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Novel Nano-Formulation Concept of Ketorolac Loaded with PLGA: Design, Development and Analysis

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ABSTRACT

The main objective of the present work is to formulate Ketorolac tromethamine (KT) loaded PLGA nanoparticles (NPs) with sustained effect and improved bioavailability. Ketorolac tromethamine (KT) is well known NSAID (Non-steroidal anti-inflammatory drug) reported with multiple pharmacological activities. Ketorolac tromethamine (KT) loaded PLGA (polylactic-coglycolic acid) nanoparticles were prepared for determining the cytotoxicity activity on the SCC 29 colon cancer cell lines. The process of KT nanoparticle fabrication includes solubility in dichloromethane, blending with (non-ionic surfactant) Pluronic F68 followed by ultrasonication and solvent evaporation. In this study five formulations were prepared (F1-F5) which are subjected to stability testing at various pHs, Drug release and in vivo pharmacokinetic studies. The prepared nanoparticles showed better oral absorption and good cytotoxic properties than KT alone.

Keywords: *Cytotoxicity, Dug stability, Ketorolac, Pluronic F68, PLGA Nanoparticles.*

INTRODUCTION

Chemically ketorolac is 5-benzoyl -2,3-dihydro-1H-Pyrrolizine 1-carboxyllic acid [1] Ketorolac is a non-steroidal anti-inflammatory agent and it is chemically related to the indomethacin. Ketorolac is a racemic mixture of [-]S and [+] R enantiomer forms with the S form having analgesic activity. Anti-inflammatory effects are believed to be inhibition of both COX-1 and COX-2 which leads to inhibition of prostaglandin synthesis leading to decreased formation of precursors of prostaglandins and thromboxanes from arachdonicacid [2-4].In terms of Ophthalmic applications of ketorolac, ocular administration of ketorolac reduces PGE2 levels in aqueous humor[5].Ketoralac is a well-known NSAID in the treatment of Rheumatoid arthritis, inflammation, Pain reliever ,in reduction of aqueous humor and post operative cancer pain[6]. Extensive literature review on ketorolac with empirical evidences revealed that along with its anti-inflammatory activity it showed promising results in treatment of various cancers. Sabinda et al has proved that ketorolac salt is a newly discovered DDX3 inhibitor for the treatment of oral cancer.[7]. *Invitro* Anticancer activity of ketorolac with rosuvastatin easy found effective against DDX3 in the form of hydrogel formulation.in oral squamous cell carcinoma has been reported by Khaggeswar et al, 2015.[8]. Ketorolac also showed its therapeutic benefit in ovarian cancer patient which has been reported by Yuna.G, 2015 [9]. By restoring above facts in mind the present research work has been designed to prepare the nanoparticles of ketorolac with PLGA for the investigation of its anticancer activity .PLGA is a bio-degradable, bio-compatible and non-toxic polymer with mucoadhesive properties[10] which have greater drug loading capabilities and able to show sustained drug release properties[11].PLGA nanoparticles are prepared by ionotropic gelation method which does involve interaction between the negative groups of

sodium tripolyphosphate and the positively charged amino groups of PLGA[12]. The following are among the important technological advan-tages of nanoparticles as drug carriers: high stability (i.e., longshelf life); high carrier capacity (i.e., many drug molecules can be incorporated in the particle matrix); feasibility of incorpora-tion of both hydrophilic and hydrophobic substances; and feasi-bility of variable routes of administration, including oral adminis-tration and inhalation. These carriers can also be designed to enable controlled (sustained) drug release from the matrix. The methods for nanoparticle preparation and characterization.

The aim of the proposed study is to prepare and evaluate PLGA nanoparticles of KT and to study the cytotoxicity effect in SCC-29 cell lines. Therefore the present research work aimed to approach one step The following are among the important technological advan-tages of nanoparticles as drug carriers: high stability (i.e., long shelf life); high carrier capacity (i.e., many drug molecules can be incorporated in the particle matrix); feasibility of incorpora-tion of both hydrophilic and hydrophobic substances; and feasi-bility of variable routes of administration, including oral adminis-tration and inhalation. These carriers can also be designed to enable controlled (sustained) drug release from the matrix ahead for further establishment of Ketorolac as an anti-cancer agent. Furthermore, drug loading, drug stability, oral pharmacokinetics and cytotoxic activity in cell lines were studied.

MATERIALS AND METHODS

PLGA (poly (lactic-co-glycolic acid) mol wt 148.11), Pluronic F68, didodecyl dimethyl ammonium bromide (DMAB) were purchased from sigma (U.S.A). Potassium dihydrogen phosphate, acetone, ethyl acetate sodium hydroxide, dichloromethane, D-mannitol, were of analytical grade. HPLC grade acetonitrile was purchased from SD FineChem, India.

HPLC Analytical Method

HPLC Instrument for quantification of Ketorolac tromethamine (KT)

A reversed phase HPLC method was developed for analysis of Ketorolac tromethamine (KT). The HPLC system consisted of two Shimadzu LC-20AD HPLC pumps equipped with SPD-20A UV/VIS detector, a Rheodyne (20 µl volume capacity) injector and Shimadzu LC Solution software was used. Chromatographic separation was performed on 25 cm RP-C18 (250mm x 4.6mm i.d.) with particle size of 5µm HPLC column. The mobile phase consisted of acetonitrile: milli pore water (65:35, v/v) was used. Freshly prepared mobile phase was filtered through 0.22 µm filter and degassed for 20 min before analysis. All samples were analyzed under Gradient elution at a flow rate of 1.0 ml/min, and effluent was monitored at 320 nm. A 25 µl of sample was injected onto the Rheodyne and analyzed at 25 ^{0}C.

Chromatographic Conditions

Column RP C18	(25.0 cm length)
Pump system	Gradient pump system
Mobile phase	ACN : Millipore Water (65:35, v/v)
Pump pressure	12.0 Mpa
Flow rate	1.0 ml/min
RT	2.35 mins
UV Wavelength	320 nm
Injection volume	20 µl
Injector volume	25 µl

Analytical Method Development

A suitable mobile phase was selected to develop simple analytical method for Ketorolac tromethamine in bulk. For selection of mobile phase, the criteria employed were sensitivity of the method, ease of sample preparation, solubility of the drug, cost of solvents and applicability of method to various purposes.

Preparation of Ketorolac tromethamine Standard Stock Solution

Standard Ketorolac tromethamine (10.0mg) was dissolved in 65.0 ml of Acetonitrile and made up to 100 ml with Millipore Water to get the final concentration of 100 µg/ml.

Calibration Standards

From the standard stock solution of Ketorolac tromethamine (100µg/ml), different concentrations were prepared in the range of 0.1, 0.5, 1.0, 5.0, 10.0 and 15.0µg/ml and chromatogram showing concentration 1.0 µg/ml (Figure 1). The calibration curve was plotted (Figure – 2) and data presented in Table –1

System Suitability Test

The system suitability test is an integrated part of the analytical method and it ascertains the suitability and effectiveness of the operating system. System suitability test of the chromatographic system was performed before validation run. Theoretical plates and tailing factor were determined and results shown in Table – 2

Analytical Method Validation

a) Specificity

The specificity of the method was determined by checking for interference with the drug from placebo components. No interfering peaks were found in the chromatogram near the retention time of the drug indicating that excipients did not interfere with the estimation of the drug by proposed HPLC method.

b) Linearity and range

From Ketorolac tromethamine stock solution (100µg/ml) was diluted to yield solutions in the concentration range of 0.1-15µg/ml. The solutions were prepared in triplicate and analysed by injecting 20µl in HPLC and results were showed in Table – 3

c) Accuracy

To determine accuracy of the proposed methods, different concentrations of Ketorolac tromethamine was prepared from independent stock solution and analysed. The percentage recoveries (±SD) for Ketorolac tromethamine was found to be within acceptable limit (Table –4). The high percentage recovery and low standard deviation values indicate that method is accurate. This result revealed that any small change in the drug concentration in the solution can be accurately determined by these proposed methods

d) Precision

Precision determined by studying repeatability and intermediate precision. Repeatability was determined by using LQC, MQC and HQC of Ketorolac tromethamine: 0.25, 2.5 and 12.5µg/ml and analysed (N = 9). Inter-day and intra-day variation was taken to determine intermediate precision of the proposed methods. Different levels of drug concentrations in triplicates were prepared three different times in a day and studied for intra-day variation. Same protocol was followed for three different days to study inter-day variation (N = 27). Percent RSD values were found to be below 2% which indicating that these methods have excellent repeatability and intermediate precision (Table – 5.).

e) Detection limit (DL) and Quantitation limit (QL)

The DL and QL of Ketorolac tromethamine by the proposed methods were determined by using calibration standards. DL and QL were calculated as 3.3 σ/S and 10 σ/S respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regression equation. The results are shown in Table – 6

f) Robustness

Robustness is a measure of capacity of a method to remain unaffected by small, but deliberate variations in the method conditions and is indications of the reliability of the method. The proposed method was determined by variation of mobile phase composition by ±2% of ACN: Millipore Water (63:37, v/v and 67:33, v/v) and flow rate by ±0.1ml/min (0.9 and 1.1ml/min) had no significant effect on the retention time and chromatographic response of Ketorolac tromethamine, indicating that the method was robust and the results are shown in Table - 7.

