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Separation of Three Oxicams: Tenoxicam, Meloxicam and Lornoxicam by Ion Pair RP-HPLC

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ABSTRACT

A rapid and stability indicating ion-pair reversed phase high performance liquid chromatographic method was developed for qualitative and quantitative estimation of three oxicam drugs: tenoxicam, meloxicam and lornoxicam. The method was validated according to ICH, FDA and USP guidelines with respect to accuracy, precision, specificity, linearity, solution stability, robustness, sensitivity and system suitability. The method was developed by using an isocratic condition of mobile phase comprising buffer pH 6.5 [tetra butyl ammonium hydroxide (0.008M) and sodium 1-heptane sulfonate (0.003M)] and acetonitrile in a ratio of 65:35 v/v ratio at a flow rate of 1.5 mL/min over C-18 (ODS, 250 x 4.6 mm) column at ambient temperature. The method showed linear response with correlation coefficient (r^2) value of 0.999. The recoveries for all drugs were found more than 99% which demonstrated the accuracy of this method. Intraday and inter-day precision studies of the new method were less than the maximum allowable limit (RSD%≤ 2.0). Forced degradation studies were carried on to check its stability indicating property. All the drugs gave sharp peaks within 7min with excellent symmetry and high resolution. Therefore, a rapid, sensitive and stability indicating ion pair RP-HPLC method was developed for simultaneous separation of tenoxicam, meloxicam and lornoxicam in their any combination or in bulk raw materials.

Keywords: Ionpair, RP-HPLC, stability-indicating, tenoxicam, meloxicam, lornoxicam

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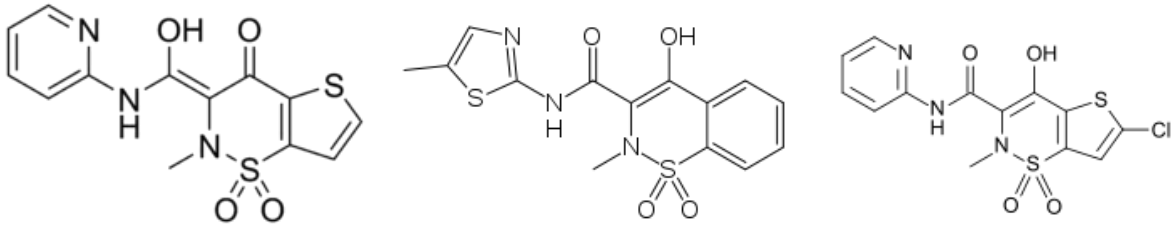
INTRODUCTION

High performance liquid chromatography (HPLC) today, has become a straightforward separation technique and it is now one of the fastest growing separation techniques in the modern laboratory. Recently, significant technological improvements in instrumentation and column packings, has made high performance liquid chromatography (HPLC) the preferred method for the separation and quantitative analysis of a wide range of samples [1-3]

Separation of drugs of closely related chemical structures can be efficiently separated and characterized by HPLC methods provided that the molecules differ in polarity, solubility profile, pKa value etc. In HPLC separation, competition for the various compounds contained in the sample is created by choosing a mobile phase and a stationary phase with different polarities. Then, compounds in the sample that are similar in polarity to the stationary phase [column packing material] will be delayed because they are more strongly attracted to the particles. Compounds whose polarity is similar to that of the mobile phase will be preferentially attracted to it and move faster. In this way, based upon differences in the relative attraction of each compound for each phase, a separation is created by changing the speeds of the analytes [4]

Based upon this principle we tried to separate three oxicam derivatives namely tenoxicam (TNX), meloxicam (MLX) and lornoxicam (LNX) in a single method. These are a class of enolic acids that have anti-inflammatory, analgesic and antipyretic activity. These three NSAIDs are nonselective COX inhibitor [5].



(a) (b) (c)

Figure1: Chemical structure of (a) tenoxicam,(b) Meloxicam, and (c) Lornoxicam

These drugs closely resemble in chemical structure and physical properties [6] For example, all three drugs has a pKa value of about 4.7, all are insoluble in water but soluble in 1N sodium hydroxide and all are pale yellow to yellow crystalline powder [7,8] For the determination tenoxicam and meloxicam, there is established method in British Pharmacopoea [9]. Lornoxicam is still an INN drug and several methods have been stated in different journals so far [10-13]. As these three drugs are identical in physical appearance, chemical properties and pharmacological actions we put an effort to separate and identify them through a single HPLC method so that they can be identified individually in raw materials as well as in formulations. Hence, an ion pair reversed phase HPLC method was developed and validated by strictly following the guidelines

of USP, FDA and ICH with respect to accuracy, precision, specificity, stressed studies, solution stability, robustness and sensitivity.

