Development and validation of RP-HPLC method for determination of Lornoxicam in rabbit's plasma

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Abstract: A simple, rapid and accurate reverse phase high performance liquid chromatographic (RP- HPLC) method was developed for the quantification of lornoxicam in oral disintegrating tablets (ODTs) and in rabbit's plasma. C18 Hypersil™ column was used as stationary phase to separate the drug. Mobile phase methanol: acetonitrile: water (60:30:10) was run isocratically at flow rate of 1 mL/min at room temperature. Mean retention time was 4.23 minutes and minimum amount of lornoxicam that can be measured was 7 ng/mL in rabbit's plasma. Good linearity was observed in concentration range of 10-100 ng/mL with regression coefficient R^2 value of 0.9989 and slope value 23773. As per ICH norms, developed method was validated in terms of interday, intraday precision, accuracy, specificity, limit of detection (LOD), limit of quantification (LOQ) and drug plasma stability studies. All the data obtained revealed that this method can be used for *in-vitro* and *in-vivo* determination of lornoxicam in various pharmaceutical preparations.

Keywords: Lornoxicam, RP-HPLC method, pharmacokinetic evaluation, validation.

INTRODUCTION

Lornoxicam an oxicam derivative (6-chloro-4-hydroxy-2-methyl-N-2-pyridinyl-2H-thieno[2,3-e]-1,2-thiazine-3-carboxamide 1,1-dioxide) having chemical formula $C_{13},H_{10}N_3O_4S_2Cl$ is a potent nonsteroidal anti-inflammatory drug (Lorenz *et al.*, 2008). Its shows strong analgesic and antipyretic effects due to nonselective inhibition of cyclo-oxygenase -1 and 2 (Atzpodien *et al.*, 1997; Prajapati *et al.*, 2012). Unlike other oxicams, it has more efficacy as it also acts as central analgesic and anti-inflammatory by increasing endogenous concentration of dinorphin and beta-endorphin (Zaid *et al.*, 2017). Lornoxicam is prescribed for indications like rheumatoid arthritis, osteoarthritis, sciatica and postoperative pain management (Hadi *et al.*, 2015; Tawfeek *et al.*, 2014).

Lornoxicam is available in tablet and injection dosage form. There is no monograph given in any pharmacopeia but literature reveals different methods for the quantification of lornoxicam including U.V spectrophotometric (Sivasubramanian *et al.*, 2010; Kondawar *et al.*, 2011; Warkar and Rele, 2011), polarographic (Çetin *et al.*, 2009), voltametric (Ghoneim *et al.*, 2002), TLC-densitometric (Ghoneim *et al.*, 2002), high performance liquid chromatographic (Patil *et al.*, 2009) and LC-MS/MS methods (Kim *et al.*, 2007; Li *et al.*, 2015). Various HPLC methods have been reported for the analysis of lornoxicam *in-vitro* (Attimarad, 2010). Dasharath *et al* developed and validated a spectrophotometric method for the simultaneous determination of Panadol and lornoxicam by using various dissolution medias (Patel *et al.*, 2012). Mahesh Attimarad developed and validated a RP-HPLC method for the simultaneous determination of lornoxicam and paracetamol in tablets by using methanol and phosphate buffer as mobile phase (Attimarad, 2011). Oral dispersible tablets of lornoxicam were developed and evaluated for different *in-vitro* characteristics. There was a task to establish different pharmacokinetic parameters and bioavailability studies for these oral disintegrating tablets as well. Purpose of current research work is to develop and validate more accurate, sensitive and simple reverse phase HPLC method for quantification of lornoxicam in oral dispersible tablets and as well as in rabbit's blood plasma to establish its pharmacokinetic parameters. This developed method was economical, simple and rapid that can be used for *in-vitro* and *in-vivo* quantitative determination of lornoxicam.

