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## DEVELOPMENT AND VALIDATION OF A HPLC METHOD FOR *IN-VIVO* STUDY OF DICLOFENAC POTASSIUM

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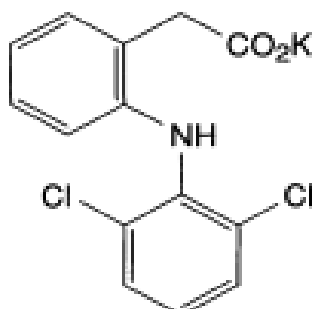
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### ABSTRACT

A High Performance Liquid Chromatographic method was developed for determination of Diclofenac Potassium in *in-vivo* study during bioavailability study. The chromatographic separation was conducted on Shimadzu Prominence LC 20 connected with PDA detector; using mixed column ODS/Cyano; ACE, (100 x 4.6 mm, 5 μm). The mobile phase was isocratic consisted of Methanol: 50 mM potassium dihydrogen phosphate buffer, in ratio of (50 : 50 v/v) and was delivered to the system at a flow rate of 1.5 ml/min, with an injection volume of 20 μl and the detection wavelength ( $\lambda_{max}$ ) was 280 nm. Diazepam was used as internal standard. All assays were performed at ambient conditions. The calibration curve of Diclofenac Potassium in plasma was linear with correlation coefficient ( $r^2$ ) = 0.9996; over a concentration range of 0.25 - 3 ug/ml. The retention times for Diclofenac Potassium and the internal standard (Diazepam) were found to be 5.95 and 7.90 minutes, respectively. The mean recovery was found to be 90.57%. The relative standard deviation (RSD) was found to be < 2. The proposed method was validated and successfully applied to the determination of Diclofenac Potassium in human plasma for bioavailability studies.

**INTRODUCTION:** Diclofenac Potassium is an effective non-steroidal anti-inflammatory drug (NSAID) indicated for various painful indications. Chemically, Diclofenac Potassium is Potassium [2-[(2,6-dichlorophenyl)amino]phenyl]acetate, (C<sub>14</sub>H<sub>10</sub>Cl<sub>2</sub>KNO<sub>2</sub>), (Molecular weight = 334.24) and having a melting point of 283-285°C. Its structure is as follows<sup>1</sup>:



**FIGURE 1: CHEMICAL STRUCTURE OF DICLOFENAC POTASSIUM**

It is official on USP<sup>2</sup>, BP<sup>3</sup>, and Ph. Eur which describe potentiometric method for estimation of the drug. Various literatures disclose UV spectrophotometric methods and different HPLC methods for estimation of Diclofenac Potassium in different dosage forms<sup>4 & 5</sup> and in different biological fluids as plasma and serum<sup>6 & 7</sup>.

The present manuscript describes a new simple, sensitive, accurate, precise, and rapid HPLC method for estimation of Diclofenac Potassium in human plasma.

**MATERIALS AND METHODS:** Diclofenac Potassium standard was supplied by Sinochem, China. Acetonitrile, Water, and Methanol were HPLC grade; supplied by Merck, Germany.

Diazepam (as internal standard) and Potassium dihydrogen phosphate were supplied by Sigma-Aldrich, Germany. The method development was performed with Shimadzu Prominence LC 20 connected with PDA detector; using mixed column ODS/Cyano; ACE, (100 x 4.6 mm, 5  $\mu$ m). Injection volume was 20  $\mu$ l.

### Analytical procedure for determination of Diclofenac Potassium in plasma:

#### 1. Assay Method Description and Chromatographic conditions:

A simple, rapid, specific and reliable HPLC assay of Diclofenac Potassium in human plasma has been developed. The analysis was conducted on Shimadzu Prominence LC 20 connected with PDA detector; using mixed column ODS/Cyano; ACE, (100 x 4.6 mm, 5  $\mu$ m). The mobile phase was isocratic consisted of Methanol: 50 mM Potassium dihydrogen phosphate, in ratio of (50 : 50 v/v) and was delivered to the system at a flow rate of 1.5 ml/min, with an injection volume of 20  $\mu$ l and the detection wavelength ( $\lambda_{max}$ ) was 280 nm. Diazepam was used as internal standard. All assays were performed at ambient conditions.