Preparation of Nanoparticles

Emulsion-Solvent Evaporation Method:

In the present study, the five formulations (F1 to F5) of Ketorolac tromethamine loaded PLGA nanoparticles were prepared by addition of 3% (F1), 5% (F2), 7% (F3), 9 % (F4) and 10% (F5) of PLGA with constant addition of 2% Ketorolac tromethamine. Accurately weighed 100 mg of Ketorolac tromethamine and 3 ml of dichloromethane was transferred in to test tube and allowed the polymer to dissolve overnight. Furthermore, 0.4% w/v solution of Ketorolac tromethamine was emulsified with dichloromethane and further emulsified with 20 ml of 1% w/v Pluronic F68. The component was placed for ultrasonication process for 10 min at 30% amplitude and subjected to solvent evaporation by stirring for 8 h. The nanoparticles distributed in sample was centrifuged at 12000 rpm, the sediment of nanoparticles were collected.

Characterization of the Nanoparticles

Dynamic light scattering (DLS) measurements

The average particle size and its distribution (polydispersity index, PDI) of nano particles were measured in triplicate at 25 ◦C by DLS using Zetasizer. The instrument utilizes a 4 mW He–Ne red laser at 633 nm. The light scattering is detected at 173◦ by non-invasive backscatter (NIBS) technology with a measuring range from approximately 0.6 nm to 6 µm. Disposable polystyrene cuvettes, 1 ml were used for measurements. Water or buffers (filtered through a 0.22 µm membrane filter) were used to dilute the formulations. The DLS measurement yields z-average mean hydro dynamic diameter of the sample, which is intensity weighted mean diameter of the bulk population. Whereas, the PI value obtained is a measure for the width of size distribution and ranges from 0 to 1. The values near to zero indicate monodispersed particle population whereas values >0.5 signifies a very broad size distribution. And the results are shown in Table – 8.

Determination of Ketorolac tromethamine entrapment efficiency and drug loading capacity of PLGA nanoparticles

Nanoparticles of Ketorolac tromethamine were further evaluated for loading capacity and entrapment efficiency by centrifuging at 10000x g at -5° C for 45 min. The amount of free Ketorolac tromethamine in the clear surface solution after centrifugation was determined by using HPLC. The drug loading and loading efficiency of KT in PLGA nanoparicles were determined from the following formulae 1 and 2 mentioned below:

Stabili

Encapsulation efficiency (% EE) = [1-(Drug in clear supernatant liquid / Total drug added)] x 100% Drug loading capacity (% LC) = (Weight of KT in NP / Weight of NP recovered) x 100

Study of Ketorolac tromethamine loaded PLGA nanoparticles at different pH

The stability of Ketorolac tromethamine loaded PLGA nanoparticles were evaluated at various pH 1.2, 3.5, 5.5, 6.8 and 7.4 respectively. Accurately weighed 10 mg of Ketorolac tromethamine loaded PLGA nanoparticles and pure Ketorolac tromethamine was transferred to 1.5 ml centrifuge tube. Furthermore, 1 ml of mentioned buffer was added to centrifuge tubes containing PLGA nanoparticles and incubated at 25° C for 24 h. The drug content was determined after exposure with various pH and the percent of drug degradation was evaluated The prepared formulations were further analyzed for drug content and pH dependent stability. Each formulation was diluted with mobile phase acetonitrile, 65% v/v at 25ºC and analyzed to obtain the HPLC area as per the requirements of linearity and amount of drug in each formulation was calculated. The solutions of pH 1.2 and 2.5 were prepared using 0.1 N hydrochloric acid and remaining pH solutions were made with phosphate buffers. The drug content was determined using HPLC method and the extent of drug degradation was evaluated and the results are shown in Table – 9.

Study of *in-vitro* release of KT loaded PLGA Nanoparticles

The in vitro drug release is very important to determine the effect of formulation components and manufacturing processes on finished product, to analyze the batch-to-batch variation, to meet the label claims, to establish in vitro in vivo correlation and further as compendia requirement. To evaluate the drug release, the nanoparticles were diluted with 1 ml of phosphate buffer, pH 7.4 and incubated at 37° C in temperature controlled automatic shaking equipment (50 rpm) for 3 h. At various time points, the selected tube was centrifuged and the amount of drug release was determined using validated HPLC method. (Table – 10).

Determination of bioavailability of KT loaded PLGA Nanoparticles

Bioavailability is the rate and extent of drug absorption and essential for any pharmaceutical molecule to elicit pharmacological action. To determine the bioavailability profile of KT and KT loaded PLGA nanoparticles, KT alone and KT loaded PLGA nanoparticles were administered orally to wistar rats. The animal experiments protocol was approved by Institutional Animal Ethics Committee of Balaji Institute of Pharmaceutical Sciences has approved animal facility with CPCSEA registration No. 1694/PO/Re/13/ CPCSEA -has approved animal facility with CPCSEA. The KT alone and formulations were taken equivalent to 5 mg of KT and administered orally to wistar rats (weighing approximately 260 g). The blood samples were withdrawn from tail vein under mild anaesthesia. The time intervals were 0.5, 1, 3, 6, 9, 12, 18, 24, 36 and 48 h respectively. The blood samples were added to tubes having EDTA. In addition to the above, protein precipitation method was performed and analyzed using validated HPLC method.(Table-11)

***In vitro* Cytotoxicity Studies**

The *in vitro* anticancer activity of KT and KT loaded PLGA nanoparticles were determined at different levels in SCC-29 cell lines. In Brief, SCC-29 cells were taken at a concentration of 1 × 10^3 cells per well in 96-well plates. After 72 h of treatment with formulations at 0.001, 0.01, 0.1, 1, 5, 10 and 20 µM, MTS reagent was transferred to the cells and incubated for two hours and the cell viability was evaluated at absorbance at 490 nm using an ELISA reader. (Table-12)

RESULTS

Analytical method development and validation:

An analytical HPLC method for Ketorolac tromethamine in bulk was developed and validated as per ICH guidelines. The basic chromatographic conditions were designed to be simple and easy to use for HPLC analysis. The RP-C18 column was used because of its advantages of better reproducibility, low-back pressure and low tailing. The proportion of the mobile phase components was optimized to reduce retention times and to produce sharp peak. Detection at 320nm resulted in good response and good linearity. The retention time of KT was found to be 2.3±0.5 min respectively.

System suitability test results of Ketorolac tromethamine shows that the system is suitable and the selected column is more efficient for the proposed HPLC method. The specificity of the method was determined by checking for interference with the drug from placebo components. No interfering peaks were found in the chromatogram near the retention time of the Ketorolac tromethamine indicating that excipients did not interfere with the estimation of the drug by proposed HPLC method and the proposed method is specific and selective for the Ketorolac tromethamine. To determine linearity a calibration graph was obtained by plotting Ketorolac tromethamine concentration against peak area. Linearity was good in the concentration range of 0.1-15.0µg/ml. The regression equation was y = 46529 x + 4194 with the correlation coefficient was 0.9999 (Fig. 3.2). DL and QL of Ketorolac tromethamine was found to be 0.0295 µg/ml and 0.0895 µg/ml respectively. The excellent mean percent recovery values (nearly 100%) and their low standard deviation values (SD<1.0) of Ketorolac tromethamine represent accuracy. This result revealed that any small change in the drug concentration in the solution can be accurately determined by this proposed method. Precision determined by studying repeatability and intermediate precision. The low percent RSD values were found to be below 2% which indicating that proposed method has excellent repeatability and intermediate precision. The analytical method of Ketorolac tromethamine remained unaffected by slight but deliberate changes in the analytical conditions. The result was not affected by varying the conditions and system suitability data were also found to be satisfactory during variation of the analytical conditions (Table 5.1.6) and therefore the proposed method is robust.

