RP-HPLC Method Development and Validation for Aceclofenac and Piperine Simultaneous Determination in Rat Plasma to Examine Pharmacokinetic Parameters

Manisha [1],Bibu Prasad [2],V.Balamani [3],Habizur Rahman [4],
Assistant professor [1,2,3,4],
Department of Pharmacy,
Samskruti College of Pharmacy,
Kondapur (V), Ghatkesar (M) Medchal Dist, Telangana, India.

ABSTRACT

Aceclofenac, a BCS class II drug, is highly metabolized in human hepatocytes and microsomes and is weakly soluble in water. Its oral bioavailability is about 15%, which is rather poor. A naturally occurring bioenhancer, piperine is utilized to boost the oral bioavailability of several pharmaceuticals. The pharmacokinetics of piperine (10 mg/kg) and aceclofenac (20 mg/kg) in combination were examined in this study's rats. In this investigation, piperine was utilized to increase the bioavailability of aceclofenac despite their different chemical and physical characteristics. This was followed by the creation and validation of an HPLC technique to assess their simultaneous measurement in rat plasma. Solid phase extraction was used to prepare the plasma samples before HPLC analysis was performed. The research revealed that the retention times for piperine and aceclofenac were 14.4 minutes and 5.3 minutes, respectively. The linear range of aceclofenac concentration in rat plasma was shown to be between 0.1 and 20 μg/mL. The calculated correlation coefficient (R2) was 0.9995. Cmax rose to 48.52 μg/mL from 23.59. Aceclofenac's oral bioavailability may be increased by including piperine into the formulation.

Introduction

Aceclofenac (NSAID) is one of the most commonly used non-steroidal pain relievers (Fig. 1a). Osteoarthritis and rheumatoid arthritis pain are treated with it. It functions by preventing the production of prostaglandins (PG), an enzyme that causes pain, swelling, inflammatory responses, and a high body temperature.[1–3] The recommended daily dosage of aceclofenac is 200 mg. A regular dose is required because aceclofenac's plasma elimination half-life is approximately 4 hours. When administered orally, about 15% of it was bioavailable. It belongs to the group of BCS Class II medicines. They have a poor bioavailability due to extremely low solubility in biological fluids. Human hepatocytes and microsomes substantially metabolise aceclofenac to produce the main metabolites 4′-hydroxydiclofenac, 4′-hydroxyaceclofenac, and diclofenac. It is likely that CYP2C9 mediates the metabolism of aceclofenac.[4–6] The prospect of increasing solubility and rate of dissolution to boost bioavailability has been researched using a variety of techniques.[7–9]

Increasing medication bioavailability is crucial for therapeutic purposes since it directly impacts plasma concentrations and therapeutic effectiveness. There are numerous ways to boost a drug's bioavailability. The most recent strategy uses bioavailability enhancers based on herbs. Plant-based bio-enhancers include piperine, gingerol, niaziridin, allicin, curcumin, capsaicin, and quercetin, to name a few.[10–12] The black pepper fruit contains piperine (*Fig. 1b*), the first bioenhancer in the world, and a significant plant alkaloid (*Piper longum* and *Piper nigrum*). Through a variety of processes, piperine increases gastrointestinal absorption and decreases medication metabolism in the stomach.[13–17] In a study that compared piperine to commercially available treatments, the bioenhancing effectiveness of piperine in combination with non-steroidal anti-inflammatory medicines was described as clinically relevant at a dosage of 5 to 20 mg/kg.[18] It has been determined that the marketed drug formulation "Risorine®" is bioequivalent to rifampicin preparations sold in pharmacies. It contains the active ingredients rifampicin, isoniazid, and piperine as a bioenhancer. The dosage of rifampicin was reduced from 450 to 200 mg when piperine was added.[19] Many medications have their bioavailability increased by piperine. The optimal bioavailable drug combination with piperine requires substantial research and an appropriate formulation technique. Currently there is no research on the aceclofenac and piperine combination. This study was designed to assess the bioavailability of aceclofenac alone, and in combination with piperine.
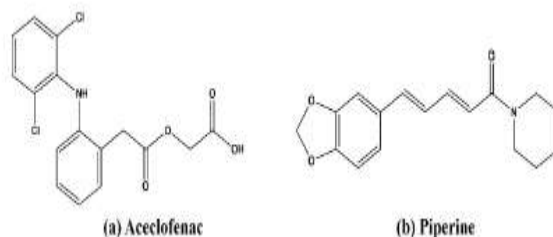
Fig. 1: Structure of a) Aceclofenac and b) Piperine