MATERIALS AND METHODS

Chemical and reagents

Acetonitrile and methanol of HPLC grade were purchased from Sigma Aldrich (Switzerland). Lornoxicam of 99.8% was gifted by Pharm-Evo Pharmaceuticals Karachi, Pakistan. Orthophosphoric acid (HPLC grade) was purchased by Merck. Double distilled water and other solvents were used after filtration by using 0.45µm Whatman filter paper.

Equipment

The HPLC system used for lornoxicam analysis was Agilent Technologies 1100 Series, Germany. Lab solution

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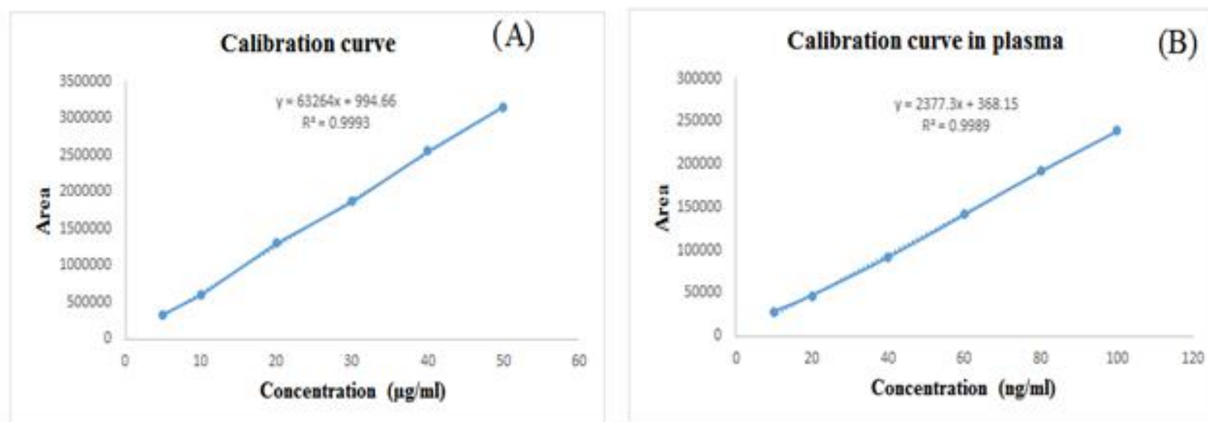

Fig. 1: A) Calibration curve for standard solutions of lornoxicam, B) calibration curve in rabbit's plasma

software was applied to monitor and integrate output signals.

Preparation of standard and sample solutions

To prepare standard stock solution of lornoxicam having strength of 100 µg/mL, 10 mg of drug was dissolved in methanol and final volume was made in 100 ml volumetric flask. From this stock solution, six dilutions of 5µg/mL to 50 µg /ml were prepared as standard solutions. For sample preparation, 20 tablets from previously optimized formulation of oral dispersible tablets of lornoxicam were taken and weighted to calculate average weight. Then these tablets were crushed with the help of pestle and mortar to make homogeneous powder. A weighted amount of powdered tablet equivalent to 4 mg of lornoxicam was transferred to 100 mL volumetric flask and dissolved in methanol. All the standard and sample preparations were filtered before further use.

Development of standard graph

After filtration, 20 µl of each dilution of standard preparation was injected (n=6). Mobile phase was run at 1mL/min and mean area of peak absorption was calculated. For calibration curve, graph was plotted between standard solution`s strength on x-axis and peak area on y-axis.