Preparation and Characterization of the Nanoparticles

Five formulations of KT loaded PLGA nanoparticles were prepared and further analysed for determination of particle size. The sizes of the nanoparticles of the five formulations (F1 to F5) were found 184 nm, 164 nm, 184 nm, 164 nm and 170 nm respectively.

Determination of KT Drug Loading Capacity Entrapment Efficiency and of PLGA Nanoparticles.

The nanoparticle size was directly proportional to the drug concentration; therefore, the nanoparticles size was increased to the increasing in the concentration of KT. The entrapment efficiency (% EE) and drug loading (% LC) with various PLGA concentrations were showed in the Figure. The maximum drug loading capacity 68% was observed for KT at 0.8 mg/mL and conclude that as the concentration of KT increases, the entrapment efficiency is decreased and drug loading is increased.

Stability study of KT and KT loaded PLGA Nanoparticles at Different pH

KT and KT loaded PLGA nanoparticles of KT i.e. F1, F2, F3, F4 and F5 were subjected to stability study at pH 3.5, 5.5 and 6.8. The formulation F2, F3, F4 and F5 were showed greater

stability i.e. the percentage of drug remaining was found more than 95% at all the pH levels in comparison to F1. The drug product and formulation exposure to various pH levels to reveals the stability at various gastric climates and this helps in understanding the drug degradation against pH.

In vitro release of KT from Nanoparticles

The drug release from the nanoparticles enables to understand the drug release behaviour and suitable for estimation of amount of drug release over a period of time. Dissolution is very much important for identification of pattern of drug release. Drug release pattern is the key feature of any formulation for understanding the type of release like sustained or immediate release. The drug release from the formulation helps in determining the batch to batch uniformity and also helps in evaluating any process change parameters. KT and PLGA nanoparticle formulations like F1, F2, F3, F4 and F5 were showed different drug release profile. F2, F3, F4 and F5 were shown sustained drug release pattern.

In vivo Study

The drug release into the blood can be determined using pharmacokinetic analysis. The blood samples were processed and plasma was separated. The plasma samples were subjected to protein precipitation technique to remove the matrix effect and analyzed using validated HPLC method. The all PLGA formulations were showed greater drug release in comparison to KT alone. The heights plasma concentrations of KT were found at 3 h and all PLGA formulations (F1 to F5) were 6 h respectively.

In vitro Cytotoxicity Studies

The cytotoxicity of KT and KT loaded P nanoparicles F1, F2, F3, F4 and F5 were evaluated to determine the effectiveness on SCC-29 cells. SCC-29 cells were used to determine the cytotoxic activity of KT, F1, F2, F3, F4 and F5 formulations. SRB assay was used to determine cell viability. The supernatant component was taken out and washed with PBS and images taken under 40X. The KT and KT loaded P loaded nanoparticles treated cells are showed in Figure 7. The cells applied with formulations F1, F2, F3, F4 and F5 showed better anticancer activity over KT alone. The cells were showed blebbing.

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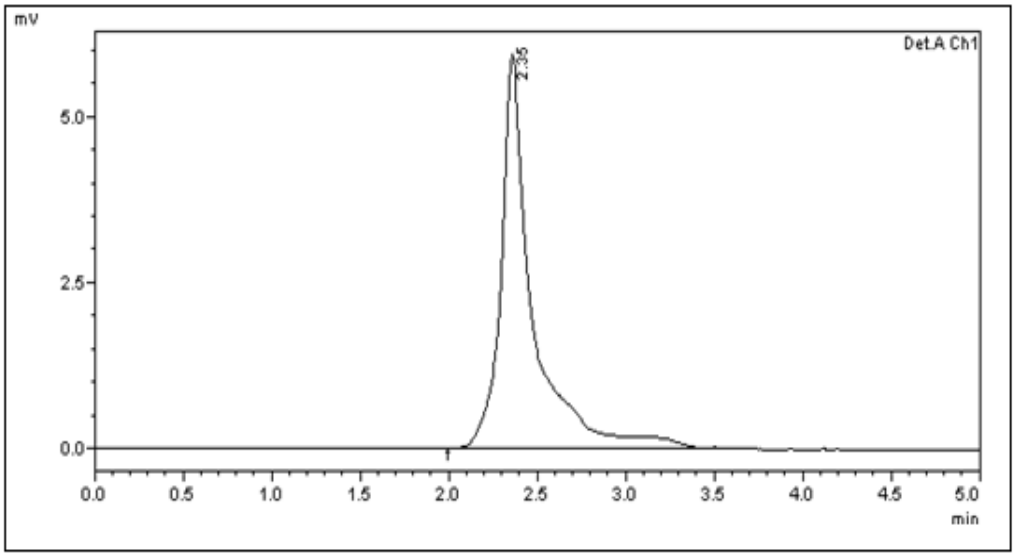

Fig.1. The chromatogram shows concentration of Ketorolac Tromethamine (1µg/mL) was being analyzed using acetonitrile: water (65:35 v/v)

