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Original Research Article

Formulation and Evaluation of Ketorolac Loaded Pluronic f 127 Hydrogel and Investigation of Cytotoxic Activity in SCC-29 Cell Lines

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Abstract

Aim: The present research work was planned to prepare pluronic f 127 (poloxamer 407) based hydrogel formulations of ketorolac and to evaluate the parameters like swelling behaviour, drug P^H stability, invite and *in vivo* drug release and *in vitro* cytotoxic activity. ***Methodology:*** Two different strengths of ketorolac hydrogel formulations were prepared using pluronic f127 and were analysed by validated HPLC method for drug content, PH stability and *in vivo* drug release. Further invitro anticancer activity was evaluated using sulphorhodamine B (SRB) assay in SCC29 cell lines. ***Result:*** Both the formulations F1 and F2 showed better PH stability at PH 3.5, 5.5 and 6.8. *In vitro* and *in vivo* drug release pattern showed half life at 3 hours, AUC_{0-t} 669 and 667ng h/ml, C_{max} 884 and 872 ng/ml for F1 and F2 respectively. ***Conclusion:*** Hydrogel formulation F1 showed better percentage control growth when compared to F2 hydrogel formulation and ketorolac alone.

Keywords: Ketorolac, pluronic f127, hydrogel, cytotoxicity, dug stability.

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INTRODUCTION

Ketorolac which is chemically 5-benzoyl -2,3-dihydro-1H-Pyrrolizine 1-carboxyllic acid [1], is a non-steroidal anti-inflammatory agent and it is chemically similar to the indomethacin. The ketorolac [-]S form have analgesic activity. Ketoralac is a well known NSAID, used in the treatment of Rheumatoid arthritis, inflammation, Pain reliever, reduction of aqueous humour and post operative cancer pain [2]. Extensive review of literature on ketorolac along with empirical evidences clearly revealed that along with its anti-inflammatory activity various formulations of ketorolac were available for its potential anticancer applications [3]. Sabinda et al has proved that ketorolac salt discovered as newly DDX3 inhibitor for the treatment of oral cancer [4]. *In vitro* Anticancer activity of ketorolac with rosuvastatin hydrogel was found effective against DDX3 in oral squamous cell carcinoma has been reported by Khaggeswar *et al*., [5]. Ketorolac also showed its therapeutic benefit in ovarian cancer patient which has been reported by Yuna. G [6]. By restoring these important facts in mind the present research work has been planned to prepare the pluronic f127 based hydrogels of ketorolac for the investigation of anticancer activity. Hydrogels retain large amounts of water or biological fluids are three-dimensional (3D) hydrophilic polymeric networks, characterized by soft and rubbery consistence in analogy to living tissues [7, 8]. Pluronic f127 are recently received a huge attention in the field hydrogels. It is an amphiphilic synthetic copolymer with a hydrophobic poly (Oxypropylene) (POP) block between two hydrophilic poly (Oxyethylene) (POE) blocks [9-11]. This molecule can readily self- assemble to form micelles base on the temperature and concentration uue to their amphiphilic nature. These hydrogels are characterized by their ability to carry a significant quantity of drug. They are non toxic, biodegradable and stable, therefore found suitable to use as controlled release agents [12]. Therefore the present work aimed to approach for further establishment of Ketorolac as an anti-cancer agent in hydrogels.

MATERIALS AND METHODS

Pluronic f 127 was purchased from Sigma Aldrich, USA. Water purified by reverse osmosis, MilliQ, USA and further filtered by 0.22 µm membrane filter. HPLC grade methanol was purchased from SD FineChem, India and all other chemicals were used of analytical grade. Pure Ketorolac (99.34% purity) was obtained as a gift sample from Hetero Labs, Hyderabad.