Material And Methods

Chemicals and Reagents
From Yucca Laboratories, Mumbai, Piperine was bought. Additionally, a gift sample of aceclofenac was received from Alkem Laboratories Pvt. Ltd., Mumbai. Sigma Aldrich chemicals Pvt. Ltd. was the source for every additional chemical and reagent.

Analytical Instruments
JASCO HPLC with UV/VIS detector (Jasco UVS 2075) was used for analysis. All glassware used were of A grade and Eppendorf Centrifuge 5424 R, Celfrost CF300 Horizontal Deep Freezer, and weighing balance of METTLER TOLEDO New Classic MS were used in the study.

Animals
The Institutional Animal Ethics Committee (IAEC) [Bharati Vidyapeeth, Poona College of Pharmacy [IAEC/PCP/PCH02/2020-2021] approved the pharmacokinetic investigation. Male Wistar rats [weight (180-220 gm; n = 12)] were provided by the National Institute of Biosciences, Sinhgad road, Pune. The animals were kept in ventilated cages for one week. The room was kept at a constant temperature of between 22 to 25℃, relative humidity in the 55 to 60% range. The cycle of 12-hour dark and 12-hour light was maintained. Animals were given food pellets (

Table 1: HPLC method reported in literature for analysis of piperine and aceclofenac in rat plasma

Column	Mobile phase	Extraction method	Retention time (min)	Reference
Aceclofenac:				
Hypersil BDS C18 (250 mm × 4.6 mm, 5)	Methanol-triethylamine (pH 7.0: 0.3% v/v in Milli-Q water) (60:40, v/v)	Protein precipitation using ACN	8.6	21
Atlantis C18, 100 × 2.1 mm internal diameter, 3-m particle size, Waters)	Acetonitrile/0.1% formic acid (aq.) (9:1 v/v)	Protein precipitation using ACN	1.54	22
Piperine				
Symmetry C(18) column (25Rx4.6 mm)	25 mM KH(2)PO(4) (pH 4.5)-acetonitrile (35:65)	Liquid – liquid extraction with ethyl acetate	6.6	23
RP-18, 5 µm, 250 X4.6 mm (Supelco) column	Methanol (HPLC grade) and water (Millipore), 70:30	Liquid – liquid extraction with DCM	7.808	24

New Maharashtra Chakan Oil Mills. Ltd., Sangali, India). Tap water was given *ad libitum*.

Calibration Standards (CS) and Quality Control (QC) Samples Preparation
In 10 mg of drug is solubilized in 10 mL of solvent, a stock solution of 1000 ppm concentration has been formed. This stock solution has been serially diluted further. To prepare calibration standards in concentration range 0.1 to 20 µg/mL, drug solutions were spiked in 100 µL blank plasma. Using the same process QC samples had been mapped out (Concentration of QC samples 1, 8, and 16 µg/mL).

Preparation of Buffer
Ammonium formate buffer (15 mM, 1 L) was prepared by dissolving 0.95 g of ammonium formate (NH4COOH, purity > 99%) in 800 mL HPLC grade water. pH was adjusted to 4.5 by using HPLC grade formic acid (98–100%). Water was added until the limit line of measuring flask. Solution was homogenized by agitation.

