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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-UPLC METHOD FOR THE ASSAY AND RELATED SUBSTANCES OF DICLOFENAC SODIUM IN ITS INJECTABLE FORMULATION

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ABSTRACT

Development and validation of a challenging and multipurpose stability-indicating RP-UPLC assay and related substances method for the quantification of diclofenac sodium and its impurities in its injection, in the presence of its degradation products. Various stress conditions, e.g. acid, base, oxidation, thermal and UV radiation has been employed to assess the stability indicating nature of the method. The desired chromatographic separation was achieved on a reverse phase ACQUITY UPLC® BEH SHIELD RP18 50mm x 2.1mm, 1.7µm under isocratic condition with a short run time of 5 minutes. A mixture of Triethylamine buffer of pH 2.0 and Methanol was used as Mobile phase with a flow rate of 0.35mL/min. Quantitation was achieved by UV detection at 254 nm. The method was validated for Linearity, Accuracy, Precision, Specificity, LOQ and Robustness. A linear response (r^2>0.99) was observed for Diclofenac and its impurity in the range of 0.2µg/mL – 230 µg/mL and 0.3µg/mL – 4.8µg/mL respectively. The Limit of Quantitation (LOQ) for diclofenac sodium and its impurity were 0.2µg/ml and 0.3 µg/ml respectively. The proposed method can be used in quality control for the determination of the diclofenac sodium and its

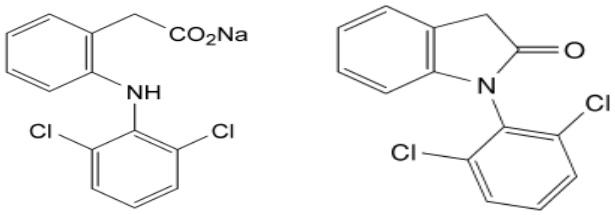
impurity in its injection and for the stability studies as the method separates the drug from its related/degradation products and excipients.

KEYWORDS: UPLC, Diclofenac sodium, Stability indicating.

INTRODUCTION

Diclofenac sodium [N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide] is one of the analgesic-antipyretic-nonsteroidal anti-inflammatory drug. Diclofenac is a widely used for the treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis osteoarthritis, musculoskeletal injuries, and post surgery analgesia in human and veterinary medicine.[1] The molecule is practically water insoluble, but it is readily absorbed from the gastrointestinal tract as the salt form.

Various analytical techniques have been reported for the quantification of diclofenac sodium (DS) in different matrices. High-pressure liquid chromatography detection (HPLC) is the most common used method for the determination of DS in biological sample or dosage forms.[2-3] Analytical methods keep on updating with time as per the requirements so as to develop a simple, reliable, cost effective, reproducible and above all a method bearing a high level of accuracy and precision. Our study aimed to develop a rapid, robust, selective, sensitive, and precise HPLC method for the determination of DS. The assay method was validated using by USP 26 [4-6] by the ICH guidelines.[7-12] The linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) and used for in determination of drug content of the DS in different pharmaceutical commercial products.[13-19]



A **B**

Fig. No. 1: Chemical structure of A) Diclofenac sodium structure and Impurity-A.

MATERIALS AND METHODS

Instruments used

UPLC : Waters Acquity^{TM} UPLC (Waters, USA)

UPLC column : BEH SHIELD-RP 18 column 50 mm x 2.1 mm, 1.7 µm

UPLC detector : PDA Detector

UPLC software : Empower-2

Analytical Balance : Mettler Toledo

Solvent filtration unit: Millipore (Rankem)

Sonicator : Bandelin Sonorex Digitec

pH meter : Lab India pH meter

Graduated Cylinder : 1000ml, 500ml, 250ml, 50ml (Borosil, Rankem)

Volumetric Flasks : 50ml, 25ml, 20ml,10ml (Borosil, Rankem)

Volumetric Pipettes : 5ml, 2.5ml, 2ml, 1ml (Borosil, Rankem)

Graduated Pipettes : 5ml, 2ml, 1ml (Borosil, Rankem)

Reagents used

Water : Milli Q

Methanol : Merck (HPLC grade)

Triethyl amine: Fischer scientific (HPLC grade)

Working Reference Standards:

Drug : Mylan Laboratories Ltd, Hyderabad

Percentage purity : 99.34% (on anhydrous basis)

Impurities used

Impurity-A : Amoli Organics Pvt.Ltd.