#### 2. Preparation of stock and working standard solution:

Stock solution of Diazepam (internal standard) solution was prepared by dissolving 50 mg of Diazepam in 100 ml methanol; then sonication for 5 minutes (500  $\mu$ g/ml). The working internal standard was prepared on each day of analysis by diluting the stock solution to contain (1.5  $\mu$ g/ml). Stock solution of Diclofenac Potassium was prepared by dissolving 50 mg Diclofenac Potassium in 100 ml methanol; then sonication for 5 minutes (500  $\mu$ g/ml). The working solution was prepared on each day of analysis by diluting the stock solution with methanol to give serial dilutions containing 0.25, 0.5, 1, 1.5, 2, 2.5 and 3  $\mu$ g/ml of Diclofenac Potassium; which were shaken well and filtered over 0.45  $\mu$ m syringe filter and injected onto HPLC.

#### 3. Plasma sample preparation for determination of Diclofenac Potassium:

The extraction procedure was applied in the preparation of plasma samples and standards, where 1 ml of each human plasma was transferred into 5 ml tube fitted with polyethylene cap.

1 ml of internal standard working solution and 1 ml methanol were added. The mixture was vortexed for 2 minutes and centrifuged at 6000 rpm for 30 minutes. The upper layer was transferred to another tube and filtered through 0.45  $\mu$ m syringe filter. A 20  $\mu$ l volume of the supernatant was injected onto the HPLC column. Concentrations of Diclofenac Potassium in unknown samples were calculated with reference to the prepared calibration curve. Retention time of Diclofenac Potassium was 5.95 minutes.

#### 4. Preparation of *in-vivo* standard calibration curve:

For calibration curve, plasma standards were prepared by spiking 1 ml of blank plasma with 1 ml of the internal standard working solution and appropriate volumes of Diclofenac Potassium working solution to produce concentrations ranging from (0.25, 0.5, 1, 1.5, 2, 2.5 and 3  $\mu$ g/ml). The spiked plasma standards were processed as described above.

The calibration curve was obtained by plotting chromatographic peak area ratios (Diclofenac Potassium/Diazepam) against the corresponding nominal Diclofenac Potassium concentration added. Samples were prepared and injected on the same day.

#### 5. Sample calculation:

The unknown sample concentration was calculated from the following formula:  $C = [(R - Y) / S]$ ; where C is Diclofenac Potassium concentration, R is the peak area ratio (drug/internal standard), S is the slope of the calibration curve and Y is the Y-intercept.

#### 6. Assay method Validation:

a. **Selectivity:** Plasma samples (four blanks) were chromatographed prior to use to check for endogenous components, which might interfere with Diclofenac Potassium or (Internal standard) Diazepam. Spiked plasma samples representing a low, medium and high Diclofenac Potassium concentration were also analyzed to verify the selectivity of the method of analysis.

b. **Linearity and linear working range:** The linearity and linear working range were determined from the constructed standard calibration curve.

- c. **Intraday Accuracy and Precision:** The intraday accuracy and precision evaluations were assessed by repeated analysis and human plasma samples containing different concentrations of Diclofenac Potassium on separate occasions in the same day (3 times on the same day). The analytical precision of the method was determined by the relative standard deviation of the percentage drug recovered.
- d. **Inter-day Reproducibility:** The day to day reproducibility of the assay for plasma samples was evaluated by comparing the least squares linear regression analysis of three standard plots obtained from spiked plasma standard at three different days over three weeks period.
- e. **Freeze and Thaw Stability in Human Plasma:** Diclofenac Potassium stability in human plasma was determined after three freeze-thaw cycles. In order to determine the effect of storage on the stability of frozen samples, three concentrations were selected representing the low, medium and high spiked plasma samples of the calibration curves. These concentrations were analyzed when fresh and after storage just prior to analysis (storage at  $-20^{\circ}\text{C}$  for 24 hours). The freeze-thaw cycle were repeated two more times, and then analyzed using the previously validated method.

- f. **Extraction Recovery:** Relative recoveries of all Diclofenac Potassium concentrations used in the plasma standards were evaluated by comparing their peak area ratios with those obtained from direct injection of unprocessed reference solutions of the same concentrations.