Extraction of Rat Plasma Sample for Analysis

The SPE method was applied to extract rat plasma samples. This technique gives better recovery and peak shape in HPLC. In 100 μL rat plasma was mixed with 100 μL ammonium formate buffer (15 mM, pH 4.5). The mixture was vortexed for 30 seconds and centrifuged at 14000 rpm, at 10℃ for 5 minutes. SPE cartridge (Strata-X 33 um polymeric Reverse Phase 30 mg/1-mL) were conditioned with 1-mL methanol. Then 1-mL water was used for equilibration after that rat plasma samples loaded and allowed to drain slowly. After draining the plasma, 1-mL water followed by 1-mL of 10% methanol (prepared with water) was used to wash cartridges, then allowed to dry. Drugs were eluted by 1-mL eluent (60% acetonitrile (ACN) mixed with 40% ammonium format buffer (15 mM, pH 4.5)). It was collected and injected onto the HPLC column. Chromatograms were recorded.

Chromatographic Conditions

JASCO HPLC system with UV-vis (JASCO UV 2075) detector was used to carry out analysis of samples. NAVCHROM software was used to analyze the data. Thermo Synchronous C18 column of 250 mm length and 4.6 mm internal diameter (ID) was used along with Phenomena security guard column (C18, 4 x 3.0 mm ID). 60% acetonitrile (ACN) mixed with 40% buffer and filtered through a 0.22 μ membrane filter. The ammonium format buffer was used (15 mM, pH 4.5). pH of buffer was maintained with formic acid. Using ultrasonication prepared mobile phase was degassed for 30 minutes. Detection wavelength 288 nm was used, which is the isosbestic point of aceclofenac and piperine UV spectra. 1-mL/min flow rate kept constant. The run time of 20 minutes was set.

Bioanalytical Method Validation

The bioanalytical method was validated by following United States Food and Drug Administration (USFDA) guidelines.[25-27]

Selectivity

Six blank samples of rat plasma were utilized alongside the rat plasma samples at LLoQ (spiked with drug) to evaluate selectivity.

Carryover effect

For this, the samples were injected in the following order: first the blank plasma sample was injected, followed by LLoQ sample, then ULoQ sample, and again the same blank plasma sample.

Linearity

The peak area of aceclofenac versus its concentration in the 0.1 to 20 μg/mL range was plotted to determine linearity. To evaluate the linearity of plasma samples, the correlation coefficient was used.

Accuracy and precision

Three concentrations of QC samples were examined in six replicates to measure interday and intraday precision and accuracy.

Extraction recovery

Blank plasma samples and plasma spiked QC samples were extracted. After extraction the drug was spiked in extracted blank plasma to get post spiked QC samples. Blank plasma (post) spiked with QC samples and extracted analytical QC samples were compared to check extraction recovery.

Stability

One of the most important aspects in the development of a bioanalytical method and its validation is the solute's stability under different conditions. For bench-top stability the spiked QC samples were kept at room temperature. After keeping them on a bench for 8 hours. the samples were processed and studied. In case of processed sample stability, plasma samples were first processed and then kept on bench at room temperature. After 6 hours. These samples were loaded onto HPLC. Spiked QC samples were kept at −20℃ for 15 days to study long-term stability. In case of freeze-thaw stability spiked QC samples were removed from deep freezer and kept on bench to thaw for 12 hours. The same samples were again placed in a deep freezer. This cycle was continued three times. After three cycles the samples were processed and loaded on to HPLC for analysis.

