Development and validation of HPLC analytical method for Nepafenac in ophthalmic dosage form (suspension)

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Abstract: The aim of the present study was to develop and validate an analytical method for the estimation of nepafenac as a raw material as well as in dosage form (suspension) by using reverse phase high performance liquid chromatographic (RP-HPLC). The target was to obtain an easy, rapid, reproducible as well as a rugged method. The HPLC system that was used in the proposed study was LC-20AD liquid chromatograph equipped with SPD-20A UV-VIS detector. The separation was performed on C18 column which was attached with loop 20µl. Elution was done at ambient temperature with a mobile phase consisting of acetonitrile: Water (40: 60v/v) at a flow rate of 1ml/min and at a wavelength of 254 nm. The proposed method was validated as per the ICH guidelines. The retention time for nepafenac was 7.49 minutes (% CV=0.0076). The percentage coefficient variation (CV) of six consecutive peak areas of injections was 0.34% with tailing factor 1.76. The peak area responses were linear within the concentration range of 0.078-20.0µg/ml (R^2=0.9993). The sensitivity of the method could be evaluated by limits of detection (LOD) (0.0195µg/ml) and limits of quantitation (LOQ) (0.039µg/ml). Nepafenac drug is s in its diluent that could see by intra-day (% CV =0.45-1.96) and inter-day variation (%CV=0.173-1.898%). The accuracy and recovery results of 80%, 100% and 120% were 97.40% to 102.10% with % CV of 0.3201% to 1.3496%. The robustness and ruggedness of the method are significantly broader and is reproducible. It could be used as a more convenient, efficient, easy and time saving method for the analysis of drug in raw material as well as in dosage form (ophthalmic suspension).

Keywords: Anti-inflammatory drug, NSAIDs, High Performance Liquid Chromatography, ophthalmic suspension, Quantitative analysis, Validation.

INTRODUCTION

With the increased influx of new and partially modified forms of existing drugs into the market, there is an element of hiatus between the introduction to the market and insertion in the pharmacopoeias, possible unaccreditation in continuous and wider usage being the reasons Additionally it is mandatory according to ICH guideline that before regulatory acceptance of any drug, it should have the identification and qualification test. Along with it the control of impurities in the drug substances needs to be clearly defined and same needs to be done in the formulated finish products (ICH, 2003; ICH- Q3A, 2006; ICH- Q3B, 2006).

Under the given scenario the pharmacopoeia may not establish the presence of conventional and analytical procedures for the stated drugs, hence, making the development of newer analytical entity a necessity. So HPLC method makes it easier to determine or to separate a mixture of compounds in pharmaceutical dosage forms and are also used to identify, quantify or purify the individual components of the mixture (USP-2013; BP-2012).

Nepafenac is a prescription ophthalmic non-steroidal anti-inflammatory solution for topical, ocular administration intended for post cataract surgery associated pain and inflammation. Nepafenac is a precursor for amfenac, conversion being facilitated by intraocular hydrolases that inhibits the activity of cyclooxygenase COX-1 and COX-2. Its side effects may include decreased visual acuity, a feeling that something is in the eye, increased eye pressure or a sticky sensation, as well as other side effects (dailymed.nih.gov).

Nepafenac is designated chemically as 2-amino-3-benzoylbenzeneacetamide with an empirical formula of $C_{15}H_{14}N_2O_2$. The structural formula of nepafenac is (dailymed.nih.gov)

The aim of the present study was to develop and validate a simple and economical HPLC method for the quantitative analysis of nepafenac according to ICH guideline. That will help to determine the label amount of drug in its dosage form (ophthalmic suspension) and to maintain the quality control. There is not enough literature available for the estimation of nepafenac in any dosage form as well as it is not mentioned in USP-36 and BP-2012.

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MATERIALS AND METHODS

Instruments

The HPLC system consisted of a LC-20AD Shimadzu liquid chromatography equipped with SPD-20A UV-VIS detector. Chromatographic separations were performed on C18 5µm (4.6mm x 250mm) column, which is attached with loop 20µl and connected to CBM Communications Bus Module Shimadzu with Dell system. Also used electronic balance and pH meter during study.