## RESULTS AND DISCUSSION:

### Assay Method Validation of Diclofenac Potassium:

1. **Selectivity:** Chromatograms obtained at the lower of sensitivity for drug free plasma showed no interfering peaks at the retention times of Diclofenac Potassium and the internal standard. Typical chromatograms obtained from a processed blank human plasma sample alone, with Diclofenac Potassium and with Diclofenac Potassium/Diazepam are illustrated in **figures 2, 3, and (4)** respectively.

The retention times of Diclofenac Potassium and the internal standard (Diazepam) are approximately 5.95 and 7.9, respectively. Both peaks were sharp with good baseline resolution and minimal tailing, thus facilitating accurate measurements of the peak area ratios. No interferences by the metabolites or normal constituents of plasma were observed. The overall chromatographic time is 10 minutes.

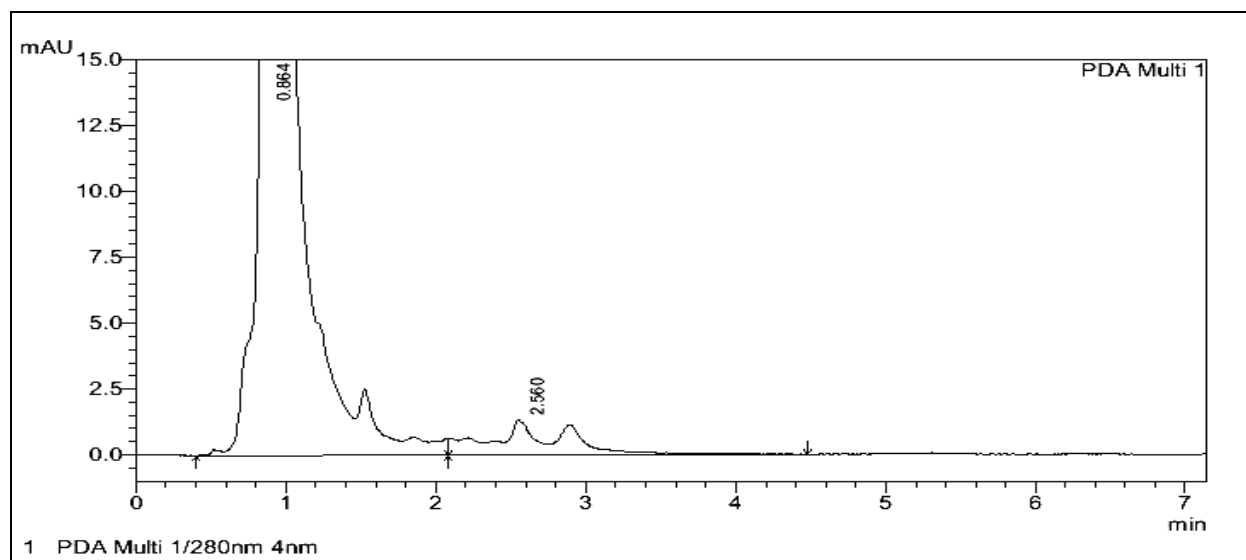


FIGURE 2: A TYPICAL CHROMATOGRAM OBTAINED FROM A PROCESSED BLANK HUMAN PLASMA SAMPLE

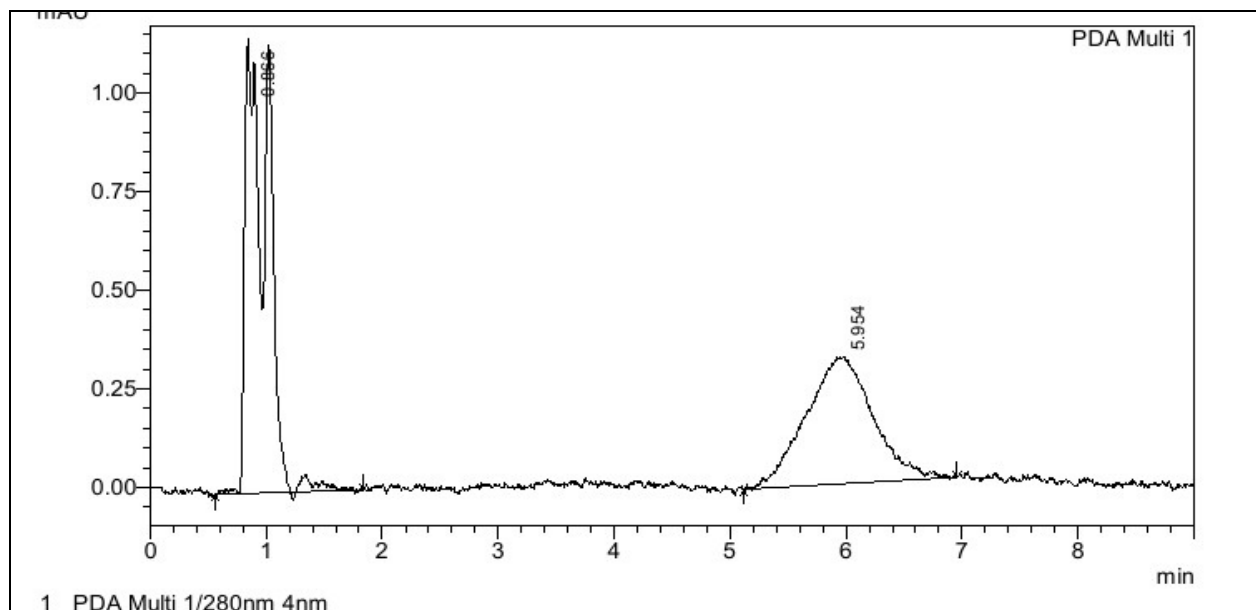


