a period of 12 hours 24 minutes stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards and quality control samples. The % mean stability for HQC and LQC was found to be 99.65% and 105.79% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

3.2.3 Auto sampler Stability:
Auto sampler stability of the processed quality control samples was determined for a period of 80 hours 05 minutes by storing them in auto sampler maintained at temperature 5 ±3 °C. Stability was assessed by comparing against the freshly spiked calibration standards and quality control samples. The % mean stability for HQC and LQC was found to be 98.24% and 97.63% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

3.2.4 Wet Extract Stability at Room Temperature:
Wet extract stability of the spiked quality control samples was determined for a period of 24 hours 06 min by storing them at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards and quality control samples. The % mean stability for HQC and LQC was found to be 98.28% and 106.30% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

3.2.5 Wet Extract Stability at Refrigerated Temperature:
Wet extract stability of the spiked quality control samples wa888s determined for a period of 97 hours 55 min by storing them at 5 ±3 °C. Stability was assessed by comparing them against the freshly spiked calibration standards and quality control samples. The % mean stability for HQC and LQC was found to be 99.48% and 105.78% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

3.2.6 Dry Extract Stability
Dry extract stability of the spiked quality control samples was determined for a period of 49 hours 55 min by storing them at -28 ±5 °C. Stability was assessed by comparing the stability samples against the freshly spiked calibration standards and quality control samples. The % mean stability for HQC and LQC was found to be 100.06% and 93.21% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

3.2.7 Stability of Analyte in blood at Room Temperature
Stability of analyte in blood was determined at room temperature for a period of 03 hours and 05 minutes. Stability was assessed by comparing them against the freshly prepared samples in blood. The % mean stability for HQC and LQC was found to be 101.00% and 98.43% respectively at room temperature, which is within the acceptance limit of 85.00% - 115.00 %. The data of stability were summarized below in Table 1.

Table 1: Stability of trandolapril under various storage conditions

	Concentration (pg/ml)	RSD (%)	%mean stability
Room temperature at 7 hrs 10 mins	380.89	1.38	100.20
	14.25	3.99	99.49
Storing at a period of 11 days 1 hr	380.89	1.19	100.18
	14.25	1.88	101.52
Freeze thaw stability stored at -28˚±5˚c	380.89	1.90	99.48
	14.25	5.94	101.31
Bench top stability stored at a period of 12hrs 24mins	380.89	3.43	99.65
	14.25	10.36	105.79
Auto sampler stability at 80 hrs 5mins	380.89	2.70	98.24
	14.25	5.78	97.63
Wet extract stabilityat 24 hrs 6 mins	380.89	3.69	98.28
	14.25	5.68	106.30
Wet extract stability at refrigerated temperature	380.89	1.07	99.48
	14.25	9.64	105.78
Dry extract stability at 49hrs 55mins	380.89	1.81	100.06
	14.25	11.98	93.21
Stability of analyte in blood at room temperature	380.89	8.24	101.0
	14.25	0.57	98.43

3.3 Anticoagulant Effect

Anticoagulant Effect was assessed by the spiked calibration standards and freshly prepared quality control samples in K3 EDTA human plasma. The % mean stability for HQC and LQC was found to be 103.83% and 106.30% respectively, which is within the acceptance limit of 85.00% - 115.00 %.

4. Results and Discussion

4.1 Method development

4.1.1 Optimization of chromatographic and mass conditions

In order to optimize MS condition, a standard solution of the trandolapril and IS were directly infused along with the mobile phase into the mass spectrometer withelectrospray ionization(ESI) asthe ionization source. Compared with the response observed in positive ion mode and negative mode, we discovered that trandolapril and IS had stronger signal intensity and lower background noise in positive ion mode. Therefore, detection was performed in positive ion mode in this study.