Material

Nepafenac (standard) powder was gifted by Barrett Hodgson Pharmaceutical Pakistan (Pvt) Ltd. Methanol, acetonitrile, double distilled water and all other solvents used, were of HPLC grade and reagents of analytical grade.

Preparation of diluent

Nepafenace is practically insoluble in water. In purposed method for accurate and complete dissolution of the drug, the mixture of methanol: Acetonitrile (50: 50) was used as diluent.

Preparation of mobile phase

Mobile phase consisted on acetonitrile: water (40:60v/v). All solvents and solutions were filtered through filtration unit (Millipore, 0.45µm pore size) and degassed before use. The flow rate was maintained at 1 ml/min and volume of injection was 20µl. Detection was performed at a wavelength of 254 nm and analysis was carried out at ambient temperature.

Preparation of stock solution

10mg of nepafenac standard powder was weighed accurately and transferred to 50ml volumetric flask. Diluted with diluent to volume and mixed. The concentration of stock solution was 200µg/ml (on dried basis).

Preparation of sample solution

Accurately pipette out 1ml from 0.1% ophthalmic suspension into a 100ml volumetric flask dissolved and diluted to volume with diluents (10µg/ml).

Preparation of system suitability solution

solution for system suitability test was prepared by dissolving 0.5ml of stock solution in to 10ml of diluent (10µg/ml).

Furthermore series of dilution were prepared for the estimation of calibration curves (0.078µg/ml-20µg/ml) in diluents according to the study design.

Validation procedure

The aim of the study was to obtain a simple, lucrative and convenient HPLC method for the determination of nepafenac in its dosage form. The experiment was carried-out according to the official specifications of United State Pharmacopeias (USP-36), International Conference on Harmonization (ICH-1996) and Centre of Drug Evaluation and Research (CDER-1994). The parameters considered for validation in this method were system suitability, specificity, range and linearity, limit of detection, limit of quantification, accuracy, precision, ruggedness and robustness.

The assessment for the suitability of the system was done using six (6) drug replicas at concentration of 10g/ml. It was used to confirm that the resolution and reproducibility of the chromatographic system is adequate for the analysis to be done. The method was evaluated by analyzing the repeatability, retention time, peak area, tailing factor, theoretical plates (Tangent) of the column.

Selectivity of the method was used to ensure separation of pharmacologically active ingredient from excipients that incorporated in their dosage forms.

The linear relationship was evaluated by estimating the time lag between the upper and lower analytical concentrations of a sample. To evaluate the linearity, LOD and LOQ of the method (standard substance), serial dilutions were made from the stock solution in the range of 0.0097-20µg/ml.

To validate the accuracy and recovery of the purpose assay method from ophthalmic suspension, six different known concentrations of the nepafenac standard (range from 0.0625-20µg/ml) in mobile phase were analyzed. Samples for recovery studies (in ophthalmic suspension) were also prepared by known amount of drug at three different concentration levels (in triplicate) i.e. 80%, 100% and 120% levels of the target nepafenac and were analyzed according to the procedures.

The method precision was analyzed under the attribute of repeatability that had been done by six (6) determinations at 100% of the test concentration (system suitability), intermediate precision (intra-day and inter-day variation), and reproducibility (an inter-laboratory trial).

Robustness studies were done on method precision using a sample concentration of 10µg/ml by making slight variations in flow rate, concentration of acetonitrile and change in pH of mobile phase. To estimate the ruggedness of the method, the procedure was repeated in the laboratory of Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi, Pakistan.

For stability studies, the stability of drug in diluents was evaluated, by keeping different concentration of drug in refrigerator.

RESULTS

The presented method is a simple, sensitive, and an easy HPLC method to operate, using UV detection for the determination of nepafenac in raw material and in the pharmaceutical dosage form (suspension). This might not be used only to analyze the chemical purity and assay of nepafenac, but also for the pharmacokinetic study.

The proposed method is selective and reproducible that allows to separate all possible excipients from pharmaceutical dosage form (suspension) and to quantitate the nepafenac amount precisely.

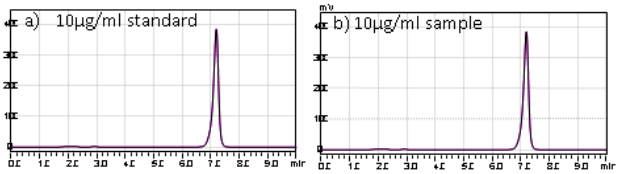

Fig. 1: a) 10µg/ml standard (nepafenac) in mobile phase. b) 10µg/ml concentration of nepafenac from ophthalmic suspension.