Percentage purity : 99.7%

Preparation of Diluent

Mixture of Water and Methanol (35:65 v/v)respectively.

Preparation of diluted standard solution

Preparation of standard stock solution

75.00mg of DCSI RS was transferred in 50 ml volumetric flask and then 35ml of diluent was added, sonicate to dissolve, cool and make up volume with diluent.

Preparation of diluted standard solution

Pipette out 2.5ml of above solution and transferred into 50ml volumetric flask and adjusted the volume with diluent and mix well.

Further transfer 2ml of above solution and transferred into 50ml volumetric flask and adjusted the volume with diluent and mix well.

Preparation of DCSI Related substance 'A' stock solution

1.5 mg of DCSI Related Substance 'A' was transferred in 10 ml volumetric flask and then 2.5ml of methanol was added, sonicate to dissolve, cool and make up volume with diluent.

Preparation of Diethyl pthalate solution

8.00 mg of Diethyl phthalate was weighed and transferred into 50ml flask then added 35 ml of diluent, sonicate for dissolving, cool and diluted to volume with diluent and mix.

Preparation of system suitability solution

Pipette 1ml of DCSI Related compound 'A'stock solution, 10ml of DCSI standard solution and 2.5ml of Diethyl pthalate solution into 20ml volumetric flask and dilute to volume with diluent and mixed.

CHROMATOGRAPHIC CONDITIONS

Column: BEH Shield RP18 (50mm×2.1mm) 1.7 µ

Mobile phase - A: Triethylamine buffer pH 2.0

Mobile phase - B: Methanol

Diluent: Water and Methanol (35:65 v/v)

Mode: Isocratic MP-A: MP-B(35:65)

Flow Rate: 0.35 ml/min

Column Temperature: 35^0C

Sample Temperature: 5^0C

Injection Volume: 2µL

Run Time: 5 min

Needle Wash: 80% methanol

Wave Length: 254nm

Detector: PDA detector

Preparation of Mobile phase

Prepared a mixture of Buffer and Methanol in the ratio of 35:65 v/v respectively and degassed.

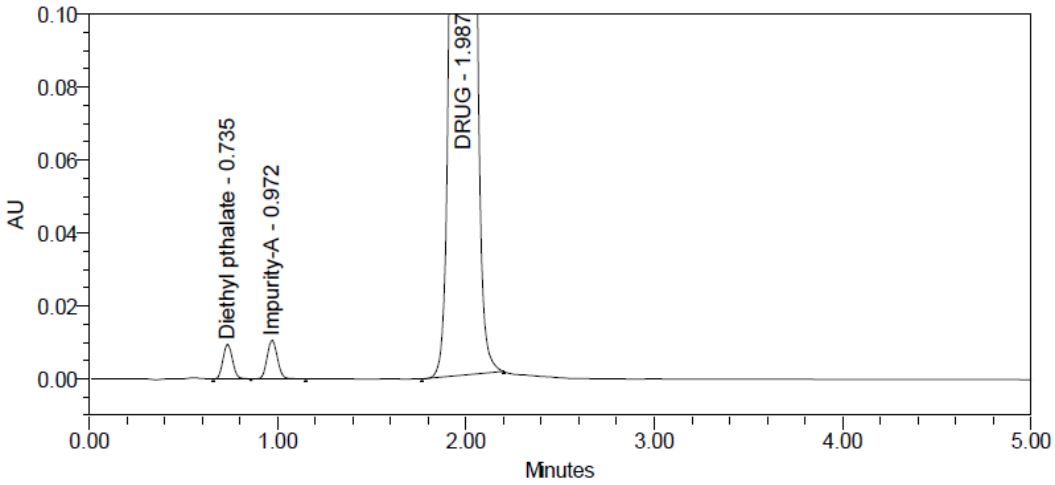


Fig No.2: Chromatogram of optimized method.