HPLC Analytical Method

For the determination of the concentration of KT in hydrogel, developed valid analytical method [13] was utilized. Standard ketorolac (10.mg) was dissolved in 65.0 ml of methanol and made up to 100 ml with Millipore water to get the final concentration of 100 mcg/ml. From the standard stock solution of ketorolac (100mcg/ml) different concentrations were prepared in the range of 0.01, 0.05, 1.0, 3.0, 5.0, 8.0, 10, 12, 15 mcg/ml and injected in to HPLC system. 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 a 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 methanol and 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 25^0C

Preparation of Ketorolac loaded Pluronic f 127 hydrogel.

Two formulations (F1 and F2) of ketorolac loaded hydrogels [14] were prepared by dissolving 180mg pluronic f127 in Millipore water. Dissolved the total content and freeze it. 10 mg of accurately weighted KT was added to dissolve in gel base. Required amount of Millipore water was added, freeze the total content in refrigerator until the clear gel was formed to obtained 18% hydrogel formulation (F1). Similarly 25% hydrogel formulation (F2) was prepared by dissolving 250 mg pluronic f127 and 10 mg of ketololac, in required amount of Millipore water.

Swelling behavior of hydrogels in Pseudo Extra Cellular Fluid (PECF)

Swelling properties [15] of prepared hydrogels (F1 and F2) was investigated using PECF solution. The PECF solution and simulated wound fluid which is consists of 0.22 g of KCl, 0.68 g of NaCl, 2.5 g of NaHCO3, and 0.35 g of NaH_2PO_4 in 100 ml of deionized water. The pH of PECF and ionic strength of the solution were 7.4±0.2 and 0.48M respectively. To investigate their swelling behaviour fresh hydrogels were left to swell in PECF solution at 25°C. At room temperature hydrogels were accurately weighed and then soaked in PECF solution. After 10 minutes hydrogels were removed from solvent carefully, wiped by filter papers, weighed, and placed in PECF solution [15, 16]. Until a constant weight was reached for each sample this procedure was repeated several times. The percentage swelling of hydrogels was calculated from the difference between the initial and the final weight of the sample divided by the initial weight of the sample.

Stability study of KT **and hydrogel at different pH**

Stability of hydrogel formulations were investigated at various pH (3.5, 5.5 and 6.8), 0.1 M hydrochloric acid, phosphate buffers were selected. 10 mg of KT and KT loaded hydrogels were accurately weighted and transferred to 2 ml centrifuge tube and 1 ml of each buffer was added to tubes containing hydrogels and incubated at 25° C for 24 h. The content of drug was determined using developed HPLC method and the extent of drug degradation was also evaluated.

In Vitro Release of KT **from Hydrogels**

1 ml aliquots of KT loaded hydrogel formulations were centrifuged at 10000 x g. Decanting the supernatant and the obtained pellet was diluted with 1 ml phosphate buffered saline at pH 7.4 followed by incubation at 37°C under shaking equipment at 50 rpm for 3 h. A tube was selected and centrifuged at 10000 x g for 17 min at various time intervals. The released KT was determined using validated HPLC analytical method.

Determination of bioavailability of KT **loaded Hydrogel formulations.**

For the bioavailability determination of KT loaded hydrogels, KT alone and hydrogels have been administered orally to wistar rats to determine the amount of KT reaching systemic circulation. Institutional Animal Ethics Committee (IAEC), Balaji Institute of Pharmaceutical Sciences, Narsampet, Warangal, has approved animal facility with CPCSEA registration No.1694/PO/Re/S/13/cpcsea. KT and KT loaded hydrogels were taken equivalent to 5 mg of pure KT and administered orally to male wistar rats (~270 g) and blood samples were withdrawn from tail vein under mild anaesthesia at the intervals of 0.5, 1, 3, 6, 9, 12, 18 and 24 h respectively. The blood samples were withdrawn and transferred to EDTA tubes. Then the matrix were removed from the samples using protein precipitation and amount was analyzed using validated high performance liquid chromatographic method.