RP-HPLC method development in rabbit's plasma

This method was also applied to evaluate *ex-vivo* pharmacokinetic parameters of lornoxicam in rabbit's plasma. Method reported by Li *et al*. (2015) was slightly modified. Blood sample taken in heparin containing tubes was centrifuged at 5000 rpm for 5 mins and obtained plasma was stored at -40°C for further use. 1 mL of 1% orthophosphoric acid (HPO_3) was added to 0.5mL of plasma. This solution was diluted with100 µl of standard drug solutions ranging from 10 ng/mL to 100 ng/mL and shaken for 5 mins. A solution of hexane, dichloromethane-isopropanol in ratio of (20:10:1) was added to extract lornoxicam from plasma by centrifugation at speed of 4000 rpm for 15 mins. The supernatant was collected in tubes and then organic solvents were subjected to evaporate under reduced pressure. The dried residue was diluted with 100µl of mobile phase, mixed vigorously for 1 min followed by centrifugation at 10,000 rpm for 5 mins. From supernatant, 20 µl was injected to HPLC each time for analysis. Peak area was calculated to construct calibration curve by plotting concentration against peak area (Moutasim *et al*., 2017).

Drug plasma stability studies

Instability of drug in biological samples is a key problem as pH, presence of different enzymes and freeze and thawed cycles can degrade the drug. So long term, short term and freeze and thawed stability studies were performed to demonstrate the stability of drug (Nováková and Vlčková, 2009). To analyze the drug plasma stability studies, blank plasma was spiked with standard drug solutions of strengths 20, 40 and 80 ng/mL. Then these freshly prepared spiked plasma solutions were analyzed and remaining were stored at freezing temperature of -20°C for four weeks. At specific time intervals a set of samples were taken, thawed and analyzed while keeping the remaining samples frozen (Radhofer-Welte and Dittrich, 1998).

RESULTS

Chromatographic conditions

In current work, different mobiles phases with various concentrations were tried to get smooth chromatograph. Initially methanol water (50:50, 65:35, 80:20), methanol and acetonitrile (50:50, 65:35, 80:20) and methanol, acetonitrile and in water in different ratios were used, but no clear resolution was achieved. The optimized conditions used to obtain clear chromatogram are given in table 1.