Triethyl amine Buffer preparation

5 ml of triethyl amine was added to 1000ml of water and pH was adjusted to 2.0 with orthophosphoric acid and filtered through 0.45 µm membrane filter.

Preparation of Diluent

Mixture of Water and Methanol (35:65 v/v) respectively.

Preparation of standard stock solution

75.00mg of DCSI RS was transferred in 50 ml volumetric flask and then 35ml of diluent was added, sonicated to dissolve, cool and made up the volume with diluent.

Preparation of diluted standard solution

Pipette out 2.5ml of above solution and transferred into 50ml volumetric flask and made up the volume with diluent and mixed well.

Further transfer 2ml of above solution and transferred into 50ml volumetric flask and made up the volume with diluent and mixed well.

Preparation of placebo solution

Pipetted out 2ml of placebo in to a 50ml volumetric flask and then added 35 ml diluent mixed well and sonicated for 5min and made up the final volume with diluent and mixed.

Preparation of test solution

Pipetted out 2ml of sample soution in to a 50 ml volumetric flask and then added 35 ml diluent mixed well and sonicated for 5 min and made up the final volume with diluent and mixed.

Preparation of spiked sample

Pipetted out 2ml of sample solution and 1ml of DCSI Related compound 'A' solution into 50ml volumetric flask and added about 35ml of diluent and sonicated for 5 minutes and made up the final volume with diluent and mixed.

Preparation of standard stock solution

75.00mg of DCSI RS was transferred in 50 ml volumetric flask and then 35ml of diluent was added, sonicated to dissolve, cool and make up volume with diluents (RS stock solution)

Preparation of diluted standard solution

Pipetted out 5ml of above solution and transferred into 50ml volumetric flask and adjusted the volume with diluent and mix well.

Preparation of test solution

Pipetted out 2ml of sample solution in to a 50 ml volumetric flask and then added 35 ml diluent mixed well and sonicated for 5 min and adjusted the final volume with diluent and mixed (RS stock solution).Pipette out 2ml of above solution and transferred into 20ml volumetric flask and adjusted the volume with diluent and mix well.

Preparation of spiked sample

Pipetted out 1ml of sample solution and 1ml of DCSI Related compound 'A' solution into 25 ml volumetric flask and added about 35ml of diluent and sonicated for 5 minutes and adjusted the final volume with diluent and mixed. Pipette out 2ml of above solution and transferred into 20ml volumetric flask and adjusted the volume with diluent and mix well.

METHOD VALIDATION

SYSTEM SUITABILITY

System suitability testing is an integral part of any analytical procedure. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constituting an integral system factor are parameters that are normally used in assessing the column performance. These parameters include column efficiency, resolution,

tailing factor, relative standard deviation, number of theoretical plates, relative retention time and capacity factor.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at two levels: repeatability and intermediate precision. Precision should be investigated using homogeneous, authentic samples. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision was measured in terms of repeatability of application (System precision) and measurement (Method precision). The precision of the method was ascertained from the peak areas of six replicate injections of a fixed concentration.

System precision

The system precision was carried out to verify that the analytical system was working properly and could give accurate and precise results. Standard solution preparation was injected six times into the chromatograph and chromatograms were recorded. The results are given below.

Table 1.0 Results of System Precision.

Injection number	Peak area
1	18935
2	18951
3	18934
4	19031
5	18923
6	18823
Mean	18923.66667
% RSD	0.4

Method Precision (Repeatability)

Precision of the test method is performed by injecting six test samples prepared by spiking the impurity at specification limit to the target concentration. Inject the solutions in the chromatographic system as per the method then calculate the % of individual impurity, % Relative standard deviation of diluted standard and all the impurities. Tabulate the results in the table given below.

Area of impurity in Sample Dilution factor of Std

$$\% \text{ of Impurity} = \frac{\text{Avg Area of Impurity}}{\text{Avg Area of Standard}} \times \frac{\text{Dilution factor of Sample}}{\text{Dilution factor of Sample}} \times \text{Potency of std}$$

Table No. 2: Results for Repeatability of Impurities.

