Cytotoxic activity of ketorolac-loaded chitosan nanoparticles in SCC-29 cell lines

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Received 8 January 2018
Accepted 14 March 2018

Egyptian Pharmaceutical Journal
2018, 17:53–59

Context
Ketorolac (KT) is a NSAID, with multiple pharmacological activities having been reported.
Aim
To formulate chitosan nanoparticles of KT for assessing their potential in decreasing the growth of colon cancer cells.
Materials and methods
Chitosan nanoparticles of KT such as F1 and F2 have shown anticancer activity against SCC-29 cell lines. Furthermore, nanoparticles have been subjected to stability test at various pH, drug-release study, and in-vivo pharmacokinetics in Wistar rats.
Results
The nanoparticles were in the size range between 164 and 210 nm. The oral absorption of nanoparticles was relatively higher than the KT alone.
Conclusion
The prepared nanoparticle formulations showed better activity than KT alone.

Keywords:
chitosan, cytotoxicity, dug stability, ketorolac, nanoparticles

Egypt Pharmaceut J 17:53–59

1687-4315

Introduction

Chemically ketorolac (KT) is 5-benzoyl-2,3-dihydro-^{1}H-pyrrolizine 1-carboxylic acid [1] KT is a NSAID and is chemically related to indomethacin. KT is a racemic mixture of [–]-*S* and [+]-*R* enantiomer forms with the S form having analgesic activity. Anti-inflammatory effects are believed to inhibit both COX-1 and COX-2, which leads to inhibition of prostaglandin synthesis, leading to decreased formation of precursors of prostaglandins and thromboxanes from arachidonic acid [2–4] In terms of ophthalmic applications of KT, ocular administration of KT reduces PGE2 levels in aqueous humor [5]. KT is a well-known NSAID in the treatment of rheumatoid arthritis and inflammation, in relieving pain, and in reduction of aqueous humor and postoperative cancer pain [6]. Extensive literature review on KT with empirical evidences revealed that along with its anti-inflammatory activity, it showed promising results in the treatment of various cancers. Samal *et al*. [7] has proved that KT salt is a newly discovered DDX3 inhibitor for the treatment of oral cancer. In-vitro anticancer activity of KT with rosuvastatin was found to be effective against DDX3 in the form of hydrogel formulation in oral squamous cell carcinoma, as reported by Khaggeswar *et al*. [8]. KT also showed its therapeutic benefit in patients with ovarian cancer as, reported by Guo *et al*. [9]. While keeping the aforementioned facts in mind, the present research work has been designed to prepare the nanoparticles of KT with chitosan for the investigation of anticancer activity. Chitosan is a biodegradable, biocompatible, and nontoxic polymer with mucoadhesive properties [10], which have greater drug-loading capabilities and the ability to show sustained drug-release properties [11]. Chitosan nanoparticles are prepared by an ionotropic gelation method which does involve interaction between the negative groups of sodium tripolyphosphate and the positively charged amino groups of chitosan [12]. Therefore, the present research work aimed to approach one step ahead for further establishment of KT as an anticancer agent.

Materials and methods

Chitosan was purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium tripolyphosphate was purchased from Central Drug House, Delhi, India. Water was purified by reverse osmosis, MilliQ (USA), and further filtered by 0.22-μm membrane filter. HPLC grade acetonitrile was purchased from SD FineChem (India). Lutrol F 127 was purchased from Himedia, A-406, Bhaveshwar Plaza, LBS Marg, Mumbai (India). Potassium dihydrogen phosphate

infocom network limited, New Delhi, India and all other chemicals used were of analytical grade.

HPLC analytical method

For the determination of the concentration of KT in various polymeric and biological matrices, the developed valid analytical method [13] was utilized. Accurately weighed quantity (10 mg) of KT was transferred to 10-ml volumetric flask, dissolved with 2 ml of acetonitrile, and volume was made up to the mark with mobile-phase acetonitrile : water (60 : 40 v/v). Further concentrations (1, 3, 5, and 10 μg/ml) were prepared by diluting the stock solution (1 mg/ml) with the mobile phase and injected into Shimadzu Binary Prominence HPLC system (250 mm×4.6 mm C-18 column having 5 μm). The chromatographic conditions include mobile-phase acetonitrile : water (60 : 40 v/v), flow rate 1 ml/min, and detection at wavelength of 320 nm. The calibration curve was prepared by plotting concentration versus peak area, and the linearity was established. The linear equation was found to be area=68 214×concentration+16 134, retention time was 1.88 min, and correlation coefficient was found to be 0.999 with relative SD less than 2%, which also justified the precision of the used method.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by ionic cross linking of chitosan solution with sodium tripolyphosphate prepared in the presence of Lutrol F 127 as a suspending agent to avoid aggregation, at 25°C under continuous stirring. Chitosan nanoparticles were prepared by dispersing 10 mg of KT in 10 ml of chitosan solution, containing 0.5% Lutrol F 127, and by further adding tripolyphosphate (0.2% w/v). The tubes containing nanoparticles were centrifuged at 10 000*g* for 45 min using cooling centrifuge, and supernatant was discarded. Various strengths of KT formulations such as F1 and F2 were prepared by using chitosan as a polymer, where F1 contains 2% w/v of chitosan loaded with 2% w/v of KT and F2 contains 4% w/v of chitosan loaded with 2% of KT. The solubility of chitosan in water was increased by maintaining temperature at 6°C for 24 h. Furthermore, these are analyzed for drug content and pH-dependent drug stability.