Pharmacokinetic Study

The animals were fasted for 24 hours and separated into control group, group A [aceclofenac, 20 mg/kg; given orally] and group A+P [aceclofenac, 20 mg/kg along with piperine, 10 mg/kg, given orally] (n=12). After dosing, 0.5 mL blood per animal was collected using retro-orbital venous plexus (ROP) technique at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hours. After 4 to 5 hours, the animals were given food. Ca-EDTA tubes were marked and used to collect blood. It helps to prevent blood coagulation. The tubes were centrifuged at 4°C and 10,000 rpm for 2 to 5 minutes to collect plasma. In 100 μL of rat plasma was taken in prelabeled eppendorf tubes. SPE technique was used to extract rat plasma samples and prepare the sample for analysis. In 20 μL of sample was loaded onto the HPLC column. Chromatograms were recorded.

Statistical Analysis

A Win Nonlin noncompartmental analysis (NCA) tool was utilized to estimate various pharmacokinetic parameters from plasma concentration and time profile. The trapezoidal rule was followed here. In these studies, the pharmacokinetic parameters Cmax (the highest point drug level), t1/2 (half-life), Tmax (the duration to reach peak concentration), AUC (area underneath the curve), and Kel (elimination rate constant) were calculated and compared. The data were presented as mean followed by standard deviation. The student's t-test was used to analyze the data statistically. *p-values* less than 0.05 were considered statistically significant.

Results

Method Development

Many isocratic and graded mobile phase compositions with multiple acids and buffers were tested to attain the optimum separation between the specified analytes. The finalized method developed for the simultaneous determination of aceclofenac and piperine in the biological matrix (rat plasma) is summarized in Table 2.

Method Validation

The HPLC bioanalysis method to determine the aceclofenac concentration in biological matrix (rat plasma) was validated as per the USFDA guidelines.

Selectivity

During method development, the selectivity of the method was tested to ensure that analytes did not interfere. Chromatograms of six blank plasma samples and the same of spiked plasma sample were compared. Selectivity was verified by this comparison. At retention times of 5.3 minutes (aceclofenac) and 14.5 minutes (piperine), there was no interference observed. (Fig. 3)

Carryover effect

To determine this, interference of analyte at retention time was evaluated. The samples were injected in the following order: first the blank plasma sample was injected, followed by LLoQ sample, then ULoQ sample and again the same blank plasma sample. The absence of interference showed that the carryover effect was absent.

Table 2: Summary of developed HPLC method

Drug	Aceclofenac and piperine
Biological matrix	Rat Plasma
Sample preparation	Solid Phase Extraction (SPE)
SPE cartridge	Strata-X 33 μm polymeric reverse phase (30 mg/1-mL)
System	JASCO HPLC with UV detector
Detector	UV-vis (Jasco UVS 2075)
Column	Thermo Synchronious C18 column (250 X 4.6 mm X 5 μm)
Mobile Phase	ACN : Buffer (60:40 v/v)
Buffer	15 mM ammonium formate, pH 4.5 (adjusted with formic acid)
Flow Rate	1 mL/minutes
Detection Wavelength	288 nm – Isosbestic point (Fig. 2)
Injection Volume	20 μL
Run Time	20 minutes
Software	NAVCHROM
Retention Time	Aceclofenac – 5.3 minutes and piperine – 14.4 minutes
Linearity range	0.1–20 μg/mL

optimum separation between the specified analytes. The finalized method developed for the simultaneous determination of aceclofenac and piperine in the biological matrix (rat plasma) is summarized in Table 2.
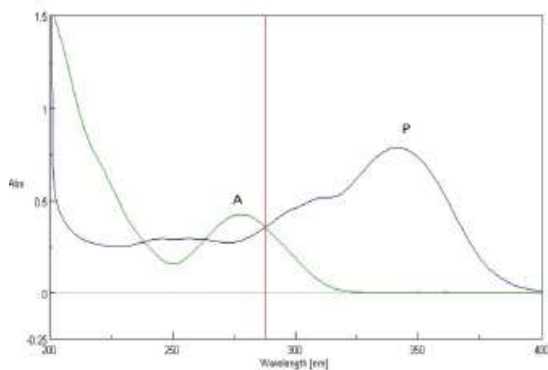
Method Validation

The HPLC bioanalysis method to determine the aceclofenac concentration in biological matrix (rat plasma) was validated as per the USFDA guidelines.