S.no	% Impurity-A	%Total impurities
1	0.20632	0.22632
2	0.20478	0.22478
3	0.20627	0.22627
4	0.20679	0.22679
5	0.20746	0.22746
6	0.20777	0.22777
Avg	0.20657	0.22657
% RSD	0.5	0.4

Intermediate precision (Ruggedness)

Analyse six spiked sample solutions at specification level by different analyst on different day, by using different instrument and different column.

Table No. 3: Results for Intermediate precision of Impurities.

S. no	% Impurity-A
1	0.25331
2	0.25325
3	0.25307
4	0.25330
5	0.25164
6	0.25263
Avg	0.25287
% RSD	0.26

ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or as an accepted reference value and the value found. Prepare stock solution of known impurity, prepare sample solutions in triplicate by spiking test preparation with impurity stock solutions at 50%, 100%and 150 % (preferred) of specification limit to the target concentration. Inject the solutions in the chromatographic system as per the method. Calculate the % recovery of all the impurities. Tabulate the results in the table given below and accuracy at 150% level should be used in range. Accuracy determination is done for every impurity while doing related substances validation to determine the amount of impurity found.

$$\% \text{ of Impurity} = \frac{\text{Area of impurity in Sample}}{\text{Avg Area of Standard}} \times \frac{\text{Dilution factor of Std}}{\text{Dilution factor of Sample}} \times \text{Potency of std}$$

Result obtained in above calculation

$$\% \text{ of recovery} = \frac{\text{Result obtained in above calculation}}{\text{Theoretical value}} \times 100$$

Table No. 4: Recovery % of Impurity-A in Recovery Solution at different Levels.

Sample No.	Spike level	'µg/ml' added	'µg/ml' found (recovered)	% recovery	Mean % recovery
1.	50%	0.09970	0.09522	95.5	95.6
2.	50%		0.09596	96.2	
3.	50%		0.09488	95.2	
1.	100%	0.19940	0.17961	90.1	90.0
2.	100%		0.17841	89.5	
3.	100%		0.17996	90.3	
1.	125%	0.24955	0.22750	91.3	91.9
2.	125%		0.23043	92.4	
3.	125%		0.29935	92.0	
1.	150%	0.31904	0.29232	91.6	92.0
2.	150%		0.29407	92.2	
3.	150%		0.29412	92.2	

LIMIT OF QUANTIFICATION

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be determined quantitatively with suitable precision and accuracy. Determine the limit of Quantification of DCSI and its impurity based on signal to noise ratio method. Determine the signal to noise ratio at analyte concentration of 0.005%. If signal to noise ratio is 10 or more, qualify this concentration as limit of quantification. If signal to noise ratio is less than 10, increase the concentration but not beyond 0.05%, to achieve the signal to noise ratio 10 and then qualify the concentration as limit of quantification. Determine the Precision and Accuracy at Limit of quantification level.

Result obtained in above calculation

$$\% \text{ of recovery} = \frac{\text{Result obtained in above calculation}}{\text{Theoretical value}} \times 100$$

Table No. 5: Results of LOQ.

Name of the Compound	LOQ		
	Concentration (ppm)	Area	S/N Ratio
Impurity-A	0.299	1491	10.2

LINEARITY OF DETECTOR RESPONSE

Linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. To demonstrate the linearity of detector response for DCSI and its impurity prepare not less than five solutions with concentrations ranging from Limit of quantification level to 200 % of the target concentration at specification limit, inject in to the chromatographic system by following concentration in the test method. (preferred levels are LOQ,40%,100%,150% and 200%). Inject the solutions in to the chromatographic system and the calibration curve was plotted using peak area ratio Vs concentration of the standard solution. From the calibration curve, the slope and intercept were calculated**.** Summarize the results in the table given below.

Table No. 6: Results for Linearity of Detector Response for Diclofenac.