System suitability was achieved by injecting six (6) replicated injection of standard (nepafenac). The percentage coefficient variation (% CV) of the retention times and the peak areas of nepafenac from the six consecutive injections were 0.102 % and 0.34 %, respectively. The Mean theoretical plate count, based on USP tangent calculations (USP-2004) for nepafenac peak was 7718.895 (table 1).

Linearity

For the calibration of the method a linear curve was constructed by using known amounts of nepafenac in the range of 0.078-20.0 µg/ml (fig. 2).

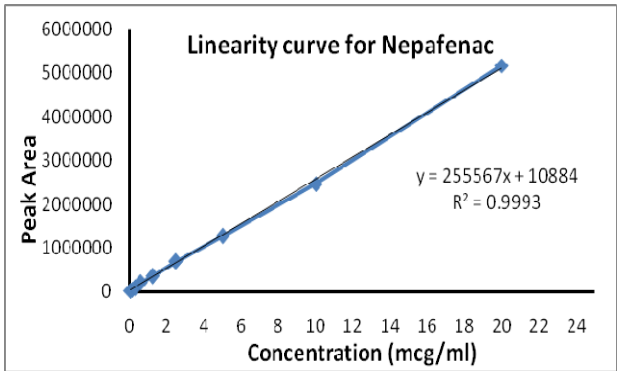

Fig. 2: Calibration curve shows linearity over the concentration range

Specificity

Specificity test was performed to establish the separation of pharmacologically active ingredient from the excipients that are present in the nepafenac suspension (0.1%).

Accuracy and recovery test

Samples solution (for 0.1% suspension) were prepared in triplicate at three levels over a range of 80-120% and analyzed according to the procedure. The percentage recovery ranged from 97.40 % to 102.10 % of the label claim of nepafenac at all three levels of recovery study, and % CV values for each level ranged from 0.32% to 1.35 % (table 2).

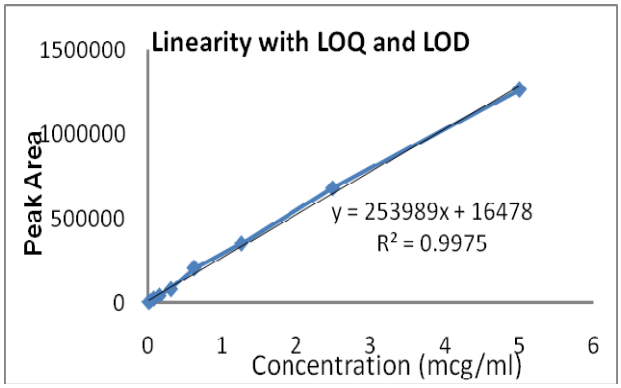

Fig. 3: Estimation of LOD and LOQ of Nepafenac

Limit of detection and Limit of quantification

The calculations for limit of quantification (LOQ) and Limit of detection (LOD) were done on the basis of peak responses of nepafenac standard solution (range = 0.0097-5.0 µg/ml) in which LOQ (0.039µg/ml) was three times and LOD (0.0195µg/ml) was nine times the base line noise, respectively with $r^2 = 0.9975$ (fig. 3).

Precision

The reproducibility (intra-day precision) CV was 0.4545-1.9595% and repeatability (inter-day precision) CV was 0.1715-1.898% by injecting the different concentrations of standard solution (0.625-10 µg/ml) on the same day and different days respectively (tables 3 and 4).

Robustness and Ruggedness test

The robustness of the purposed method was checked by making slight variations in flow rate, change in amount of acetonitrile and change in pH (table 5). The results showed no significant changes with the changes in flow rate from 0.8 ml/min. to 1.2 ml/min, with percentage coefficient variation 1.65 and in case of the change in pH from 6.6 to 6.8, the recovery variation was 0.53 % whereas the acetonitrile variation from 38% to 44 % showed a recovery of 99.58% and 100.78% with % CV 0.847.