Selectivity

During method development, the selectivity of the method was tested to ensure that analytes did not interfere. Chromatograms of six blank plasma samples and the same of spiked plasma sample were compared. Selectivity was verified by this comparison. At retention times of 5.3 minutes (aceclofenac) and 14.5 minutes (piperine), there was no interference observed. (Fig. 3)

Carryover effect

To determine this, interference of analyte at retention time was evaluated. The samples were injected in the following order: first the blank plasma sample was injected, followed by LLoQ sample, then ULoQ sample and again the same blank plasma sample. The absence of interference showed that the carryover effect was absent.

(A-Aceclofenac and P-Piperine)

Fig. 2: Overlay of aceclofenac and piperine UV spectra (Isosbestic point)
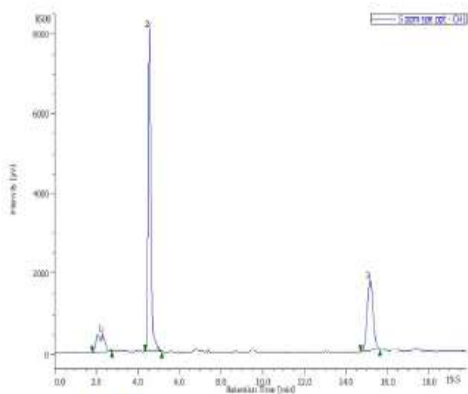
(1-Rat plasma, 2-Aceclofenac, and 3-Piperine)

Fig. 3: HPLC Chromatogram

Linearity

Aceclofenac had a linear range of 0.1 to 20 μg/mL in rat plasma. Using calibration curve data, the correlation coefficient (R^2) was calculated ($R^2 = 0.9995$). Aceclofenac's regression equation was discovered to be $y = 9791.9x + 1834.9$ (*Fig. 4*).

Accuracy and precision

Quality control samples (1, 8, and 16 μg/mL) were used in the interday and intraday precision investigations. The %RSD (relative standard deviation) was observed to fall within the range of all levels tested. The %RSD for

intra-day was found to be 1.65 to 2.57% and for inter-day was found to be 2.22 to 4.44% (acceptance limit 15%). This demonstrated that the method for aceclofenac in the biological matrix was repeatable and precise (Table 3).

Stability

QC samples 1, 8, and 16 μg/mL were used to study the stability of aceclofenac in rat plasma and the results showed that there was no evidence of degradation. The % RSD for benchtop stability samples was between 1.61 to 2.98 %, for processed samples, stability was between 1.59 to 3.22%, for long-term stability samples was between 1.65 to 4.42% and 2.16 to 3.56% for freeze-thaw stability samples. All results are within the acceptance limit. This concludes that plasma samples of aceclofenac are stable when kept for 8 hour on the bench for bench top stability and for 6 hour on bench after processing for processed sample stability at room temperature. It remained stable for 15 days in plasma when stored at -20°C. And it is also stable after three cycles of freeze-thaw (Table 3).

Pharmacokinetic Study

After oral administration of drug, 0.5 mL blood was collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hours, per animal in marked tubes containing Ca-EDTA. After extraction of plasma, 20 μL of filtered supernatant was
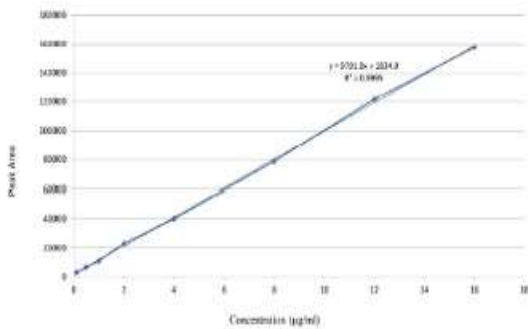