S.no	% Level	Concentration (ppm)	Peak Area
1	LOQ	0.417	2588
2	50	1.19	7702
3	100	2.98	18152
4	125	3.725	22303
5	150	4.619	27942

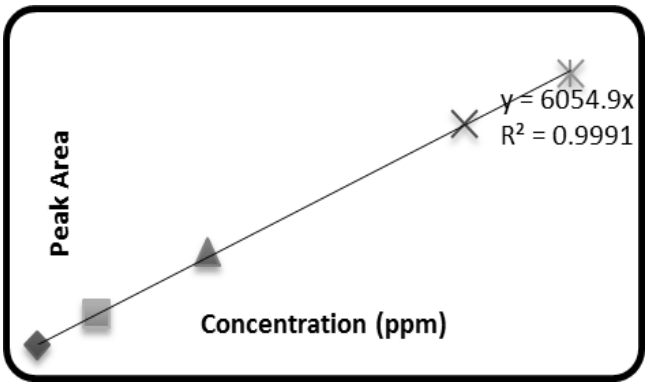


Fig. No. 3: Linearity Graph of DCSI.

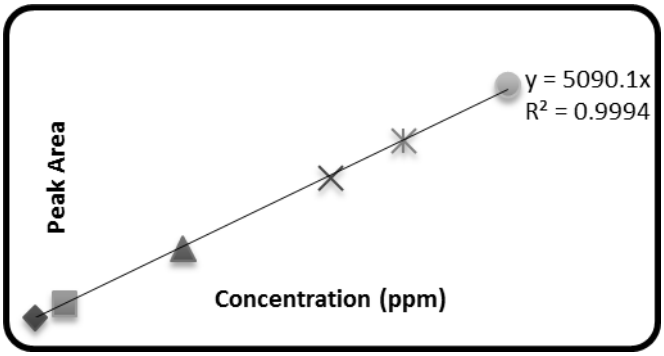


Fig. No. 4: Linearity Graph of Impurity-A.

SPECIFICITY

Specificity is to ensure that the signal measured comes from the substance of interest, and that there is no interference from excipient, degradation products and impurities. Prepare the placebo solution in duplicate as per the test procedure. Inject into chromatographic system and check the interferences due to placebo and blank peaks at the retention time of DCSI and known impurity.

ROBUSTNESS

It is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and indication of its suitability during normal usage.

Table No. 7. Robustness.

S.no	Parameter	Rt of impurity a	Rt of DCSI	R.r.t	Resolution Between impurity a and DCSI
1	CONTROL	0.976	2.02	0.483	8.22
2	pH PLUS	0.985	2.031	0.485	7.76
3	pH MINUS	1	2.115	0.473	8.12
4	TEMP PLUS	0.969	1.985	0.488	7.91
5	TEMP MINUS	0.986	2.065	0.477	7.93
6	FLOW PLUS	0.877	1.814	0.483	7.47
7	FLOW MINUS	1.072	2.223	0.482	8.29

SUMMARY AND CONCLUSION

The scope and objective of the present work is to optimize the chromatographic conditions to develop a RP-UPLC method for the estimation of Diclofenac sodium and its related substances in the selected injectable dosage form (75mg/2ml) and the same is validated. The literature review indicates that there are few methods reported for the determination of

impurities in Diclofenac sodium's injection by HPLC, HPTLC and UV in human plasma and its bioanalytical applications. There is no method reported for the determination of potential impurities of the Diclofenac sodium by UPLC. The RP-UPLC method was developed using BEH SHIELD-RP 18 column 50 mm x 2.1 mm, 1.7µ; detection carried out at 254 nm with flow rate of 0.35mL/min. The mobile phase was Triethylamine buffer of pH 2.0 and Methanol which gives good resolution and good peak shapes for Diclofenac sodium and its related substances, diluent used was Water: Methanol (35:65). The total run time required was 5 minutes. The linearity and range was established over the range of LOQ – 150 % concentration range for Diclofenac sodium and its related substances. The correlation coefficient of Diclofenac sodium and its related substances was found to be 0.999.

The developed method was validated for specificity, accuracy, precision, recovery, linearity, robustness, ruggedness and system suitability. The low standard deviation values and good recoveries indicate the reproducibility and accuracy of the developed method. As well the % RSD values for precision study also were within acceptable limit**.** A Stability indicating, Rapid, Accurate, Precise and Economical UPLC method has been developed for estimation of Diclofenac sodium and its impurities. The proposed method can be used for the routine analysis with less run time.

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CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest related to this research.

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