Chromatographic conditions played a critical role in achieving good chromatographic behavior. In this study, acetonitrile was chosen as the organic phase because of the advantage of higher responses and lower background noise by comparison with formic acid. Previous study indicates that low concentration of acid solution can facilitate the protonation and improve the intensity of basic compounds in the positive mode. Different concentrations of formic acid (0.05%, 0.1% and 0.2%) were added to acetonitrile as different mobile phases. The shape of peak and intensities of trandolapril and IS were improved significantly when 0.1% formic acid was added in the mobile phase.

4.1.2 Optimization of sample preparation

Sample preparation is an important step for accurate and reliable LC–MS/MS assay. Several sample pre-treatment methods were investigated in this study, including protein precipitation and liquid–liquid extraction (LLE). However, the liquid–liquid extraction with tertiary butyl methyl ether showed limited extraction recovery efficiency, considering the polarity of the analytes. Thus, the protein precipitation method which was simpler and less time consuming was finally used. Different precipitation organic solvents were evaluated. Results showed that a precipitation with acetonitrile provided a higher recovery and perfect peak shape for both trandolapril and IS.

4.1.3 Selection of internal standard

According to the US Food and Drug Administration (FDA) guidance, any IS used in biological analysis should be a structurally similar analog of the analytes or a stable labeled compound. In this study ramipril and meloxicam were tested. Where ramipril has given unsatisfactory response. Therefore, meloxicam was chosen as the IS, because of the high ionization response in ESI mass spectrometry and a similar chromatographic retention time.

4.2 Method validation

4.2.1 Specificity

The detection of trandolapril and IS by MRM mode was highly selective with no interference from the endogenous substances. Fig.5 represents typical chromatograms of blank plasma, blank plasma spiked with trandolapril at LLOQ and IS. Typical retention times for trandolapril and IS were 5.3 min and 1.24 min, respectively.

4.2.2 Linearity and LLOQ

The calibration curves showed good linearity over the concentration range of 0.5–5000 ng/ml in human plasma. A typical equation of the calibration curves was $y = 0.0173x + 0.253$ ($r = 0.9969$), where y was the peak area ratio of trandolapril to IS and x was the concentration of trandolapril. The LLOQ for trandolapril in human plasma was 5.061pg/ml.

4.2.3 Recovery and matrix effect

The extraction recovery was in the range of 85.0–115.0% for trandolapril, which could meet the requirements of analysis. Results of matrix effects indicated that no significant matrix effects were observed for the analytes.

4.2.4 Precision and accuracy

The results of intra- and inter-day precision and accuracy at three concentration levels of the QC samples (380.89, 209.49,14.24 and 5.27pg/ml) were summarized in Table 2. The intra- and inter-day RSD were measured to be below 13.21% and 15.64%

The results demonstrated that both the intra- and inter-day assay values were all within the acceptable range.

4.2.5 Stability

Trandolapril had no effect on stability in autosampler (80 hrs 5mins) through repeated four freeze-thaw cycles and under the frozen condition at −28±5˚C.

Table 2: Precision and accuracy

	Pg/ml	Intra day	Inter day	Accuracy	%Recovery
HQC	380.89	3.27	2.37	101.56	82.87
MQC	209.49	4.21	3.9	103.96	89.32
LQC	14.24	8.92	13.02	94.67	87.15
LLQC	5.27	13.21	15.64	107.07	86.44

5. Conclusion

5.1 Conclusion on the Chromatographic Method

Based on the experiments done during the course of validation, it can be concluded that the intended method is validated for the estimation of Trandolapril in human plasma over the concentration range of 5.061-500.682 pg/ml. The precision and mean accuracy are within the acceptable limits. Consistent recoveries are observed for LQC, MQC and HQC. The method is specific enough in the presence of K_2EDTA anticoagulant. The method is precise and accurate enough to dilute the samples, if necessary.

This method can be used for quantification of Trandolapril in human plasma for Bioequivalence studies.

5.2 Conclusion on Stability of Analyte

Based on the stability experiments carried out during the course of validation, it can be concluded that the intended analyte is stable in all the performed experiments and the stability experiments performed are within the acceptable limits.

6. Acknowledgements

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