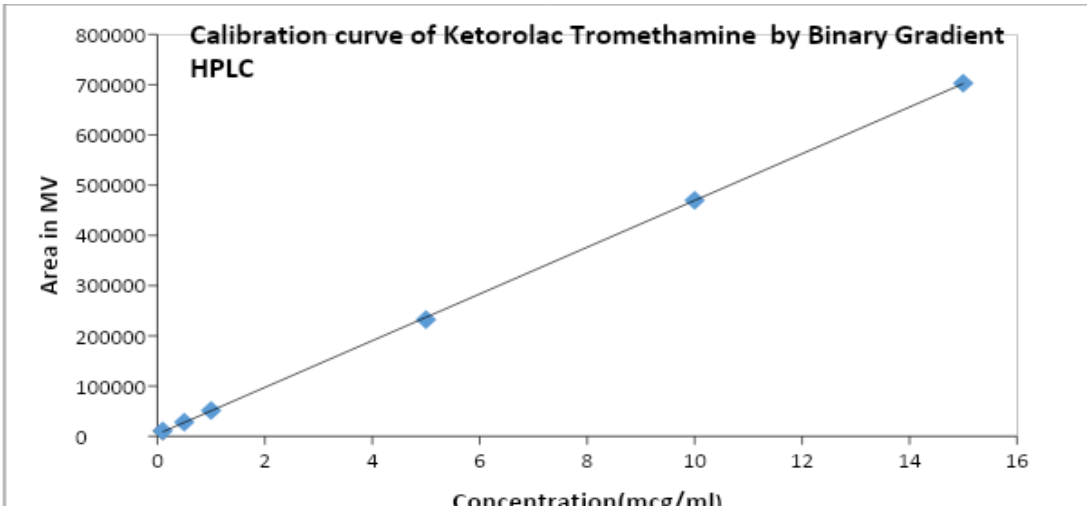

Figure2. Calibration curve of Ketorolac Tromethamine by Binary Gradient HPLC

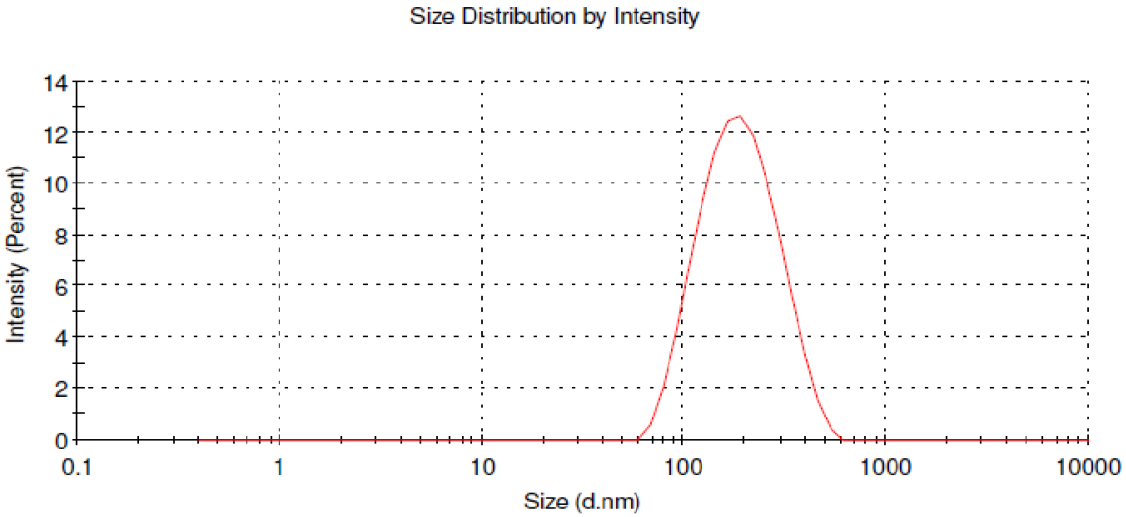

Figure 3a. The particle size distribution of formulations F1

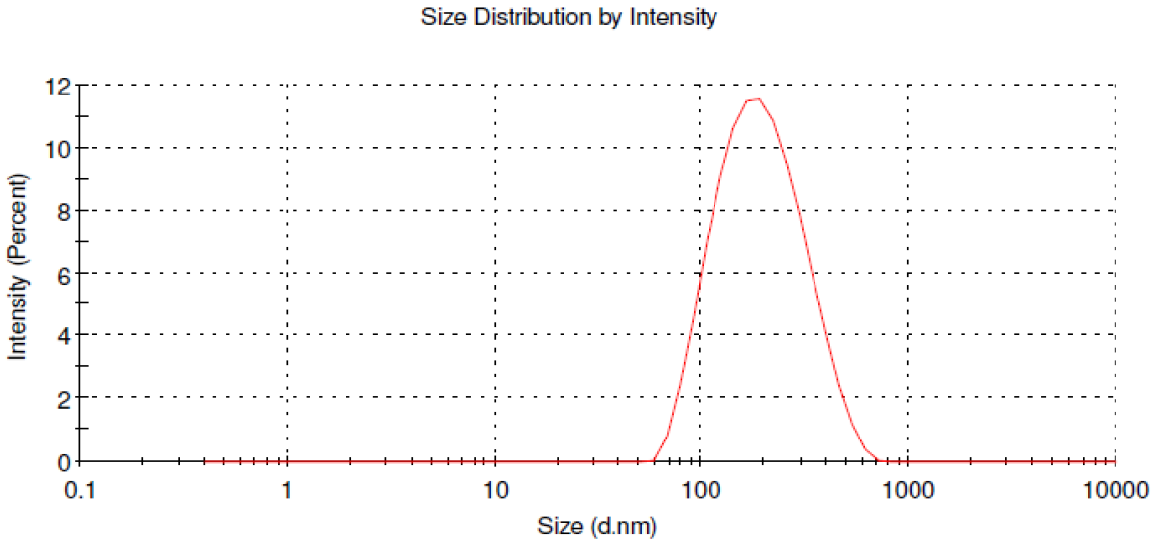

Figure 3b. The particle size distribution of formulations F2

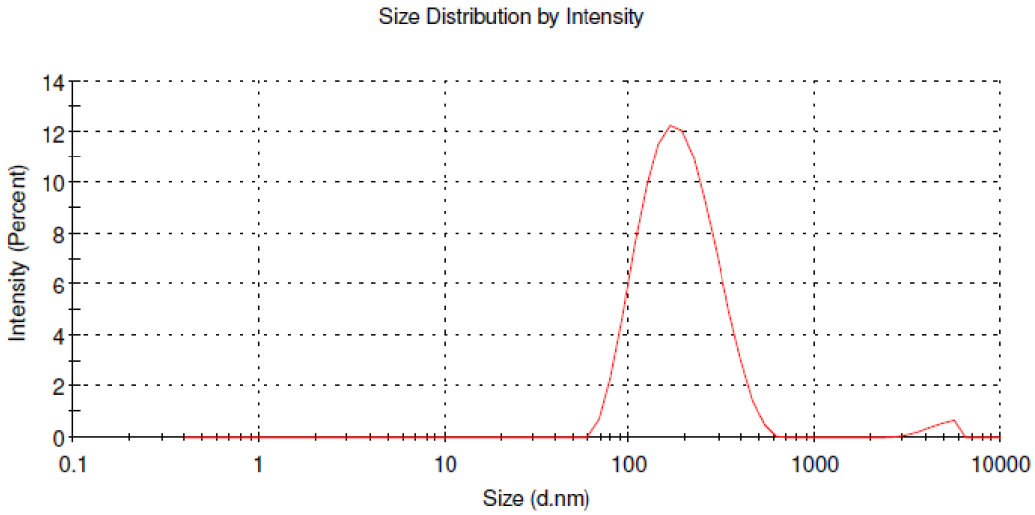

Figure 3c. The particle size distribution of formulations F3

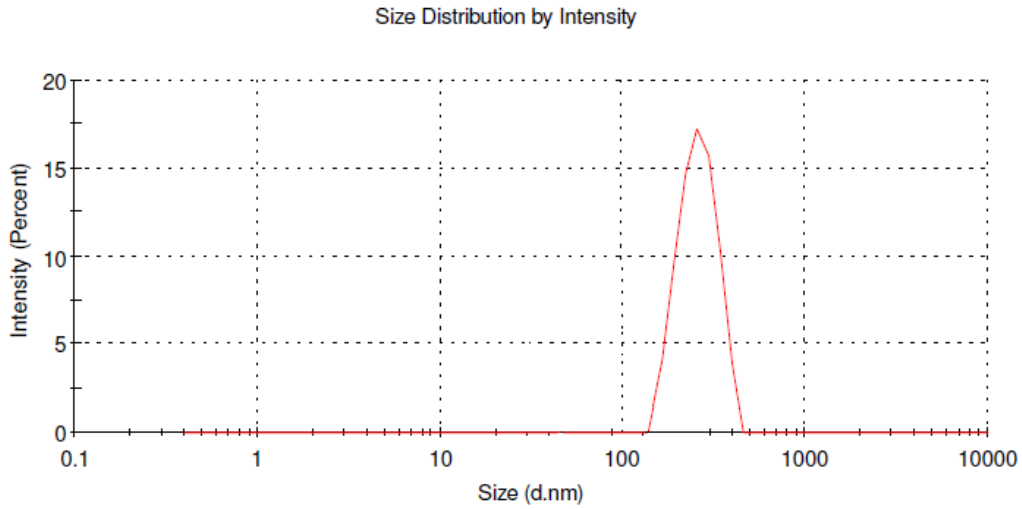

Figure 3d. The particle size distribution of formulations F4

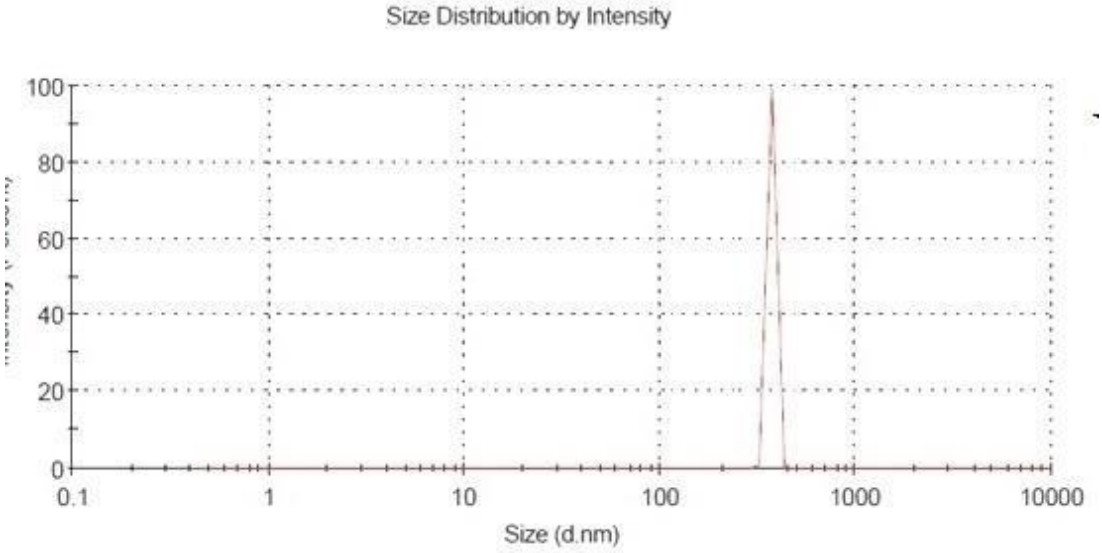