Fig. 4: Calibration curve

Table 3: Accuracy, precision, and stability study of aceclofenac in rat plasma

Concentration (μg/mL)	Mean area ± SD	%RSD	%Accuracy
Intraday			
1	11543 ± 297	2.57	97.24
8	77944 ± 1609	2.06	97.94
16	156942 ± 2587	1.65	98.35
Inter day			
1	11566 ± 333	2.88	97.12
8	80389 ± 3568	4.44	95.56
16	155497 ± 3461	2.22	97.77
Benchtop stability			
1	11578 ± 345	2.98	97.01
8	77901 ± 1699	2.18	97.81
16	156997 ± 2528	1.61	98.38

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

	Processed sample stability		
1	11551 309	2.68	97.32
8	78296 1248	1.59	98.41
16	158593 5101	3.22	96.78
	Freeze-thaw stability		
1	11708 ± 285	2.44	97.55
8	77894 ± 1684	2.16	97.83
16	155330 ± 5522	3.56	96.44
	Long-term stability		
1	11625 ± 386	3.32	96.67
8	78580 ± 1285	1.64	98.36
16	156942 ± 6931	4.42	95.58

Discussion

In the quickly evolving pharmaceutical industry, efforts should be taken to deliver safe, effective medicine in a timely manner while simultaneously taking treatment costs into account. To improve absorption, piperine can be administered concurrently with aceclofenac. The effect of the piperine on the bioavailability of aceclofenac was examined by comparing the pharmacokinetic parameters of aceclofenac. For simultaneous measurement of acelofenac and piperine in rat plasma a unique, simple, reproducible, HPLC method using UV detection was established. This method is compatible for mass spectrometry. It uses solid phase extraction technique for the extraction of rat plasma samples. The procedure was new because no previous publications on the measurement of acelofenac and piperine simultaneously in the biological matrix (rat plasma) had been discovered. The process of bio-analysis is notoriously difficult; the first major obstacle is a complex biological matrix made up of large protein molecules and the distinctive chemistry of analytes is the second major obstacle. Aceclofenac, a non-steroidal anti-inflammatory drug, and piperine, an alkaloid found in black pepper, are the analytes in this case. Because of their diverse chemical compositions, they differ from one another in terms of physicochemical properties. The sample is not suitable for routine chromatographic analysis when the plasma matrix includes big protein molecules. Finally, using Strata-X (Phenomenex) cartridges that had excellent analyte recoveries produced successful outcomes. Chromatographic conditions were optimized to provide acceptable resolution, minimize run time, avoid interferences, and perhaps build a compatible method for mass spectrometry.

The analysis identified retention time for aceclofenac and piperine to be 5.3 and 14.4 minutes respectively. No interference and carry-over effect were found at the retention time of analyses. We found the linear concentration range of aceclofenac in rat plasma between 0.1 to 20 µg/mL. The correlation coefficient (R^2) determined was 0.9995. Cmax was increased from 23.59 to 48.52 µg/mL. The accuracy and precision results suggests that the method is precise and repeatable. Stability was found within acceptable limits (%RSD less than 15%). Extraction recovery was found between 91.49 to 99.86%. According to the results of an *in-vivo* pharmacokinetic investigation, piperine significantly affected the pharmacokinetic characteristics of aceclofenac. Cmax of aceclofenac was increased from 23.59 to 48.52 µg/mL. AUC0-t increase from 130.00 to 272.63 µg/mL*h. Increase in bioavailability of aceclofenac is 2.09-fold. Including piperine with aceclofenac in formulation can improve its oral bioavailability. The optimal bioavailable drug combination with piperine requires substantial research and an appropriate formulation technique.