Content assay of nepafenac

The developed method is so simple, easy and cost effective. It could be easily used for the analysis of compound in its dosage form. Retention time is between 7-8 minutes that could also be adjusted according the needs (table 6).

Table 1: System Suitability parameters for Nepafenac

Injection number	Retention time (min)	Peak area of Nepafenac	Tailing factor	USP Tangent Plate count
1	7.48	2472779	1	7659.785
2	7.495	2481774	1.03	7728.498
3	7.491	2471888	1.03	7731.283
4	7.503	2486472	0.997	7731.283
5	7.497	2485493	0.989	7731.26
6	7.493	2493667	1	7731.26
Mean	7.493167	2482012	1.007667	7718.895
SD	0.007653	8433.84	0.017761	28.97903
CV%	0.102	0.34	1.762	0.375

Table 2: Accuracy and Recovery analysis of Nepafenac suspension

		80 %	**Level**		
Assay	Run-I	Run-II	Mean	% Recovered	% of L.C
Sample -1	2014059	1975008	1994533.5	82.26	102.82
Sample- 2	1988102	1986811	1987456.5	81.96	102.46
Sample -3	1962664	1956874	1959769.0	80.82	101.03
Mean			1980586.3	81.68	102.10
% CV					0.9276
		100 %	**Level**		
Sample -1	2347457	2392330	2369893.5	97.74	97.74
Sample- 2	2272004	2438020	2355012.0	97.12	97.12
Sample -3	2368782	2351505	2360143.5	97.33	97.33
Mean			2361683.0	97.40	97.40
% CV					0.3201
		120 %	**Level**		
Sample -1	2927550	2957161	2942355.5	121.35	101.12
Sample- 2	2808791	2966397	2887594.0	119.09	99.24
Sample -3	2967300	2961577	2964438.5	122.26	101.88
Mean			2931462.7	120.90	100.75
% CV					1.3496

Table 3: Intra-day variation

Std. Conc. µg/ml	8.3am	1.0am	5.0am	Mean	± SD	% CV
10	2442158	2459896	2472779	2458278	15374.51	0.625
	2438198	2461833	2481774	2460602	21814.08	0.886
Mean	2440178	2460865	2477277	2459440	18594.3	0.7555
5	1268408	1263278	1276638	1269441	6739.676	0.53
	1267804	1271362	1277353	1272173	4825.882	0.379
Mean	1268106	1267320	1276996	1270807	5782.779	0.4545
1.25	340728	352807	353086	348873.7	7055.733	2.02
	341295	351652	353630	348859	6624.854	1.899
Mean	341011.5	352229.5	353358	348866.3	6840.294	1.9595
0.625	204438	207307	209601	207115.3	2586.831	1.25
	209949	207004	208421	208458	1472.849	0.706
Mean	207193.5	207155.5	209011	207786.7	2029.84	0.978

Table 4: Inter day variation

Conc. µg/ml	1st day	2nd day	3rd day	mean	± SD	% CV
10	2442158	2342158	2459896	2414737	63478.18	2.629
	2438198	2405251	2461833	2435094	28418.42	1.167
Mean	2440178	2373705	2460865	2424916	45948.3	1.898
5	1268408	1254504	1265499	1262804	7333.411	0.58
	1267804	1267408	1275761	1270324	4712.453	0.37
Mean	1268106	1260956	1270630	1266564	6022.932	0.475
1.25	352807	353092	352628	352842.3	234.0093	0.069
	351652	352936	353571	352719.7	977.6197	0.277
Mean	352229.5	353014	353099.5	352781	605.8145	0.173
0.625	207307	209547	214438	210430.7	3646.703	1.733
	207004	208315	210478	208599	1754.326	0.841
Mean	207155.5	208931	212458	209514.8	2700.515	1.287

Table 5: Results for Robustness Test

Parameters	Changes	% Recovery	% of Target	% CV
Target	Conditions	100.32	100.0	
Flow rate	0.8 ml/min	99.07	98.75	1.65
	1.2ml/min	101.41	101.08	
Change in pH	6.6	101.88	101.56	0.53
(Mobile phase)	6.8	101.12	100.8	
Acetonitrile	38%	99.90	99.58	0.847
variation	44%	101.10	100.78	

Table 6: Assay of Nepafenac in ophthalmic suspension

	Content Assay	Average	± STD	± SEM
Sample 1	98.75	99.915	1.65	1.167
	101.08			
Sample2	101.56	101.18	0.54	0.381
	100.8			
Sample 3	99.58	100.18	0.85	0.601
	100.78			

Stability of content

The most important thing required for pharmaceutical and pharmacokinetics study is the stability of drug in its diluent. The present study shows that the drug is stable in purposed diluents that could be evaluated by Inter day variation (table 4), the % CV values were less than 2%.