Characterization of the nanoparticles

The particle size of the KT formulations and polydispersity index were determined using dynamic light scattering equipment (Zetasizer ZS 90). The polydispersity index has a value between 0 and 1. The value close to 0 represents particle distribution is narrow and having minimum particle size.

Determination of ketorolac loading capacity and efficiency

Loading efficiency and loading capacity of nanoparticles of KT were evaluated by taking the samples at 10 000*g* at −5°C for 45 min. The amount of free KT present in the clear supernatant after centrifugation was determined by using HPLC validated analytical method. The loading capacity and loading efficiency of KT in nanoparticles were determined from the following equations 1 and 2:

$$\text{Entrapment efficiency} = \left[1 - \left(\frac{\text{Drug in clear supernatant liquid}}{\text{Total drug added}}\right)\right] \times 100,$$

$$\text{Percentage loading capacity} = \left(\frac{\text{Weight of KT in NP}}{\text{Weight of NP recovered}}\right) \times 100,$$

where NP, nanoparticles, KT, ketorolac.

Stability study of ketorolac and nanoparticles at different pH

For the determination of the stability of nanoparticle formulations at various pH (3.5, 5.5, 6.8, and 7.4), 0.1 mol/l hydrochloric acid and phosphate buffers were selected. Accurately weighed 10 mg of KT and KT-loaded nanoparticles were transferred to 2-ml centrifuge tube and 1 ml of each buffer was added to tubes containing nanoparticles and incubated at 25°C for 24 h. The drug content was determined using HPLC method, and the extent of drug degradation was evaluated.

In-vitro release of ketorolac from nanoparticles

Aliquots of 1-ml KT-loaded nanoparticle formulations were centrifuged at 10 000*g*. The pellet obtained after decanting the supernatant was diluted with 1 ml PBS (pH 7.4) and incubated at 37°C under shaking equipment (50 rpm) for 3 h. At various time intervals, a tube was selected and centrifuged at 10 000*g* for 15 min. The released KT was determined using validated analytical method.

Determination of bioavailability of ketorolac-loaded chitosan nanoparticles

Bioavailability is the rate and extent of drug absorption into systemic circulation. To elicit any pharmacological activity, the maximum amount of drug should reach systemic circulation. For these reasons, KT alone and nanoparticles have been administered orally to Wistar rats to determine the amount of KT reaching systemic circulation. Vageswari College of Pharmacy, Karimnagar, has approved animal facility with CPCSEA registration no. 1720/po/a/14/cpcsea, and protocol was approved by Institutional Animal Ethics

Committee. KT and KT-loaded nanoparticles were taken equivalent to 5 mg of KT and administered to male Wistar rats (~280 g) orally, and blood samples were withdrawn from tail vein under mild anesthesia at intervals of 0.5, 1, 2, 3, 4, 6, and 24 h, respectively. The withdrawn blood samples were transferred to tubes containing EDTA. Furthermore, samples were subjected to protein precipitation and analyzed using validated HPLC method.

In-vitro cytotoxicity study

The SCC-29 cancer cell lines were collected from National Cancer Institute (USA). These cells were cultured in complete growth medium (RPMI 1640) supplemented with 10% fetal bovine serum (Sigma), 1% 100 U/ml streptomycin (Sigma), and 1% 100 U/ml penicillin at 37°C, 5% CO_2, and 98% relative humidity. The human cancer cell lines were allowed to grow routinely in 75-cm^2 canted-neck tissue culture flask and passaged regularly by using trypsin/EDTA. Further subculture was performed when confluence of 90% was reached. The in-vitro cytotoxicity of KT and prepared formulations (F1 and F2) were performed by using sulforhodamine B (SRB) assay [14] according to the standard protocol. In summary, 5×10^3 cells/well of SCC-29 cells were seeded in 96-well plates and incubated for next 24 h. Different concentrations (10–80 μg/ml) of KT formulations were used. The plates were incubated for 48 and 72 h and fixed with ice cold trichloroacetic acid for 1 h at 4°C. The plates were washed with distilled water (three times) and air dried. The SRB dye (0.4%) was added in the plates and kept at room temperature for 30 min. The plates were washed with 1% (v/v) glacial acetic acid for the removal of unbound SRB dye. The Tris buffer (10 mmol/l, pH 10.4) was added to each of the well and solubilized by keeping on a shaker. Microplate reader was purchased from Goregaon East, Mumbai, India (BioTek Synergy HT) at 540 nm was used to measure the values.