MATERIALS AND METHOD

Working standard of tenoxicam, meloxicam and lornoxicam were a kind gift of ACI Pharmaceutical Ltd., Narayanganj, Bangladesh. HPLC grade acetonitrile and methanol were obtained from Active Fine Chemicals Ltd., Bangladesh.

HPLC system

High Performance Liquid Chromatographic system (Shimadzu-UFLC Prominence), equipped with an auto sampler (Model- SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) was used for the analysis. The data was recorded using LC-solutions software. Analytical reversed phase C-18 column (Vydac 218TP C18 250mm x 4.6mm, 5µ) was used to analyze the standard and samples.

Preparation of mobile phase

0.7 g of sodium 1-heptane sulfonate was dissolved in 900 mL of nano pure water. Then 5 mL of tetra butyl ammonium hydroxide solution (40%) was added to it. pH was adjusted to 6.5± 0.1 with dilute potassium hydroxide or dilute ortho-phosphoric acid and volume was made up to 1000 mL with water of same quality. Then this buffer and HPLC grade acetonitrile were mixed together at a ratio of 65:35 v/v. Finally it was filtered through a 0.22µm millipore filter and sonicated to degas.

Preparation of standard solutions

20mg of each drug were taken in 50mL volumetric flask separately and 5mL methanol and 5mL 1.0N sodium hydroxide were added to each flask and sonicated for 5 min. Then volume was made up to the mark with diluent (Water: Acetonitrile=65:35). 5mL of each solution were taken together in to a 50mL volumetric flask and diluted with diluents up to the mark. Thus we got the stock solution of 40µg/mL for each drug. This solution was further diluted to get nominal concentration (20 µg/mL) and 80% ~120% of nominal concentration.

Chromatographic conditions

All analyses were done at ambient temperature under isocratic condition. The mobile phase was run at a flow rate of 1.5 mL/min for 10 minutes. The injection volume was 20 µL for standard and samples. Before analysis, every standard and sample was filtered through 0.2 µm filter tips. The column eluent was monitored with UV detection at 380nm.

METHOD VALIDATION [14-16]

Specificity

The specificity of the LC method was evaluated to ensure that there was no interference from the degradation products, excipients or other impurities in the region of actives. The specificity was

studied by injecting the unstressed and stressed standard solutions, placebo and blanks.

Stability indicating specificity

Solution stability

Stability of all three drugs in diluting solvent and mobile phase was checked by rendering the test solutions in tightly capped vials at room temperature and in refrigerator at 5ºC for 48 hrs. The solutions were analyzed by HPLC at 0 hr, 24 hr and 48 hr.

Forced degradation

Forced degradation studies are undertaken to degrade the active drug deliberately. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products without interference. Samples or drug product (spiked placebo) and drug substance are exposed to acid, base, oxidizing agent, reducing agent and water. The degraded samples were then analyzed using the method to determine if there are interferences with the active. Thus stability indicating property was evaluated.

Linearity

Five different concentration levels of standard mixture (16 µg/mL, 18 µg/mL, 20 µg/mL, 22 µg/mL and 24 µg/mL) were prepared from stock solution. Then the samples were analyzed by the proposed method and repeated three times. The average peak areas were plotted against concentrations. The linearity of the proposed method was evaluated by using calibration curve to calculate coefficient of correlation, slope and intercept values.

Accuracy

The accuracy of an analytical method expresses the nearness between the expected value and the value found. In present study, successive analysis (n = 3) for three different concentrations of standard mixture (16 µg/mL, 20 µg/mL, 24 µg/mL) were carried out to determined the accuracy of proposed method.

Precision

Precision of the assay was assessed with respect to repeatability and reproducibility. The precision of an analytical method is the degree of agreement among individuals test results where the method is applied repeatedly to multiple samplings. The precision of the proposed method was checked by intra- and inter-day repeatability of responses after replicate injections and expressed as %RSD amongst responses using the formula [%RSD = (Standard deviation/Mean) x 100 %].

Robustness

Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in the parameters of the method. Robustness provides some indication of reliability of the analytical method during normal usage. The effect of the following changes in

chromatographic conditions is usually determined: flow rate ±50%, solvent ratio ±15%, pH of buffer solution ±0.2, temperature ±10ºC and detector wavelength ±3.