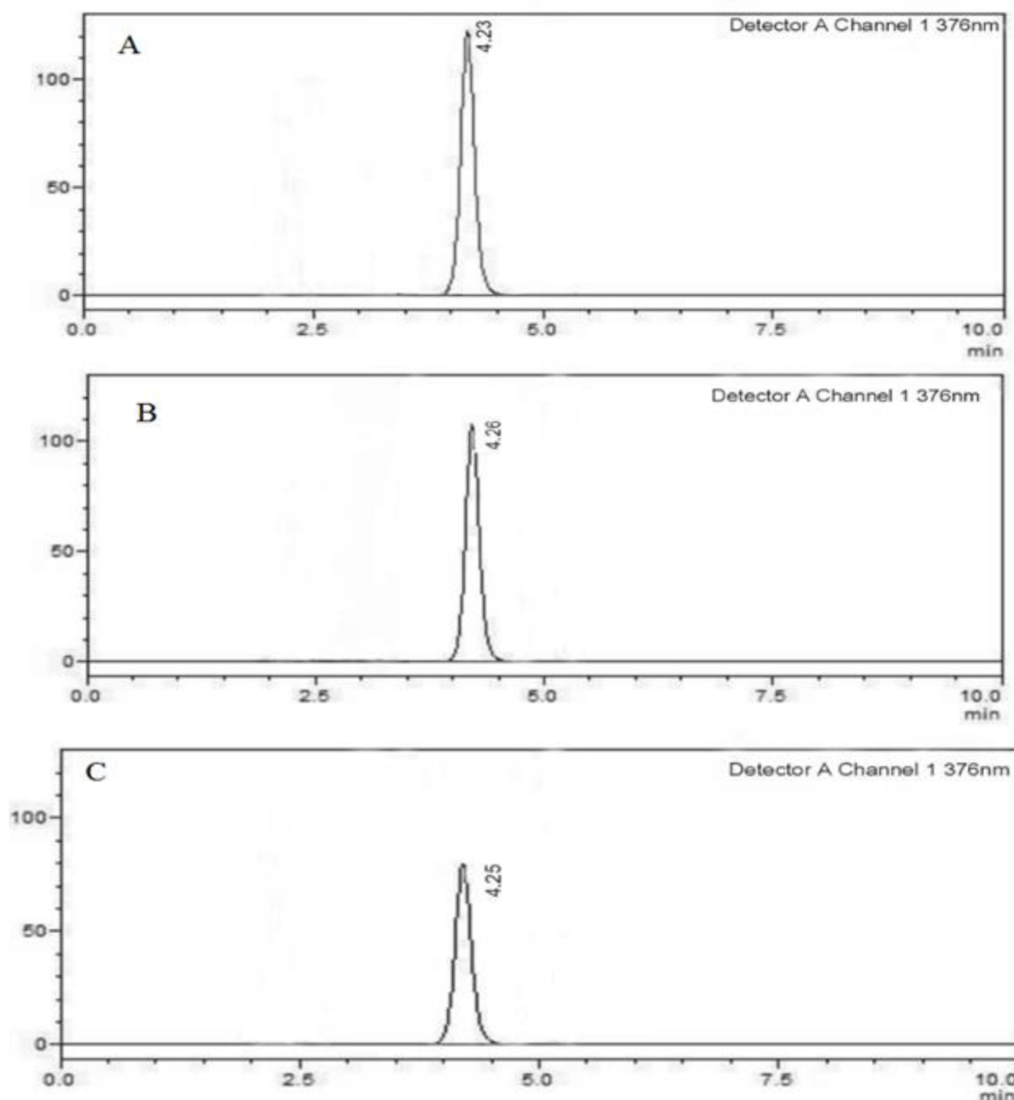

Fig. 2: Chromatogram of A) standard drug solution, B) sample formulation, C) standard drug in rabbit's plasma

Linearity of calibration curve

As mentioned above, different mobile phases in different concentrations were tried to get clear chromatogram. Among all, a satisfactory chromatogram was obtained when methanol, acetonitrile and water in ratio of 60:30:10 was used. Linearity in calibration curve was obtained with regression coefficient R^2value of 0.9993 and slope 63264 as shown in figure 1 by plotting a graph of data given in table 2. In rabbit's plasma good linearity was observed in concentration range of 10-100 ng/mL with regression coefficient R^2 value of 0.9989 and slope value 2377.3 (n=3). Statically analysis of calibration curves in-vitro and ex-vivo in rabbit's plasma has been elaborated in table 3.

System suitability

According to USP recommendations, system suitability is a crucial parameter to validate a method. 6 replicates of each of two doses 30 μg/mL *in-vitro* and 30 ng/mL in blood plasma were injected to demonstrate system suitability. Values of number of theoretical plates, tailing factor and %RSD given in table 4 proved system suitability for developed methods of lornoxicam quantification *in-vitro.*

Precision and accuracy

Intra-day and inter-day precision were calculated to check the precision of the developed method. 6 samples of 3 different strengths each in mobile phase (3.2, 4, 4.8μg/mL) and in plasma (20, 40 and 60ng/mL) were analyzed at different time intervals of same day for inter-day precision and on 3 consecutive days for intra-day precision. %RSD values ranging from 0.291 to 0.829 and 0.706 to 1.426 for inter-day and intra-day precision respectively given in table 5 has proven the precision of this method.

Table 1: Optimized conditions for reverse phase chromatography of Lornoxicam

Parameter	Conditions
Mobile phase	Methanol : acetonitrile: water (60:30:10)
Pump mode	Isocratic (Reverse Phase)
Flow rate (mL/min)	1
Stationary phase	C18 Hypersil™ BDS C18 250 x 4.6 mm
Run time (min)	15
Wavelength (nm)	376
Volume of injection (µl)	20

Table 2: Regression analysis data for development of calibration curve

Concentration (µg/mL)	Mean Peak Area ±SD (n=6)	% RSD
5	331432 ± 2623.87	0.792
10	597361± 3462.47	0.579
20	1304149 ± 519.16	0.0398
30	1871732± 1097.39	0.059
40	2553878± 1690.53	0.066
50	3153346± 505.74	0.016