Figure 3e. The particle size distribution of formulations F5

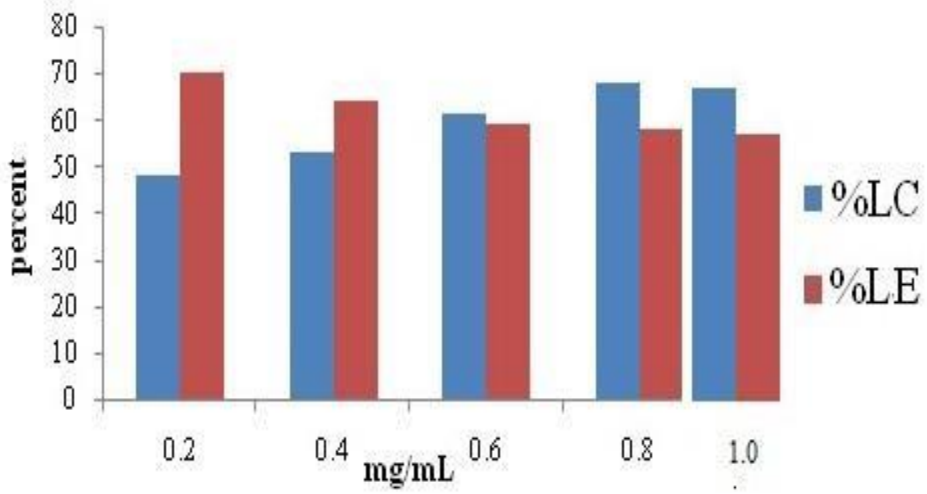

Figure 4. Loading Capacity and loading efficiency of KT loaded P nanoparticles

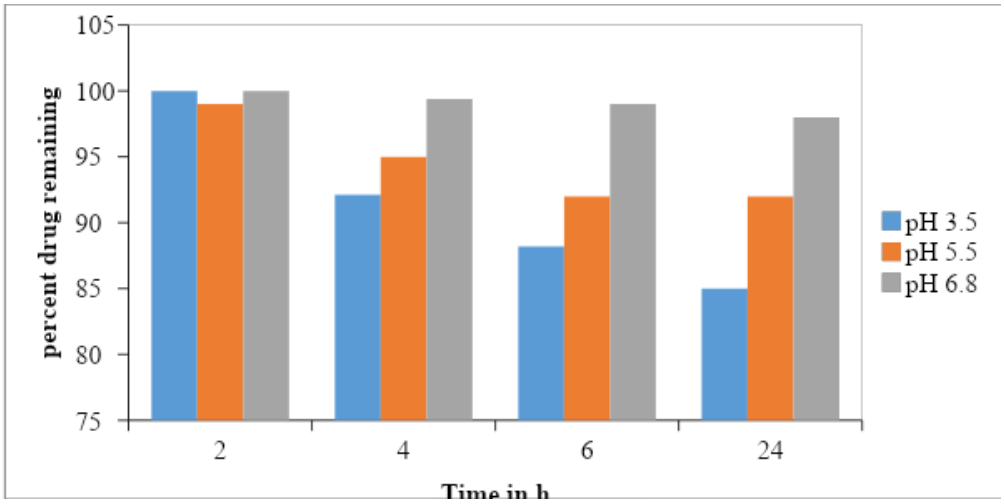

Figure 4a. pH stability profile of F1

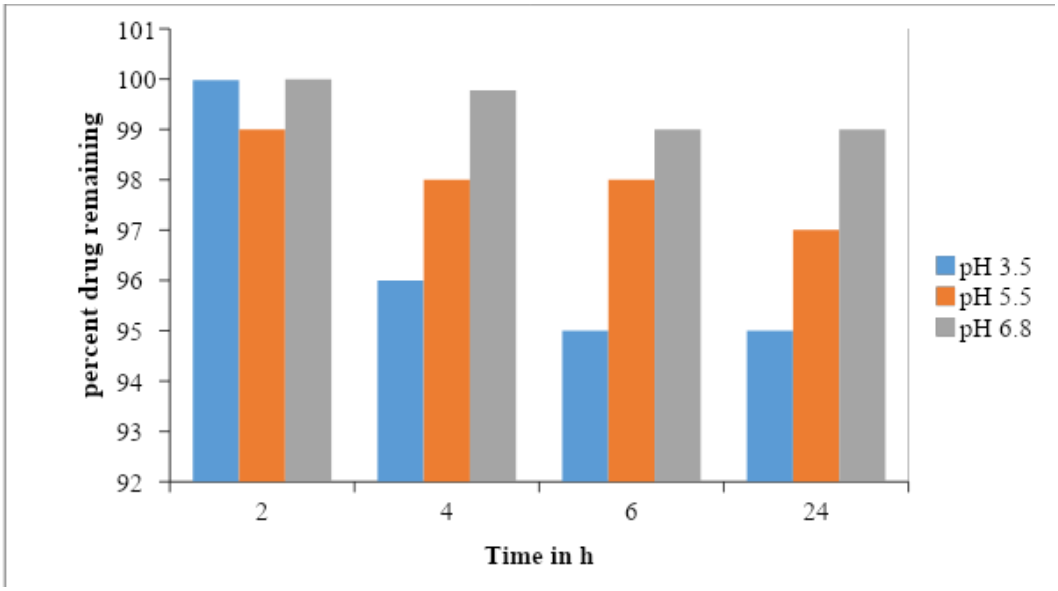

Figure 4b. pH stability profile of F2

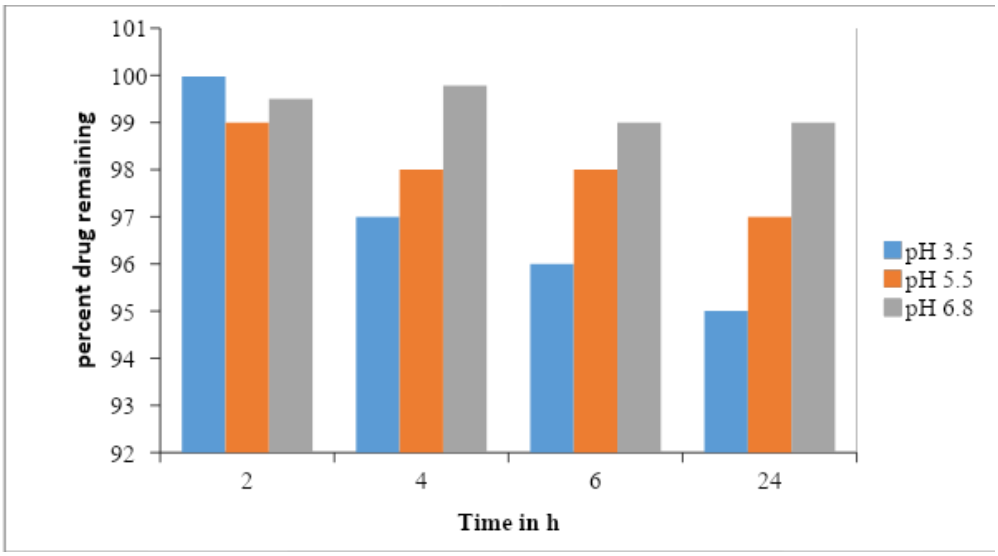

Figure 4c. pH stability profile of F3

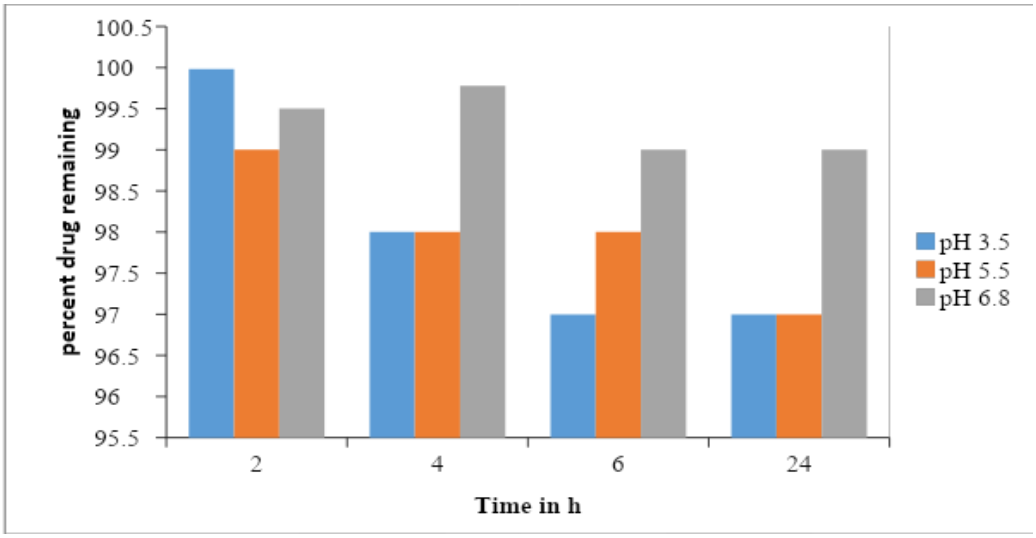

Figure 4d. pH stability profile of F4

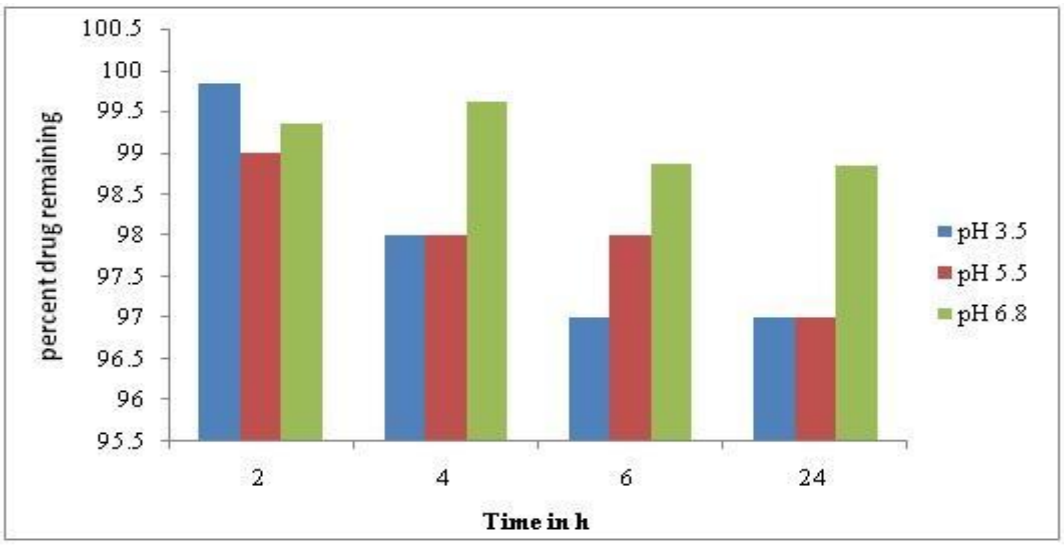

Figure 4e. pH stability profile of F4

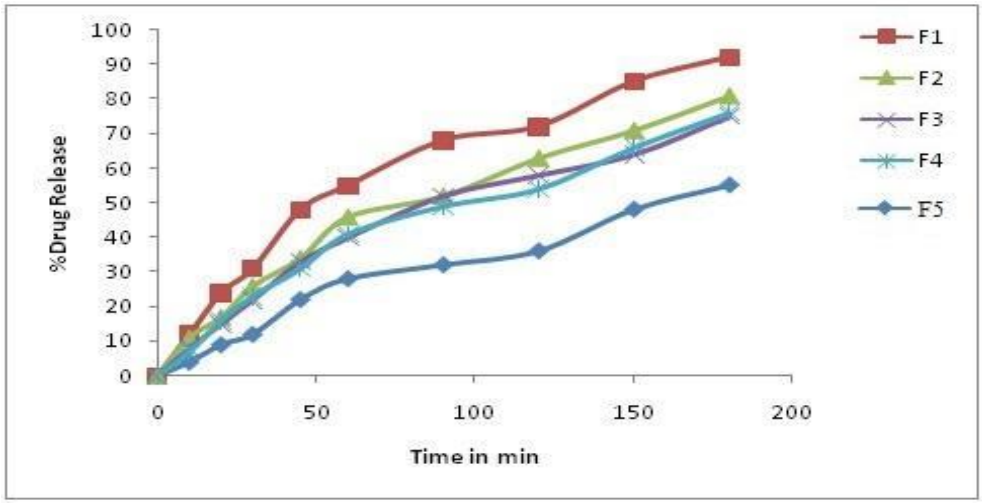