Conclusion

The HPLC method proved effective, repeatable and precise for identifying specific analytes simultaneously in spiked biological matrix (rat plasma) and *in-vivo* sample sizes. The SPE technique was successfully developed using Phenomenex Strata-X 33m polymeric reverse phase (30 mg/1 mL) cartridges. The piperine was utilized to investigate the influence on the bioavailability of aceclofenac. Concurrent administration of piperine with aceclofenac is a good option for increasing bioavailability. When combined with piperine, aceclofenac Cmax and AUC0-t values increased 2.07 and 2.09 fold, respectively. Our outcomes deliver insight into administration

of piperine in combination with aceclofenac as a better alternative to improve bioavailability while reducing medication burden on patients. This idea can switch the emphasis from substances with numerous harmful effects to the combination therapy of nutrients/fewer toxic substances. Increased oral bioavailability may help in increasing patient compliance with lower dose options.

References

1. *Dooley M, Spencer CM, Dunn CJ. Aceclofenac: a reappraisal of its use in the management of pain and rheumatic disease. Drugs. 2001 Aug; 61(9):1351-78. Available from: doi.org/10.2165/00003495-200161090-00012*

2. *Brogden R N, Wi sema n L R . Aceclofenac: a rev iew of it s pharmacodynamic properties and therapeutic potential in the treatment of rheumatic disorders and in pain management. Drugs. 1996 Jul; 52:113-24. Available from: doi.org/10.2165/00003495- 199652010-00008*

3. *Moon YW, Kang SB, Kim TK, Lee MC. Efficacy and safety of aceclofenac controlled release in patients with knee osteoarthritis: a 4-week, multicenter, randomized, comparative clinical study. Knee Surg Relat Res 2014 Mar; 26(1): 33-42 Available from: doi. org/10.5792/ksrr.2014.26.1.33*

4. *Lapeyre-Mestre, M., Grolleau, S., Montastruc, J.L. Adverse drug reactions associated with the use of NSAIDs: a case/noncase analysis of spontaneous reports from the French pharmacovigilance database 2002–2006. Fundamental & clinical pharmacology, 2013; 27(2), pp.223-230. Available from: doi.org/10.1111/j.1472- 8206.2011.00991.x*

5. *Bort R, Ponsoda X, Carrasco E, Gomez-Lechon MJ, Castell JV. Metabolism of aceclofenac in humans. Drug metabolism and disposition. 1996 Aug 1; 24(8):834-41. Available from: PMID: 8869816*

6. *Noh K, Shin BS, Kwon KI, Yun HY, Kim E, Jeong TC, Kang W. Absolute bioavailability and metabolism of aceclofenac in rats. Archives of pharmacal research. 2015 Jan; 38:68-72. Available from: doi. org/10.1007/s12272-014-0350-4*

7. *Mutalik S, Anju P, Manoj K, Usha AN. Enhancement of dissolution rate and bioavailability of aceclofenac: a chitosan-based solvent change approach. International Journal of Pharmaceutics. 2008 Feb 28; 350(1-2):279-90. Available from: doi.org/10.1016/j. ijpharm.2007.09.006*

8. *Patil LD, Verma U, Patil UD, Naik JB, Narkhede JS. Inclusion of aceclofenac in mesoporous silica nanoparticles: drug release study and statistical optimization of encapsulation efficiency by response surface methodology. Materials Technology. 2019 Oct 15; 34(12):751-63. Available from: doi.org/10.1080/10667857.2 019.1624301*

9. *Rahim H, Sadiq A, Ullah R, Bari A, Amin F, Farooq U, Ullah Jan N, Mahmood HM. Formulation of aceclofenac tablets using nanosuspension as granulating agent: an attempt to enhance dissolution rate and oral bioavailability. International Journal of Nanomedicine. 2020 Nov 17:8999-9009. Available from: doi. org/10.2147/IJN.S270746*

10. *Chavhan SA, Shinde SA, Gupta HN. Current trends on natural bioenhancers: A review. Internal Journal of Pharmacognosy and Chinese Medicine. 2018; 2:2576-4772. Available from: doi. org/10.23880/IPCM-16000123*