Table 3: Statistical analysis of *in-vitro* and *ex-vivo* standard calibration curve

Parameter	*In-vitro*		*Ex-vivo* (rabbit`s plasma)	
	Mean ± SD	%RSD	Mean ± SD	%RSD
Linearity	10-50 µg/mL	-	10-100 ng/mL	-
Correlation Coefficient	0.999267±0.0001	0.005168	0.9989±0.0001	0.0058
Slope	63264± 64.52	0.10198	2377.3 ± 66.73	0.281
Intercept	994.66 ± 75.2	0.756	368.15 ± 22.57	0.613

Table 4: System suitability studies *in-vitro* and in rabbit`s plasma

Condition	Mean AUC	%RSD	Theoretical Plates	Tailing Factor	Resolution	Retention time (minutes)
In-vitro	1871860	0.146325	2846	0.72	2.33	4.2
In Rabbit Plasma	716833.5	0.084646	3081	1.38	3.12	4.25

Accuracy is a parameter that shows extraction efficiency of prepared samples (Prajesh *et al.*, 2013). To measure the accuracy of method, spiked solutions were prepared by adding known amount of standard drug solution in sample lornoxicam tablet solution. % recovery studies were performed on these spiked and standard solutions. Solution of oral disintegrating tablets were analyzed and then recovery studies were performed by addition of fixed 30µg/mL standard drug solution to 3 different concentration levels of lornoxicam tablet solutions. % lornoxicam recovered range was from 98.49 to 100.56% as given in table 6.

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

As per ICH guidelines, limit of minimum quantity of drug that can be measured (LOQ) and minimum quantity of drug that can be detected (LOD) by this method were also performed. Signal to noise ratio was used in 3:1for LOQ and 10:1 ratio for LOD determination respectively. Minimum amount of detectable lornoxicam in rabbit's plasma was 0.5 ng/mL and 7 ng/mL was the minimum quantifiable amount (LOQ).

Specificity

Specificity of the method indicates that there is no interaction of excipients used in formulation with the retention peaks of drug (Zaman *et al.*, 2018). It is an important parameter, as per ICH norms. When oral disintegrated tablets were assayed by this developed method, it was proven that the excipients used in formulation had no effect on the retention peaks of lornoxicam as no peak was detected at the retention time of pure standard drug solution. Thus, the developed method proved the specificity for lornoxicam determination.

Table 5: Inter-day and intra-day precision for lornoxicam

Condition	Actual Amount	Concentration Found			
		Inter-day AUC Mean ± SD (n=6)	%RSD	Intra-day AUC Mean ± SD (n=6)	%RSD
In-vitro mobile phase	3.2	203154.8 ± 1087.52	0.535	200761.3 ± 2199.43	1.096
	4	252890.8 ± 840.88	0.333	251309.2 ± 1774.40	0.706
	4.8	302869.4 ± 2017.19	0.666	299811.6 ± 3315.58	1.106
In plasma	20	46305.51 ± 161.82	0.349	45521 ± 649.022	1.426
	40	90380.65 ± 262.94	0.291	91965.52 ± 1137.38	1.237
	60	137134.2 ± 1137.38	0.829	134360.7 ± 1222.02	0.909

Table 6: Lornoxicam recovery studies in mobile phase

Sr. No.	Amount of sample (µg/mL)	Amount of drug added (µg/mL)	Amount of drug recovered (µg/mL)	% recovered
1	30	0	29.92	-
2	30	10	39.83	99.77
3	30	20	49.17	98.49
4	30	30	60.26	100.56

Table 7: Stability studies

Spiked plasma concentration (ng/mL)	Concentration found (ng/mL)		% Degradation
	Initial conc.	Final conc.	
20	19.8	19.3	2.52
40	39.73	38.85	2.21
80	78.85	76.59	2.86

Robustness

When there were small variations made in experimental conditions like pH, composition and flow rate of mobile phase, there should be no significant effect on retention time of drug. In current research work, small changes in methanol content (± 1%) and flow rate (±5%) of mobile phase were made deliberately and the lornoxicam std. drug solutions were analyzed. Results of recovery studies, retention time, and peak area showed that the developed HPLC method for lornoxicam was robust.