System suitability

The purpose of the system suitability test is to ensure that the complete testing system including instrument, reagents, columns, analysts etc. are adequate for the intended analysis. The following parameters are usually determined: theoretical plate count, tailing factors,resolution and reproducibility.

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

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. On the other hand LOQ is the lowest amount of analyte in a sample that may be determined with acceptable accuracy and precision.

RESULTS AND DISCUSSION

Method development

To develop a HPLC method, firstly, pKa of all drugs were investigated. In present case, all three drugs possess a pKa of around 4.7 which suggested the pH of mobile phase should be above or around 6.5. So, initially we tried with mobile phase composed of different buffer (pH 6.0 ~7.5) and acetonitrile and/or methanol at different ratio. Finally, tetra-1-butyl ammonium hydroxide buffer (pH 6.5) and acetonitrile in 65:35 v/v ratio produced well resolute peaks of tenoxicam, meloxicam and lornoxicam at 380nm wave length. To reduce tailing and increase resolution, small amount of n-heptane sulphonic acid-Na salt was added as ion pairing agent to the mobile phase. The method was summarized in Table 1. Typical chromatogram was shown in Figure 2.

Table 1: Newly Developed RP-HPLC Method

Mobile phase	pH 6.5 Buffer (0.008M tetrabutyl ammonium hydroxide 40% aquous solution and 0.003M sodium 1-heptane sulfonate) : Acetonitrile = 65:35 v/v
Column:	Vydac 218TP C18 (250mm x 4.6mm, 5µ)
Flow Rate	1.5mL/min
Wave Length:	380nm
Retention time	TNX : 3.4 min, MLX : 5.5 min, LNX : 6.9 min.
Tailing Factor	TNX:1.34, MLX: 1.30, LNX: 1.33

Specificity

The specificity was studied by injecting the unstressed and stressed standard solution, excipients and pharmaceutical preparation of all three drugs several times on several days. It was revealed that there was no interference of peak in the region of TNX, MLX and LNX in chromatogram for the stressed sample, placebo and active. Hence the method was considered specific for the product.

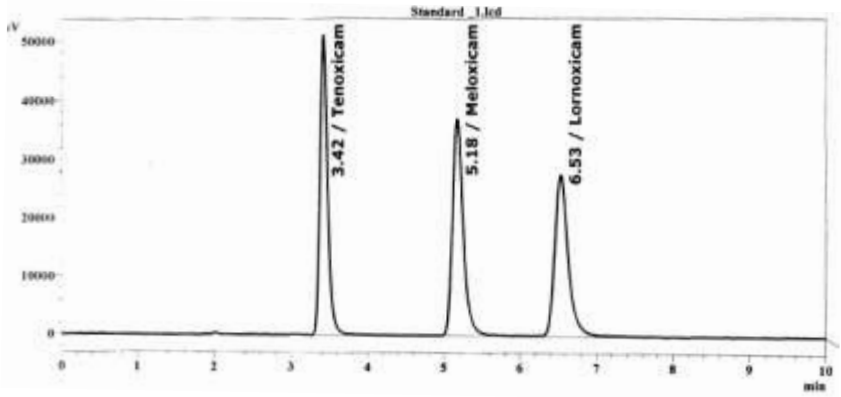


Figure 2: Chromatogram of tenoxicam, meloxicam and lornoxicam

Specificity-stability indicating

Solution stability

Two vials were prepared by dissolving the three drugs in diluting solution. One vial was kept in room temperature and another in refrigerator. Area change was investigated up to 3 consecutive days.% RSD of areas for all drugs were well between the limit (1.3%) which demonstrated that the drugs were fairly stable in diluting solution and in mobile phase. Results were shown in Table 2.

Table 2: Result of Solution Stability

Time	Room temperature			Refrigerator		
Hr	TNX	MLX	LNX	TNX	MLX	LNX
0	708879	725469	671453	708879	725469	671453
24	708462	719463	667781	708777	720063	670081
48	699897	708897	659783	708389	709877	663783
%RSD	0.7155	1.168	0.8955	0.6093	1.1019	0.6118

Forced degradation

Standard mixture were exposed to water, acid (1.0N HCl), base (1.0N NaOH), oxidizing agent (10% H_2O_2) and reducing agent (10% sodium bisulfite) for 24 hours. All three drugs exhibited more or less similar degradation pattern. Drugs were degraded significantly by oxidation and moderately by acidic environment. In aqueous and basic condition they exhibited more stability. No degradation compound was detected in the chromatogram at the proposed wave length (380nm). This result in turn substantiated our choice of mobile phase and diluting solvent with a neutral to basic pH. Result were summarized in Table 3.