FIGURE 3: A TYPICAL CHROMATOGRAM OBTAINED FROM PROCESSED HUMAN PLASMA SPIKED WITH DICLOFENAC POTASSIUM

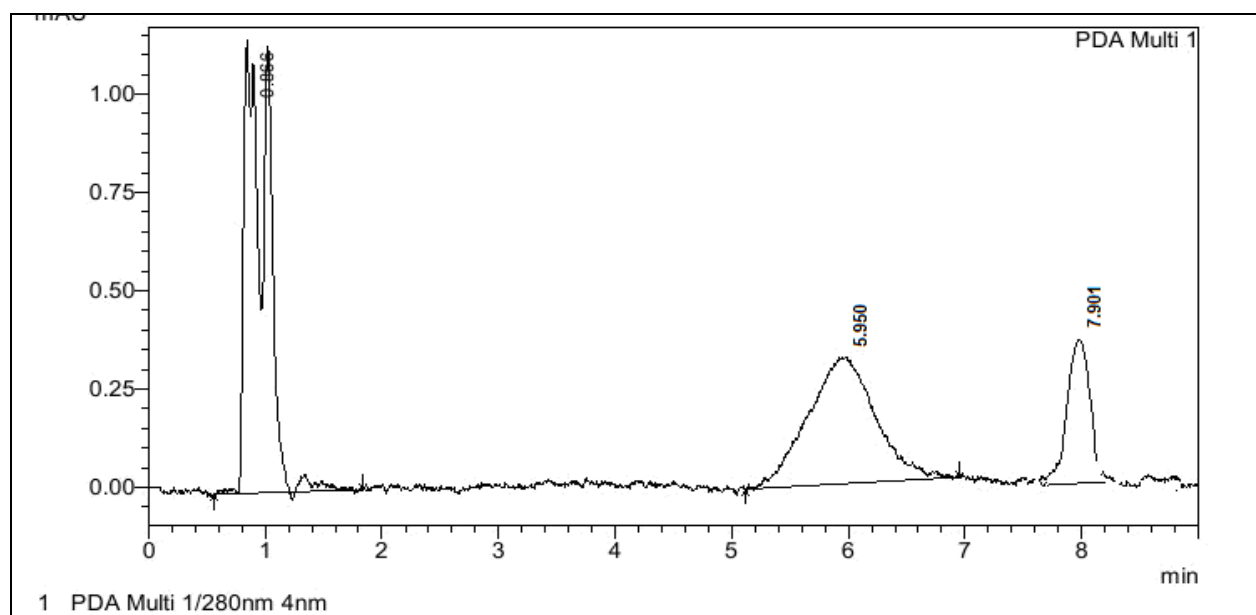


FIGURE 4: A TYPICAL CHROMATOGRAM OBTAINED FROM PROCESSED HUMAN PLASMA SPIKED WITH DICLOFENAC POTASSIUM AND DIAZEPAM

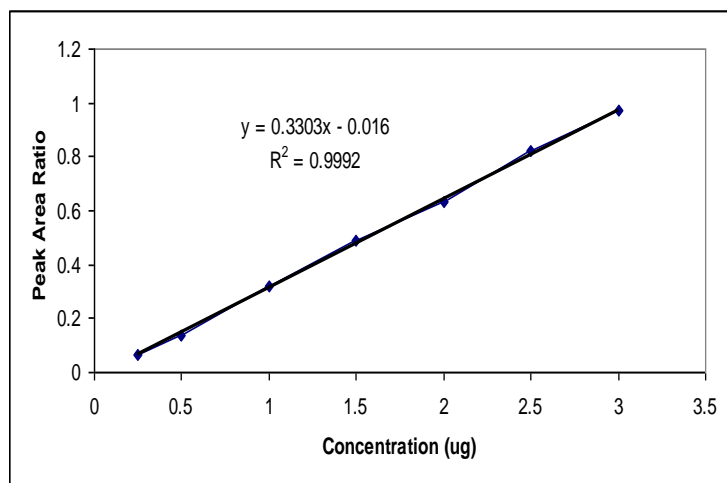
2. **Linearity and Linear working range:** Table 1 and figure 5 represents the standard curve of Diclofenac Potassium using HPLC. The regression equation:  $Y = 0.3303x - 0.016$  (where Y is the peak area ratio of Diclofenac Potassium and x is the concentration of Diclofenac Potassium in  $\mu\text{g/ml}$ ). The lower limit of quantification was  $0.25 \mu\text{g/ml}$ , with a linear response across the full range of concentrations from  $0.25 - 3 \mu\text{g/ml}$  ( $R^2 = 0.9992$ ). Standard curve obtained for Diclofenac Potassium in plasma samples was highly linear in the concentration range of  $0.25 - 3 \mu\text{g/ml}$ .