Drug plasma stability studies

Spiked plasma with three different drug concentrations were analyzed at the time of preparation and remaining was frozen at -20°C. Then set of plasma samples were taken, thawed and analyzed daily for seven days. Results of concentrations found in plasma (Table 7) proved that analyte was stable for seven days at given conditions as % degradation was less than 3%.

Analysis of lornoxicam tablets

To verify the developed method, oral disintegrating tablets containing 4 mg lornoxicam were analyzed under the conditions mentioned above. The mean assay 97.82 ± 0.99 was calculated from standard calibration curve. % RSD value was 1.014 after 6 replicates. There was no interference of excipients with the retention peaks as observed for standard drug. So, it confirmed the specificity of the method also.

DISCUSSION

ICH guidelines were followed to validate the developed method of lornoxicam quantification. Linearity, its range, precision in terms of intraday and interday, accuracy, limit of detection (LOD), limit of quantification (LOQ) and system suitability were applied to validate the analytical method (Bressolle *et al.*, 1996). Data of linearity, precision, accuracy and sensitivity validated the method. Non-interference of excipients used in oral dispersible tablets with retention peaks confirmed the specificity of method. Stability studies proved that analytical solutions used in the process were stable during whole processing time.

CONCLUSION

The developed RP HPLC method is a simple, sensitive, rapid and accurate method for the determination of lornoxicam in pharmaceutical dosage forms. This method is also applicable for the evaluation of pharmacokinetic parameters in rabbit's plasma. So, it can be concluded that this method can be used for *in vitro* and *in vivo* determination and quantification of lornoxicam in available pharmaceutical preparations.

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REFERENCES

Attimarad M (2010). Rapid RP-HPLC method for quantitative determination of Lornoxicam in tablets. *J. Basic Clin. Pharm.*, **1**(2): 115.

Attimarad M (2011). Simultaneous determination of paracetamol and lornoxicam by RP-HPLC in bulk and tablet formulation. *Pharm. Methods*, **2**(1): 61.

Atzpodien E, Mehdi N, Clarke D and Radhofer-Welte S (1997). Subacute and chronic oral toxicity of lornoxicam in cynomolgus monkeys. *Food Chem. Toxicol.*, **35**(5): 465-474.

Bressolle F, Bromet-Petit M and Audran M (1996). Validation of liquid chromatographic and gas chromatographic methods Applications to pharmacokinetics. *J. Chromat. Biomed. Sci. Appl.*, **686**(1): 3-10.

Çetin I, Koçak N and Aycan S (2009). Polarographic determination of lornoxicam in pharmaceutical formulations. *CBUJ. Sci.*, **5**(1): 11-18.

Ghoneim M, Beltagi A and Radi A (2002). Square-wave adsorptive stripping voltammetric determination of the anti-inflammatory drug lornoxicam. *Ana. Sci.*, **18**(2): 183-186.

Hadi MA, Rao R, Guggilla N and Rao AS (2015). Formulation and evaluation of mini-tablets-filled-pulsincap delivery of lornoxicam in the chronotherapeutic treatment of rheumatoid arthritis. *Pak. J. Pharm. Sci.*, **28**(1): 185-193.

Kim YH, Ji HY, Park E-S, Chae S-W and Lee HS (2007). Liquid chromatography-electrospray lonization tandem mass spectrometric determination of lornoxicam in human plasma. *Arch. Pharm. Res*., **30**(7): 905-910.