Figure 5. The drug dissolution profile of KT and PLGA loaded nanoparticles

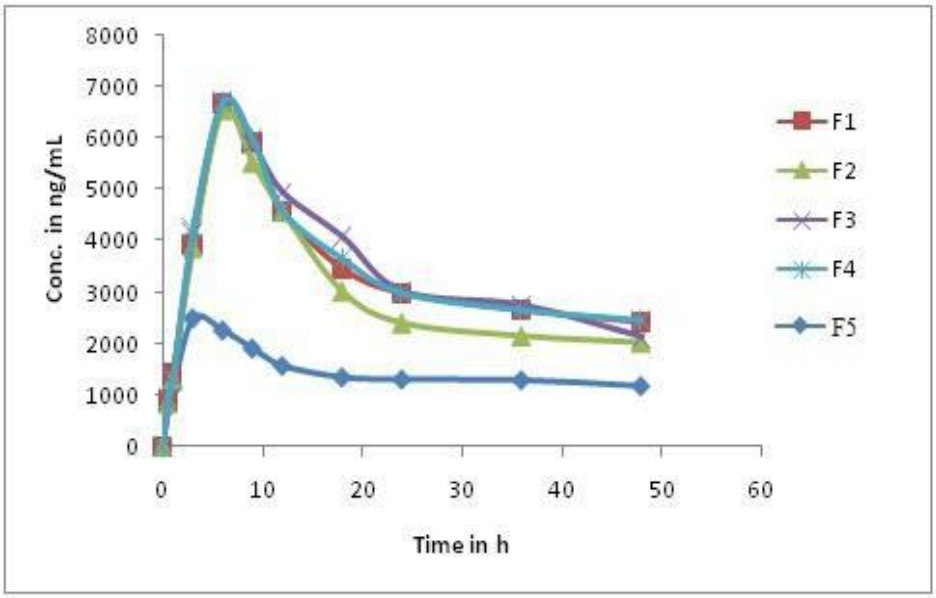

Figure 6. The oral absorption profile F1, F2, F3 F4 and F5 in wistar rats

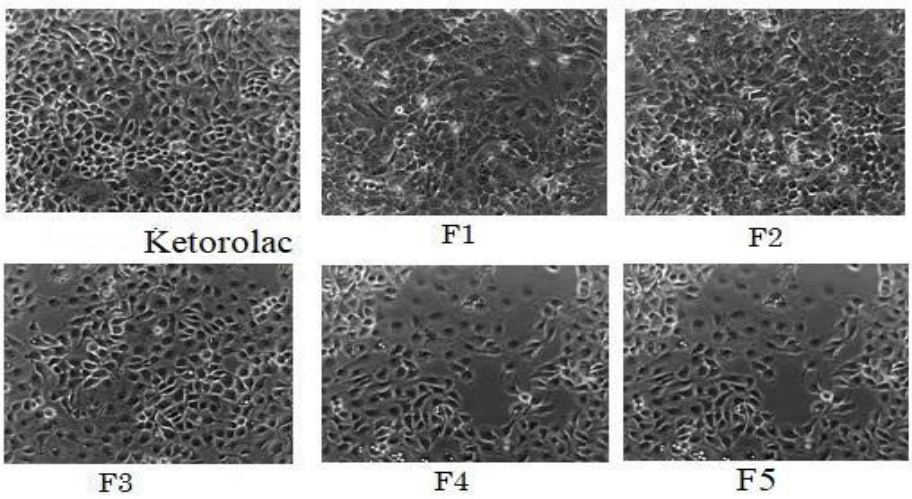

Figure 7. The SCC-29 cells treated with MX, F1, F2, F3,F4 and F5

Table 1 Calibration data of Ketorolac Tromethamine by HPLC method

Concentration (µg/ml)	Peak Area (mean ± SD, n=3)	%RSD
0.1	10420.0 ± 129.95	1.24712
0.5	27885.0 ± 513.93	1.84303
1	51608.0 ± 234.00	0.45342
5	232507 ± 2140.02	0.92041
10	469766 ± 3642.80	0.77545
15	703314 ± 3665.57	0.52119