Linear regression analysis of the standard calibration plot for human plasma was  $y = 0.1615x - 0.0225$ , where y and x are the peak area ratio and Diclofenac Potassium concentration, respectively. The correlation coefficient of the line was ( $R^2 = 0.9996$ ). The data is represented by table 2 and figure 6.

3. **c. intraday (Within Day) Accuracy and Precision:** The analytical precision of the method is determined by the relative standard deviation of the percent drug recovered as shown in table 3.

**TABLE 1: RELATIONSHIP BETWEEN THE CONCENTRATION OF DICLOFENAC POTASSIUM AND THE MEAN RELATIVE PEAK AREA USING HPLC**

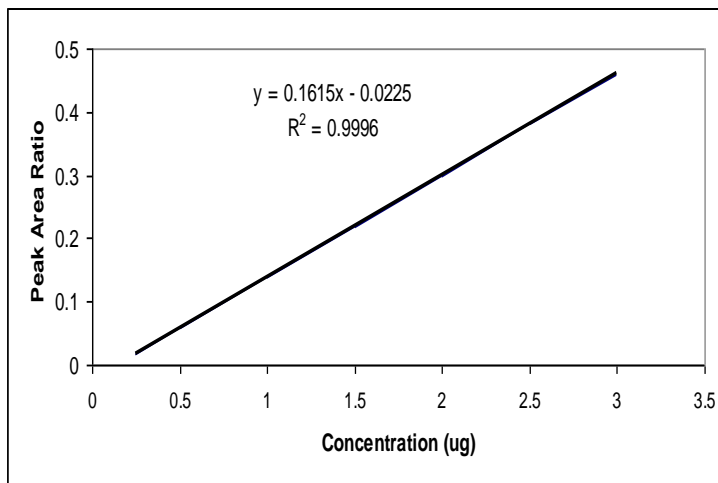
Concentration (µg/ml)	Peak area Ratio	± S.D
0.25	0.06825	1.157
0.5	0.14	0.908
1	0.32	0.409
1.5	0.49	0.514
2	0.63	0.264
2.5	0.82	0.995
3	0.97	0.561



**FIGURE 5: CALIBRATION CURVE OF DICLOFENAC POTASSIUM STANDARD**

**TABLE 2: RELATIONSHIP BETWEEN THE CONCENTRATION OF DICLOFENAC POTASSIUM IN HUMAN PLASMA AND THE MEAN RELATIVE PEAK AREA USING HPLC**

Concentration (µg/ml)	Peak area ratio	± S.D
0.25	0.0165	1.351
0.5	0.06	0.641
1	0.14	0.373
1.5	0.21975	0.552
2	0.3	0.302
2.5	0.38125	0.672
3	0.46	0.812