Kondawar M, Shah R, Waghmare J, Shah N and Malusare M (2011). UV spectrophotometric estimation of Paracetamol and Lornoxicam in bulk drug and tablet dosage form using multi-wavelength method. *Int. J. Pharm. Tech. Research*, **3**(3): 1603-1608.

Li F, Song S, Guo Y, Zhao Q, Zhang X, Pan W and Yang X (2015). Preparation and pharmacokinetics evaluation of oral self-emulsifying system for poorly water-soluble drug Lornoxicam. *Drug Deliv.*, **22**(4): 487-498.

Lorenz I, Egger K, Schubert H, Schnürer C, Tiefenthaler W, Hohlrieder M, Schocke M, Kremser C, Esterhammer R and Ischebeck A (2008). Lornoxicam characteristically modulates cerebral pain-processing in human volunteers: a functional magnetic resonance imaging study. *Br. J. Anaesth.*, **100**(6): 827-833.

Moutasim MY, ElMeshad AN and El-Nabarawi MA (2017). A pharmaceutical study on lornoxicam fast disintegrating tablets: Formulation and in vitro and in vivo evaluation. *Drug Deliv. Transl. Res.*, **7**(3): 450-459.

Nováková L and Vlčková H (2009). A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation. *Anal. Chim. Acta*, **656**(1-2): 8-35.

Patel DM, Sardhara BM, Thumbadiya DH and Patel CN (2012). Development and validation of spectro-photometric method for simultaneous estimation of paracetamol and lornoxicam in different dissolution media. *Pharm. Methods*, **3**(2): 98.

Patil KR, Rane VP, Sangshetti JN and Shinde DB (2009). Stability-indicating LC method for analysis of lornoxicam in the dosage form. *Chromatographia*, **69**(9-10): 1001-1005.

Prajapati P, Vaghela V, Rawtani D, Patel H, Kubavat J and Baraiya D (2012). Azeotropic mixture used for development and validation of Lornoxicam in bulk and its tablet dosage form by spectrophotometric method. *J. Pharm. Anal.*, **2**(4): 306-309.

Prajesh P, Vipul V and Dharmendra B (2013). Sensitive method for rapid estimation of Lornoxicam in bulk and its dosage form by RP-HPLC. *J. Ch. Pharm. Sci.*, **21**(1):110-113.

Radhofer-Welte S and Dittrich P (1998). Determination of the novel non-steroidal anti-inflammatory drug lornoxicam and its main metabolite in plasma and synovial fluid. *J. Chromatogr. B Biomed. Sci. Appl.*, **707**(1-2): 151-159.

Sivasubramanian L, Lakshmi K and Tintu T (2010). Simultaneous spectrophotometric estimation of paracetamol and lornoxicam in tablet dosage form. *Int. J. Pharm. Sci.*, **2**(4): 166-168.

Tawfeek HM, Saleem IY and Roberts M (2014). Dissolution enhancement and formulation of rapid-release lornoxicam mini-tablets. *J. Pharm. Sci.*, **103**(8): 2470-2483.

Warkar C and Rele R (2011). Estimation of lornoxicam and diacerein in dosage form by simultaneous equation and q-analysis method using UV spectroscopic technique. *Int. J. Pharm. Biosci.*, **2**(2): 124-129.

Zaid AN, Mousa A, Jaradat N and Bustami R (2017). Lornoxicam immediate-release tablets: Formulation and bioequivalence study in healthy mediterranean volunteers using a validated LC-MS/MS method. *Clin. Pharm. Drug Dev.*, **6**(6): 564-569.

Zaman M, Hanif M, Khan NUH, Mahmood A, Qaisar MN and Ali H (2018). Development and validation of stability-indicating RP-HPLC method for the simultaneous determination of tizanidine HCl and meloxicam in rabbit's plasma. *Acta Chromatogr,* pp.1-6 (Published Online: May 07, 2018).