Table 2 System Suitability Parameters of Ketorolac Tromethamine by HPLC method

Concentration (µg/ml)	Theoretical Plates (N)	Tailing Factor (T)
0.1	2025.54 1	1.375
0.5	2175.29	1.25
1	2330.41	1.294
5	2688.98	1.437
10	3138.68	1.333
15	3450.31	1.452

Table 3. Statistical data of regression equation & validation parameters for Ketorolac Tromethamine

Parameters	HPLC method
UV wavelength (nm)	320
Linearity range (µg/ml)	0.1 – 15.0
Regression equation	
Intercept (c)	4194
Slope (m)	46529
Regression coefficient (r2)	0.999
DL (µg/ml)	0.0295
QL (µg/ml)	0.0895

Table 4. Accuracy data of Ketorolac Tromethamine by HPLC Method (N=9)

Concentration (µg/ml)	Amount found (µg/ml	%Accuracy (mean ± SD, n=3)	%RSD
0.25	0.25002	100.007 ± 0.427	0.4271
2.5	2.50048	100.019 ± 0.077	0.077
12.5	12.5002	100.001 ± 0.013	0.0134

Table 5 Precision data of Ketorolac Tromethamine by HPLC Method

Concentration (µg/ml)	Intraday Precision (N=9)		Interday Precision (N=27)	
	Found Conc. (µg/ml)	%RSD	Found Conc. (µg/ml	%RSD
0.25	0.25002	0.4271	0.25055	0.6117
2.5	2.50049	0.077	2.50048	0.4573
12.5	12.5003	0.0134	12.5011	0.9561

Table 6 Robustness data Ketorolac Tromethamine (1.0µg/ml) by HPLC method

Variation in Parameters		Peak Area (with%RSD)	RT (min)	Theoretical plates (N)	Tailing factor (T)
Flow rate (ml/min)	**0.90**	51743 (0.3438)	**2.35**	2265.76	1.333
	1.10	50721 (0.6857)	**2.37**	2345.11	1.250
Mobile Phase Ratio			2.39	2217.78	1.200
	63:37	51548 (0.4984)			
	67:33	51581 (0.3643)	2.34	2158.02	1.200

Table7: Particle size &Characterization of Ketorolac Tromethamine loaded PLGA nanoparticle formuations

Sl no	Particle size & Characterization				
	F1	F2	F3	F4	F5
1	181	167	185	178	170
2	186	168	182	171	169
3	182	162	186	176	172
4	180	168	187	172	171
Mean	**182**	**166**	**185**	**174**	**170**
SD	**2.62**	**3.59**	**2.16**	**3.30**	**2.30**

Table 8.1 pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F2 Part-1)

pH	% Drug release at different time interval											
	2 hr				**Mean**	**SD**	4hr				**Mean**	**SD**
3.5	99.19	99.14	99.04	99.15	**99.14**	**0.01**	96.16	96.57	96.57	96.45	96.37	**0.20**
5.5	99.91	99.90	99.91	99.93	**99.89**	**0.012**	98.13	98.45	98.16	98.16	**97.82**	**0.15**
6.8	99.98	99.93	99.92	99.91	**99.98**	**0.03**	99.78	99.46	99.21	99.21	**99.85**	**0.29**

Table8.2.: pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F1 part-1)

pH	*% Drug release at different time interval*											
	2 hr				**Mean**	**SD**	4hr				**Mean**	**SD**
3.5	99.97	99.85	99.37	99.54	**99.68**	**0.28**	92.2	92.66	93.68	92.77	**92.9**	**0.61**
5.5	99.0	99.19	99.32	98.77	**99.08**	**0.24**	95.11	94.84	94.99	94.96	**94.94**	**0.10**
6.8	99.98	99.99	99.86	99.68	**99.88**	**0.15**	99.3	99.55	99.65	99.74	**99.58**	**0.14**

Table 8.3: pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F1 part-2)

pH	% Drug release at different time interval											
	6 hr				**Mean**	**SD**	24hr				**Mean**	**SD**
3.5	95.13	94.31	95.14	95.12	**99.94**	**0.40**	85.14	87.15	87.53	88.52	86.09	**1.44**

5.5	98.64	98.68	98.13	97.64	**98.26**	**0.47**	92.79	92.77	98.38	98.35	94.91	**3.15**
6.8	99.88	99.86	99.77	99.13	**99.64**	**0.36**	97.19	97.19	96.79	97.37	96.89	**0.53**

Table8.4 1e pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F2-Part-2)

pH	% Drug release at different time interval											
	6 hr				**Mean**	**SD**	24hr				**Mean**	**SD**
3.5	95.23	95.28	93.98	94.5	**94.93**	**0.78**	95.90	95.98	95.23	95.65	**95.67**	**0.33**
5.5	98.34	98.23	98.25	98.10	**98.22**	**0.09**	97.09	97.15	97.35	95.57	**97.19**	**0.21**
6.8	99.89	99.12	99.87	99.83	**99.63**	**0.37**	97.14	97.23	97.87	97.47	**97.52**	**0.32**

Table 8.5. pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F3 Part-1)

pH	% Drug release at different time interval											
	2 hr				**Mean**	**SD**	4hr				**Mean**	**SD**
3.5	99.98	99.10	99.23	99.36	**99.51**	**0.38**	97.3	97.20	97.50	97.35	**97.43**	**0.12**
5.5	99.12	99.37	99.23	99.35	**99.46**	**0.11**	98.58	98.58	98.38	98.23	**98.59**	**0.23**
6.8	99.15	99.35	99.65	99.32	**99.52**	**0.20**	99.78	99.45	99.35	99.2	**99.65**	**0.23**

Table 8.6. pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F3 Part-2)

pH	% Drug release at different time interval											
	6 hr				**Mean**	**SD**	24hr				**Mean**	**SD**
3.5	96.9	96.3	95.9	96.4	**96.57**	**0.41**	95.5	95.7	95.9	95.1	**95.65**	**0.34**
5.5	98.10	98.0	98.7	98.2	**98.35**	**0.31**	97.3	97.4	97.1	97.0	**97.30**	**0.18**
6.8	99.6	99.2	99.7	98.9	**99.45**	**0.36**	99.2	99.7	99.2	99.7	**99.55**	**0.28**

Table 8.7: pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals(F4-Pat-1)

pH	*% Drug release at different time interval*											
	2 hr				**Mean**	**SD**	4hr				**Mean**	**SD**
3.5	98.0	98.4	98.3	98.1	**98.12**	**0.18**	98.2	98.3	98.9	99.0	**98.6**	**0.40**
5.5	98.9	98.2	98.7	98.3	**98.42**	**0.33**	99.6	99.2	99.6	99.4	**99.65**	**0.19**
6.8	99.0	99.5	99.8	99.2	**99.54**	**0.35**	99.7	99.3	99.9	99.2	**99.62**	**0.33**

Table 8.8 pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F4 Part-2)

pH	*% Drug release at different time interval*											
	6 hr				**Mean**	**SD**	**24hr**				**Mean**	**SD**
3.5	97.5	97.2	97.0	97.3	**97.25**	**0.20**	97.9	97.8	97.1	99.7	**97.62**	**0.35**
5.5	99.7	99.3	99.2	99.1	**99.32**	**0.26**	97.3	97.8	97.1	97.3	**99.37**	**0.29**