Invitro Cytotoxicity Study

The SCC 29 cell lines were procured NCI (National Cancer Institute, USA). These obtained cells were cultured in complete growth medium (RPMI 1640) supplemented with 10% fetal bovine serum (Sigma, USA), 100 U/ml (1%) streptomycin (Sigma, USA) and 1% 100 U/ml penicillin at 37ºC, 5% CO2 and relative humidity (98%). 75 cm^2 canted-neck tissue culture flask was utilised to grown the human cancer cell lines routinely and passaged regularly by by trypsin/EDTA. when confluence of 90%, further subculture was performed. Sulforhodamine B (SRB) assay method was utilized for the *in vitro* cytotoxicity of ketorolac and prepared formulations (F1and F2) [17]. According to the standard protocol. 24 Briefly, 5×103 cells/ well of SCC 29 cells were seeded in 96 well

plates and incubated for next 24 hr's. Various concentrations (10-80 µg/mL) of ketorolac hydrogel formulations. The plates were incubated at 48 and 72 hr's with ice cold trichloro acetic acid at 4ºC for 1 hr. The plates were washed 3 times using distilled water and then air dried. The 0.4% SRB dye was added to each plates and kept at room temperature for 30 min. 1% (v/v) glacial acetic acid is used to wash the plates for the removal of unbound SRB dye. 10mM tris buffer (pH 10.4) was added to each well and solubilised by keeping on a shaker. Microplate reader (Biotek Synergy HT) at 540 nm was used to measure the values.

HPLC analysis of Ketorolac

Ketorolac was analyzed using Shimadzu LC-20AD HPLC pumps equipped with SPD-20A UV/VIS detector (250mm x 4.6mm i.d.) with particle size of 5µm), mobile phase Methanol :water (65:35 v/v), and the data was shown in Table-1. The calibration curves were prepared to calculate the drug concentration. The relative standard deviation is less than 2% indicates the precision of the HPLC analysis. The correlation coefficient was found to be 0.999 and found to be linear relationship between concentration and area. The chromatograms at 12 µg/mL was shown in the Figure-1.

RESULTS AND DISCUSSION

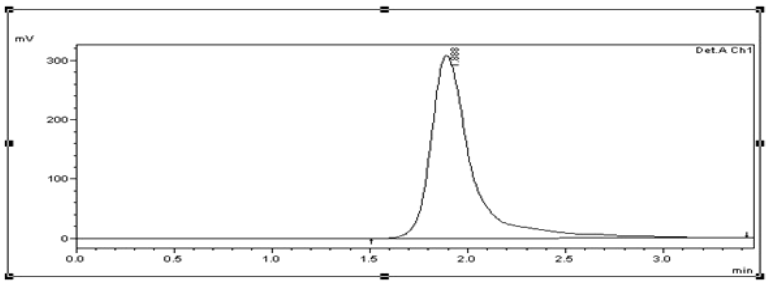

Fig-1: HPLC chromatogram of ketorolac at 12 µg/mL using mobile phase Methanol: water (65:35 v/v)

Table-1: Calibration curve data of ketorolac in selected solvent system (N=9)[b]

Concentration(mcg/ml)	Peak area (±SD)	%RSD[a]
0.1	29451±234	0.62
0.5	137847±128	0.58
1	268604±1023	0.33
3	844233±2320	0.33
5	1350123±2325	0.13
8	2055878±4352	0.88
12	3031221±1322	0.31
15	9471694±7483	0.51

[a]Relative SD or coefficient of variance. [b] Two standard stock solutions

Swelling behaviour of hydrogels in PECF

The percentage swelling of ketorolac hydrogel formulations were calculated after pre determination duration in PECF solution which were plotted as a function of time shown in Figure-2. Swelling behaviour of hydrogel formulations, F1 and F2 were checked in PECF solution as described earlier. It was observed that the formulations were able to absorb about 170% of its weight of water (F1) within 80 minutes, whereas 187.4% of its weight of water (F2) and remains constant over 100 minutes for both the hydrogel formulations. After that the swollen film were broke down in to pieces.