DISCUSSION

In order to develop and validate a simple and competent method for the analysis of nepafenac substance (pure) and in its pharmaceutical formulations (suspension), ICH guideline were used. The different phases of initial analysis were performed to select adequate and optimum parameter, such as detection of wavelength, composition of mobile phase and their adjustment, optimum pH and the effect of slight variation of these parameters on the quantitative analysis of the compound.

Only few publications were found about nepafenac during the literature survey. The documents regarding the patent of nepafenac (NEVANAC) described about the synthesis and chemical purity of the compound by HPLC- UV methods. There are a couple of articles relating to nepafenac analysis available (Elzbieta *et al*., 2014; Phani Kumar and Sunandamma, 2012; Anupama, *et al*., 2011). Among this one mobile phase consists of ammonium formate that may have a disadvantage of decomposition into hydrogen and carbon dioxide and may react with functional groups. On the other hand the second study shows that the mobile phase consisted on methanol, acetonitrile and THF, all being organic solvent, volatile and relatively costly and difficult to handle. There is then a spectrophotometeric method that is based on the oxidative coupling reaction. Moreover there is no official monograph in the pharmacopeias about nepafenac compound, supporting the present study to be more convenient with fewer drawbacks

For system suitability the columns with high tangent plate count is considered to be more efficient, whereas the tailing factor 1.0 is signified as complete symmetrical (table 1). The least square method was used to calculate the correlation coefficient (R^2 = 0.9993) of calibration curve. This correlation coefficient (R^2) confirmed that the sample solutions in specified range showed linearly proportional analyte responses to the concentration (fig. 2).

The accuracy of the method is based upon the closeness of the measured value to the true value for the sample (Global Quality Guideline-2002). The overall mean percent recovery was 100.083 % of the label claim of nepafenac with an overall CV of 0.866 % (Table 2). Precision study was performed to estimate the reproducibility of the method, done by determining the intra-day and inter-day CV percentage of the measured concentrations of nepafenac in the same samples of mobile phase (tables 3 and 4).

The reproducibility of developed method was reconfirmed in the laboratory of Pharmaceutics, Faculty of Pharmacy, University of Karachi, Pakistan, for the analysis of nepafenac under multiple conditions, such as different laboratories, analysts, instruments and at different days. It was found that the correlation coefficient (r^2) was < 1. Whereas the concentration range (0.078-20.0 µg/ml) were used that is same as the concentration range that has been used in laboratory of Pharmaceutics, college of Pharmaceutical Sciences, RAKMHSU, Ras Al-Khaimah. UAE

After validation, the developed method was applied for the analysis of nepafenac in ophthalmic suspension that is available in local market of Ras Al Khaimah (Nevanac Eye Suspension) (Table. 6). For authentication of stability test, stress testing was done by exposing the sample to hydrogen peroxide for oxidation and heat treatment for thermal degradation. The sample then was tested by same method to confirm stability of products as well as the robustness of the method.

CONCLUSION

Nepafenac is a new non-steroidal anti-inflammatory drug (NSAID). It is used to treat pain and inflammation associated with cataract surgery. Still there is no USP and BP method is available for the analysis of drugs for its purity as well as for content assay in its dosage form. The proposed method was first developed then validated. It is rapid, precise, accurate, selective, cost-effective and very flexible for its peak retention time.

Shortly the present analytical data could be used to screen the drug potential, provides help in formulation studies, quality control, monitor the stability of bulk pharmaceuticals and estimate the release of drug from final products (dosage form).

ACKNOWLEDGEMENT

The authors would like to thank Department of Pharmaceutics, RAK college of Pharmaceutical Sciences, RAK Medical and Health Sciences University, Ras al Khaimah, UAE for all financial and moral supports.

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