6.8	99.2	99.7	99.5	99.1	**99.37**	**0.22**	99.1	99.8	99.8	99.9	**99.57**	**0.37**

Table 8.9 pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals(F5-Pat-1)

pH	*% Drug release at different time interval*											
	2 hr				**Mean**	**SD**	4hr				**Mean**	**SD**
3.5	98.0	98.4	98.3	98.1	**97.12**	**0.18**	98.2	98.3	98.9	99.0	**97.5**	**0.40**
5.5	98.9	98.2	98.7	98.3	**98.42**	**0.33**	99.6	99.2	99.6	99.4	**98.75**	**0.19**
6.8	99.0	99.5	99.8	99.2	**98.84**	**0.35**	99.7	99.3	99.9	99.2	**99.52**	**0.33**

Table 8.10 pH Stability study of Ketorolac loaded PLGA Nanoparticles at different time intervals (F5 Part-2)

pH	*% Drug release at different time interval*											
	6 hr				**Mean**	**SD**	24hr				**Mean**	**SD**
3.5	97.5	97.2	97.0	97.3	**96.251**	**0.20**	97.9	97.8	97.1	99.7	**98.67**	**0.35**
5.5	99.7	99.3	99.2	99.1	**98.33**	**0.26**	97.3	97.8	97.1	97.3	**99.17**	**0.29**
6.8	99.2	99.7	99.5	99.1	**99.17**	**0.22**	99.1	99.8	99.8	99.9	**99.77**	**0.37**

Table 9: Pharmacokinetic Parameters of KT and KT loaded P Formulations (F1- F5) after Oral Administration

Pharmacokinetic parameter	**Units**	**KT**	**F1**	**F2**	**F3**	**F4**	**F5**
AUC_{0-t}	µg h/mL	67.9	164.5	229.8	169.9	166.8	167.6
$t_{1/2}$	H	10.36	29.21	12.12	24.66	25.94	25.91
C_{max}	µg/mL	2.55	6.68	6.54	6.71	6.71	6.80

Table 10.1 In-vivo Drug Release Pattern of KT and KT loaded PLGA Nanoparticle Formulations (F1-F5) (Part-1)

FLN	*% In-vivo drug release of at different time interval*																	
	0.5 mins				**Mean**	**SD**	1h				**Mean**	**SD**	3 h				**Mean**	**SD**
KT	743	744	745	742	**743**	**1.30**	1135.0	1135.9	1134.7	1134.6	**1135**	**0.59**	2485	2482	2481	2485	**2483**	**2.06**
F1	924	926.7	927.3	925.9	**925**	**1.23**	1425.9	1428.7	1427.8	1428.6	**1427**	**1.29**	3943	3942	3944	3940	**3942**	**1.70**
F2	845	843.8	849.9	844.6	**845**	**2.76**	1325.9	1326.8	1327.8	1327.7	**1327**	**0.88**	3876	3875	3873	3871	**3873**	**2.71**
F3	865	866.7	865.8	864.8	**865**	**0.65**	1398.0	1398.6	1389.3	1396.8	**1395**	**1.67**	4154	4157	4158	4152	**4155**	**2.75**
F4	941	942.8	942.1	941.0	**941**	**0.88**	1347.8	1346.3	1346.8	1346.8	**1346**	**1.39**	4258	4253	4256	4257	**4256**	**2.15**
F5	943	941.8	941.1	944.0	**942**	**0.78**	1341.8	1343.3	1349.8	1342.8	**1348**	**1.27**	4253	4254	4257	4259	**4262**	**2.27**

Table 10.3.: In-vivo Drug Release Pattern of KT and KT loaded PLGA Nanoparticle Formulations (F1-F5) (Part-3)

FLN	% In-vivo drug release of at different time interval																	
	18 h				Mean	SD	24 h				Mean	SD	48 h				Mean	SD
KT	1343	1345	1347	1341	**1344**	**2.58**	1300	1303	1307	1309	**1304**	**4.03**	1171	1174	1176	1174	**1173**	**2.06**
F1	3457	3459	3452	3450	**3454**	**4.20**	2987	2982	2984	2983	**2984**	**2.16**	2417	2415	2417	2414	**2415**	**1.50**
F2	3012	3014	3018	3015	**3014**	**2.50**	2400	2402	2399	2401	**2400**	**1.29**	2019	2017	2016	2018	**2017**	**1.29**
F3	4075	4078	4071	4072	**4074**	**3.16**	3012	3016	3013	3016	**3014**	**2.06**	2128	2123	2124	2128	**2125**	**2.62**
F4	3652	3657	3655	3650	**3653**	**3.10**	2985	2982	2980	2985	**2983**	**2.44**	2457	2453	2450	2451	**2452**	**3.09**
F5	3653	3655	3653	3651	**3654**	**3.12**	2988	2983	2981	2987	**2986**	**2.35**	2458	2452	2455	2453	**2456**	**3.21**

Table 11: Cytotoxicity Study of KT and KT loaded PLGA Nanoparticle Formulations (F1-F5)

FLN	*% Inhibition of Growth*																	
	20 µg/ml				Mean	SD	40 µg/ml				Mean	SD	80 µg/ml				Mean	SD
KT	81.3	81.5	81.8	81.3	**81.47**	**0.23**	89.0	89.7	89.5	88.9	**89.27**	**0.38**	84.9	84.2	84.5	85.0	**84.65**	**0.36**
F1	78.9	78.8	78.6	77.9	**78.55**	**0.45**	81.7	81.9	81.4	81.2	**81.55**	**0.31**	76.4	76.3	76.0	76.8	**76.37**	**0.33**
F2	79.9	79.4	79.2	79.1	**79.4**	**0.35**	79.5	79.9	78.9	79.3	**79.4**	**0.38**	76.8	76.2	76.1	76.9	**76.50**	**0.40**
F3	78.7	78.2	78.8	78.2	**78.47**	**0.32**	81.5	81.4	81.2	81.6	**81.42**	**0.17**	77.4	77.2	77.1	77.3	**77.25**	**0.12**
F4	78.4	78.1	78.0	78.4	**78.22**	**0.20**	81.2	81.5	81.4	81.0	**81.27**	**0.22**	80.9	80.2	80.9	80.2	**80,55**	**0.44**
F4	78.7	78.3	78.0	78.1	**78.31**	**0.19**	80.2	80.4	81.5	81.7	**81.31**	**0.18**	80.8	80.3	80.8	80.4	**80.57**	**0.40**

DISCUSSION

The HPLC analysis of ketorolac was conducted to determine the % of drug release for the prepared solutions of all the formulations. The % of drug release at different time interval was calculated from the calibration graph of ketorolac. The values were found satisfactory. The characterization of all formulations using dynamic light scattering technique reveals the size distribution was proper. The loading capacity and efficiency of both formulations indicates that by increasing drug loading, loading efficiency decreased and loading capacity was increased. Stability study of nanoparticles of both formulations at different pH was conducted and reveals that the formulation F1 was showed highest stability compared to other formulations. The pH stability of the formulation is very important for the drug content maintenance in the stomach and intestine. Furthermore, the stability at pH 6.8 is essential for the drug absorption while drug formulation residence in the GIT.PLGA was showed good stability at pH 6.8 and maintained for 24 h period. Drug release pattern plays a vital role in quality control. All the formulations were shown different drug release profile. The bloods Samples were estimated by using HPLC method.

All five formulations were shown higher drug release compared to KT alone. The peak plasma concentrations were reached at 6h and further the concentration of KT was started decreasing drastically. KT alone did not release effectively throughout the absorption phase compared to both formulations. *Invitro* cytotoxicity study was conducted and found that the cells treated with formulation F1have showed faintly induced cell death in SCC-29 cell lines. F1 was showed better cytotoxcity over other formulations. The cells were relieved membrane blebbing and granules.

CONCLUSION

The study confirmed that ketorolac loaded PLGA nanoparticle formulations has showed superior bioavailability and P H stability. Among five formulations F1 has shown slightly higher cytotoxicity over other formulations when compared to ketorolac alone, hence ketorolac loaded PLGA nanoparticles can be considered to be a promising system for delivery of ketorolac. These nanoparticle formulations are effective in the treatment of colon cancer compared to other chemotherapeutic agents which will lead to fewer side effects.

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