Table 3: Summary of Solution Stability Study

Drug	% of degradation against freshly prepared standard[a]				
	Acid	Base	Water	Oxidation	Reduction
TNX	5.0	1.3	2.6	14.7	1.5
MLX	8.2	5.3	6.0	21.9	5.5
LNX	5.6	2.3	2.1	15.0	2.3

[a]Mean of 3 runs

Linearity, accuracy and precision

Linearity of the standard mixture was examined on 80 ~ 120% of nominal concentration. The correlation coefficient was found more than 0.998 for all drugs indicating good linearity of calibration curve. The linearity equations for TNX, MLX and LNX were found as follows:

$y=35878x - 8933$, $r^2=1.00$ (TNX)

$y=36910x - 12594$, $r^2=1.00$ (MLX)

$y=34189x - 12554$, $r^2=0.999$ (LNX)

Average recoveries were found more than 99% indicating good accuracy of the method. The %RSD values found in precision study depicted in Table 4 showed that the proposed method provides acceptable intra-day and inter-day variation for tenoxicam, meloxicam and lornoxicam in their simultaneous determination. Results were summarized in Table 4.

Table 4: Results of Method Validation Parameters

Parameters	Limits	TNX	MLX	LNX
Slope	--------	35878	36910	34189
y-intercept	--------	8933	12594	12654
Correlation coef.(r^2)	≥0.998	1.00	1.00	0-999
Range	10 µg/m L to 50 µg/mL for all			
Accuracy [a]	98~102%	99.98%	100%	100.08%
Precision(intra-day)[b]	%RSD<2%			
Day1,Analyst1,HPLC1		0.876%	0.607%	0.521%
Day2,Analyst2,HPLC2		1.084%	0.376%	0.937%
Day3Analyst3,HPLC3		1.341%	1.104%	0.754%
Precision(inter day)	%RSD<3%	1.50%	1.88%	1.076%
LOD	S/N=3:1	10ng/mL	8ng/mL	3ng/mL
LOQ	S/N=10:1	29.5ng/mL	25.8ng/mL	11ng/mL

[a]Mean of 3 runs, [b] Mean of 6 runs

System suitability

All system suitability parameters including peak area, theoretical plate, tailing factor, retention time and resolution met the compendium acceptance limits. Results were summarized in Table 5.

Table 5: System Suitability Parameters

Parameters	TNX	MLX	LNX	Limit
%RSD of Peak area [a]	0.117	0.320	0.441	%RSD<2%
%RSD< Retention time[a]	0.562 %	1.382%	1.341%	%RSD<2%
Tailing factor	1.34	1.30	1.33	t_F <1.5
Theoretical Plate	4061	5651	6313	N >2000
Resolution [b](%)	-------	7.24%	4.52%	R_s > 2%
Capacity factor	0.988	2.04	2.8	k' > 2

[a] Mean of 6 runs, [b] Resolution with respect to former peak

Robustness

Predetermined variations were performed under the experimental conditions to assess its

robustness. Result was shown in Table 6.

Table 6: Summary of Robustness Study

Parameters	t_r (min.)			Tailing factor			Resolution Changes		
	TNX	MLX	LNX	TNX	MLX	LNX	TNX	MLX	LNX
pH ±0.2	7.0	3.8	5.7	7.2	1.4	1.3	1.4	7.3%	4.5%
	6.0	3.2	5.6	7.0	1.3	1.4	1.3	6.1%	4.0%
Flow rate	2.0	2.7	4.6	5.5	1.3	1.2	1.2	5.5%	3.7%
±0.5	1.0	4.1	6.7	7.5	1.6	1.5	1.5	8.4%	5.1%
Buffer	75%	5.0	7.5	8.9	1.7	1.8	1.7	8.3%	5.8%
±15%	55%	3.1	4.5	5.1	1.29	1.21	1.29	5.6%	3.7%

t_r=Retention time

CONCLUSION

Formulation and subsequent analysis of multiple drugs is always a challenging work. Though there is no combination product of these three oxicam drugs, but still we developed a single method for their simultaneous determination due to the fact that these three oxicams are similar in physical appearance and there is every chance that one drug may be unintentionally replaced by another in raw materials or in formulation. In this regard, we developed a rapid and sensitive RP-HPLC method so that the individual drugs can be identified as each drug had a specific retention time under the described method. The method was validated strictly maintaining the guidelines of ICH, FDA and USP. Therefore, this method can be used routine analysis of tenoxicam, meloxicam and lornoxicam individually in bulk or in their any combination dosage form.

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