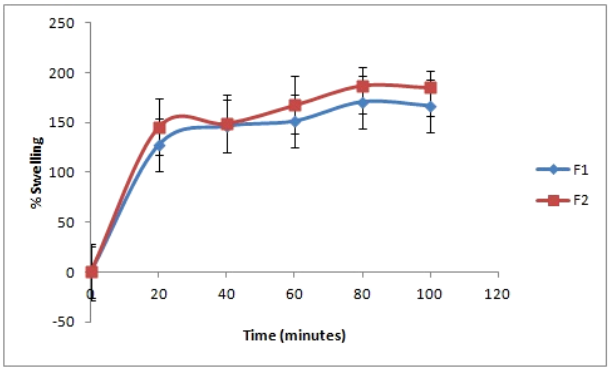

Fig-2: Swelling behaviour of hydrogel formulations (F1 and F2). (n=4, single analysis of variance, P<0.05*)

Stability study of KT and KT loaded hydrogels at various pH

Ketorolac and hydrogel formulations of KT (F1 and F2) were subjected to pH 3.5, 5.5 and 6.8 at different time internals (2h, 4h 6h and 24h). At P^H-3.5, the % of drug remaining in F1 were 96.98, 95.30, 95.34 and 95.45. At P^H-5.5, the % of drug remaining in F1 were 98.17, 95.41, 97.08 and 96.12. At P^H-6.8 the % of drug remaining in F1 were 97.06, 97.68, and 98.89. Similarly at P^H-3.5, the % of drug remaining in F2 were 97.98, 96.10 and 96.71. At P^H-5.5 the % of drug remaining in F2 were 98.0, 97.30, 96.34 and 95.27. At P^H-6.8 the % of drug remaining in F2 were 98.05, 98.18, 98.03 and 97.24. The details were graphically shown in the figure 3. The pH stability study of the hydrogel formulation is important for the drug content maintenance in the stomach and intestine. Furthermore, the stability of drug at pH 6.8 is essential for the adsorption of drug in formulation residence in the gastrointestinal tract.

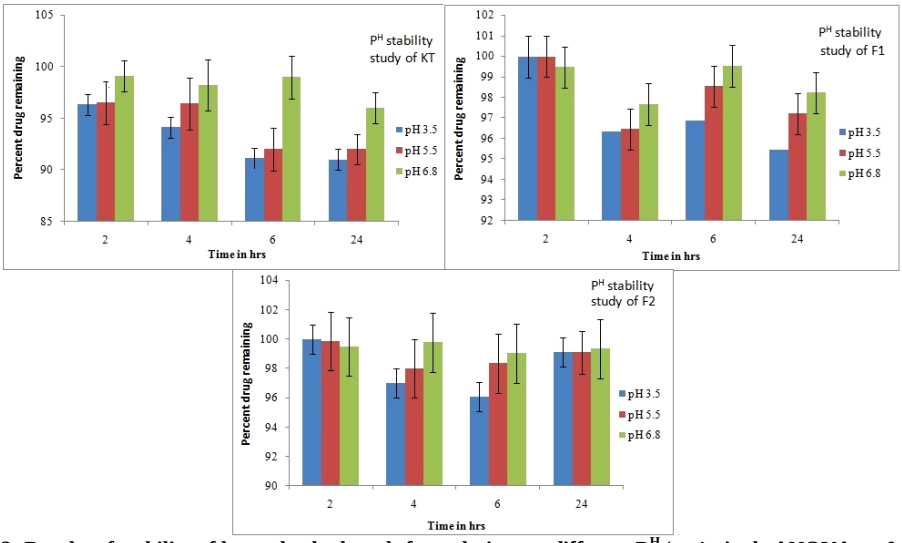

Fig-3: Results of stability of ketorolac hydrogels formulations at different P^H (n=4, single ANOVA, p<0.05*)

In vitro release of KT from hydrogel formulations

In vitro releases of drug from the formulations (F1 and F2) were evaluated for the batch to batch uniformity of drug product and to observe any change in process parameters. The F1 shows the % of drug release at 0, 0.5, 1, 2, 4, 6, hours were 42.16, 68.71, 71.3, 74.9 and 79.6 respectively. Similarly the F2 shows the % of drug release were 51.2, 77.3, 82.6, 86.3 and 91.3 respectively. The drug release pattern was shown in Figure-4.