**FIGURE 6: CALIBRATION CURVE OF DICLOFENAC POTASSIUM SPIKED HUMAN PLASMA**

**TABLE 3: ANALYTICAL PRECISION FOR THE ANALYSIS OF DICLOFENAC POTASSIUM PERFORMED ON 3 SETS OF STANDARD CURVES OF THE SAME DAY**

Spiked conc. (µg/ml)	Peak area ratio			Mean	± S.D	RSD %
	1	2	3			
0.5	0.05744	0.05675	0.05731	0.06	0.000367	0.641
1	0.13857	0.13834	0.13933	0.14	0.000518	0.373
1.5	0.21785	0.21859	0.21712	0.22	0.000735	0.337
2	0.30326	0.30614	0.3032	0.30	0.001680	0.552
2.5	0.38944	0.38467	0.38652	0.39	0.002405	0.871
3	0.45973	0.46251	0.46102	0.46	0.001391	0.302
r2	0.99938	0.99966	0.999866	0.99963		
Slope	0.162826	1.0017	0.99676	0.162907		
Intercept	-0.02389	-0.00033	0.000428	-0.024		

4. **Inter-day (Day to Day) Reproducibility:** Table 4 reveals the results of the inter-day evaluations. The results show that the correlation coefficients mean was higher than 0.99 and the coefficient of variance of the slope of the three trend lines was less than 2%. The analysis of variance of the data showed no significant difference in the slopes of the three standard plots. The results thus confirmed excellent linearity of the calibration lines and high reproducibility of the assay.

**TABLE 4: INTRA-DAY REPRODUCIBILITY FOR THE STANDARD PLOT OF DICLOFENAC POTASSIUM IN PLASMA**

Standard plot <sup>i</sup>	Slope <sup>ii</sup>	Intercept <sup>ii</sup>	Correlation Coefficient
1	0.162907	-0.0240	1.00
2	0.163049	-0.02378	0.9997
3	0.162977	-0.02396	0.9993

- i- Obtained in 3 different days
- ii- Mean of 3 determinations at each drug concentration

5. **Freeze and thaw stability in Human Plasma:** Table 5 shows the peak area ratio of three Diclofenac Potassium concentrations, namely 0.25, 2 and 5 µg/ml; representing low, medium and high spiked

plasma samples of the calibration curve when fresh and stored at -20°C. Slight difference between spiked drug concentration in both fresh and stored samples were observed.

**TABLE 5: THE PEAK AREA RATIO OF DICLOFENAC POTASSIUM TO THE INTERNAL STANDARD OF THREE DIFFERENT CONCENTRATIONS, (FRESH AND STORED AT -20°C)**

CONC. (µG/ML)	PEAK AREA RATIO					
	Fresh Sample			Stored Sample		
0.25	0.0165	0.0167	0.0166	0.01646	0.0166	0.0165
2	0.3	0.299	0.303	0.2997	0.2988	0.3
5	0.786	0.7849	0.7858	0.7858	0.7846	0.7856

6. **Extraction Recovery:** Data of the extraction recovery of Diclofenac Potassium from plasma samples fortified with the drug in the same concentrations of the drug used in the calibration curve in human plasma are shown in table 6. The extraction recovery ranged from (82.69 – 96.12%)

with a mean percent recovery 90.576% and the average coefficient of variation was 0.717%. The previous outcomes revealed the suitability of the method for the determination of Diclofenac Potassium in the plasma samples.

**TABLE 6: WITHIN DAY ACCURACY FOR DETERMINATION OF DICLOFENAC POTASSIUM IN PLASMA**

Diclofenac Potassium (µg/ml)	n = 3	Mean Recovery (µg/ml)	± S.D	Recovery %	CV%
0.5	3	0.41345	0.00675	82.69	2.00
1		0.8575	0.00587	85.75	0.838
1.5		1.33095	0.00758	88.73	0.697
2		1.9224	0.00262	96.12	0.167
2.5		2.36775	0.007	94.71	0.364
3		2.8629	0.0055	95.43	0.236

**CONCLUSIONS:** The developed method showed adequate accuracy, precision, sensitivity and reproducibility. From which, it can be concluded that the proposed method can be used for in-vivo analysis of Diclofenac Potassium in plasma.

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