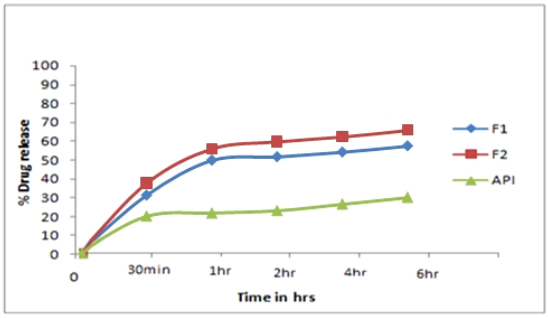

Fig-4: *In vitro* drug dissolution profile of KT and KT loaded hydrogel formulations

In Vivo Pharmacokietic Study

It was conducted using wistar rats through oral administration of both the formulations. The drug content of F1 was found in the plasma samples were 338.4, 580.2, 860.3, 755.7, 580.6, 371.2, 215.4 and 142.6ng/mL at 0.5, 1, 3, 6, 9, 12, 18 and 24hrs respectively. Similarly he drug content of F2 was found in the plasma samples were 352.2, 587.6, 847.4, 707.3 593.6, 377.8, 224.3 and 145.1 ng/mL at 0.5, 1, 3, 6, 9, 12, 18 and 24 hrs respectively. The calculated parameters were depicted in figure 5 and Table-2. The collected blood samples were analyzed using HPLC method. Both formulations were shown higher drug release in compared to KT alone. The peak plasma concentrations were observed at 3h and further the concentration of KT was started in decreased order. When compared to both formulations F1 and F2, KT alone did not release effectively throughout the absorption phase.

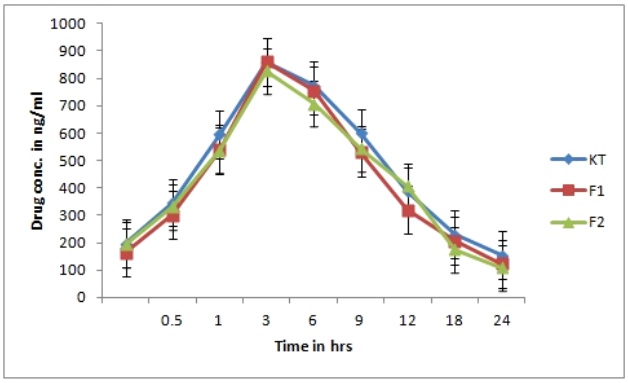

Fig-5: The pharmacokinetic profile of KT, and KT loaded hydrogel formulations (F1and F2) in wistar rats (n=4, single ANOVA, p<0.05*)

Table-2: Pharmacokinetic parameters of KT, F1 and F2 after oral administration

Pharmacokinetic parameters	Ketorolac	F1	F2
AUC_{0-t} (ng h/ml)	673	669	667
$t_{1/2}$ (h)	3	3	3
C_{max} (ng/ml)	849	884	872

***In Vitro* Cytotoxicity Study**

The cytotoxicity of KT and both hydrogel formulations F1 and F2 were evaluated to determine the effectiveness on SCC-29 cells. Cell viability was determined using SRB assay. The supernatant component was taken out and washed with PBS and images were taken under 40X. The KT and hydrogel formulations treated cells are showed in Figure-6. The cells applied with formulations F1 and F2 showed better anticancer activity over KT alone. Formulation F1 have showed faintly induced cell death in SCC-29 cell lines. F1 was showed quite better cytotoxcity over other F2. The cells were showed blebbing and granules. The growth curve was shown in the Figure-7.

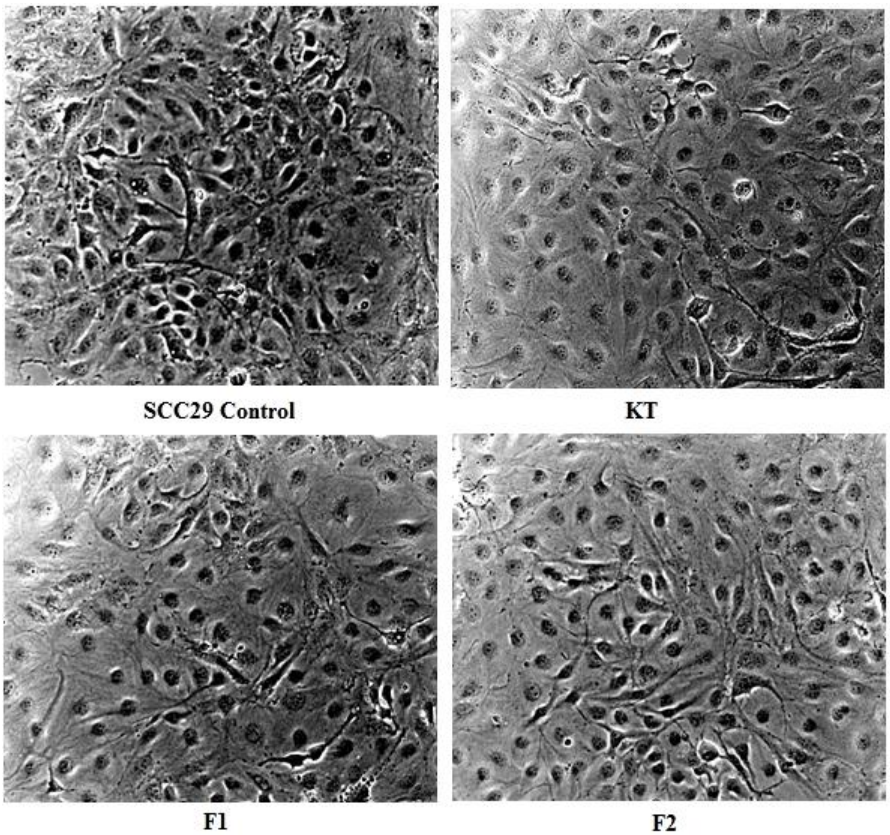

Fig-6: The SCC-29 colon cancer cell lines treated with KT and hydrogel formulations (F1-F2)

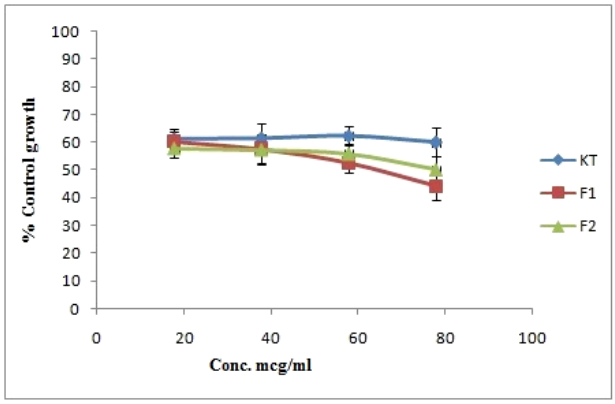

Fig-7: **Growth Curve of ketorolac and two hydrogel formulations (F1-F2) on SCC 29 cell lines. (n=4, single ANOVA, p<0.05*)**

CONCLUSION

The present study confirmed that prepared ketorolac hydrogel formulations has showed improved bioavailability and P^H stability. Among two formulations F1 has shown little better cytotoxicity over F2 formulation when compared to ketorolac alone. Hence ketorolac loaded hydrogel formulations was considered to be a promising way for the delivery of ketorolac.

Conflict of Interest

The author does not have any conflict of interest.

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