Results

HPLC analysis of ketorolac

KT was analyzed using Shimadzu HPLC C-18 (250 mm×4.6 mm, 5 μm), mobile phase acetonitrile : water (60 : 40 v/v), for various concentrations of KT, and the data are shown in Table 1. The calibration curves were prepared to calculate the drug concentration. The relative SD is less than 2% indicates the precision of the HPLC analysis. The correlation coefficient was found to be 0.999 and found to have a linear relationship between concentration and area. The chromatograms at 10 μg/ml are shown in Fig. 1.

Preparation and characterization of the nanoparticles

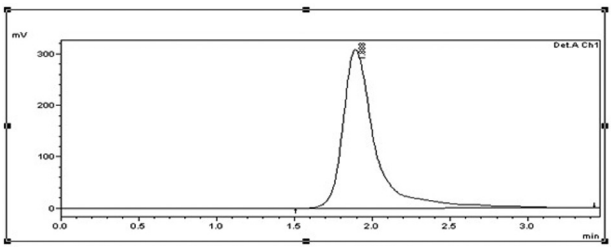
Two formulations of KT nanoparticles were prepared, and the dynamic light scattering technique was adopted for the characterization of the nanoparticle formulations F1 and F2. The mean particle hydrodynamic diameter of F1 and F2 was 164±13 and 210±11 nm, respectively. The particle size distribution of KT nanoparticles is shown in the Fig. 2.

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

Drug concentration (μg/ml)	Area (±SD)	%R (SD[a])
1	75721.8±1387.7	1.63
3	234405.9±3634.9	1.77
6	427572.8±1078.7	1.04
9	645415.7±9563.9	0.37
12	815479.4±16544.8	1.22

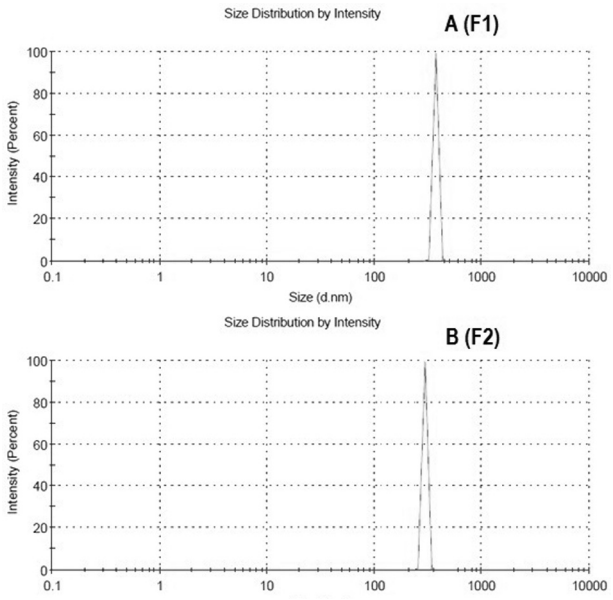
[a]Relative SD or coefficient of variance. [b]Two standard stock solutions.

Figure 1



HPLC chromatogram of ketorolac at 10 μg/ml using mobile-phase acetonitrile : water (60 : 40 v/v).

Figure 2

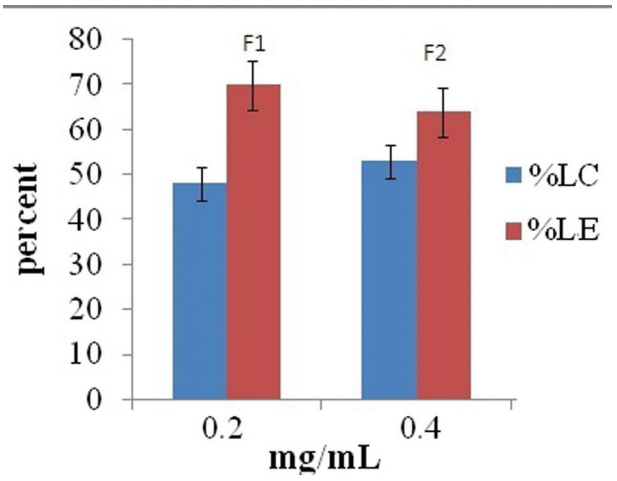


Particle size distribution of ketorolac-loaded chitosan nanoparticle formulations. (a) 2% Chitosan. (b) 4% chitosan with 2% ketorolac in each formulation.

Determination of ketorolac loading capacity and efficiency

The nanoparticle size was directly proportional to the drug concentration; therefore, the nanoparticles size was increased to the increase in the concentration of KT. The entrapment efficiency and drug loading with various chitosan concentrations are showed in Fig. 3. The maximum drug loading capacity of 67.3% was observed for KT at 0.4 mg/ml. Percentage drug loading and loading efficiency (LE) were affected by molecular weight of the polymer used. As the molecular weight of the polymer (chitosan) increased, the drug content was decreased. Increasing the molecular weight of the chitosan causes reduction in drug polymer interaction which may be responsible for reduction in drug content.

Figure 3



Loading capacity and loading efficiency study of ketorolac-loaded chitosan nanoparticles. F1–F2, ketorolac-loaded chitosan nanoparticle formulations; %LC, percentage loading capacity; %LE, loading efficiency (n=4, single analysis of variance, P<0.05*).

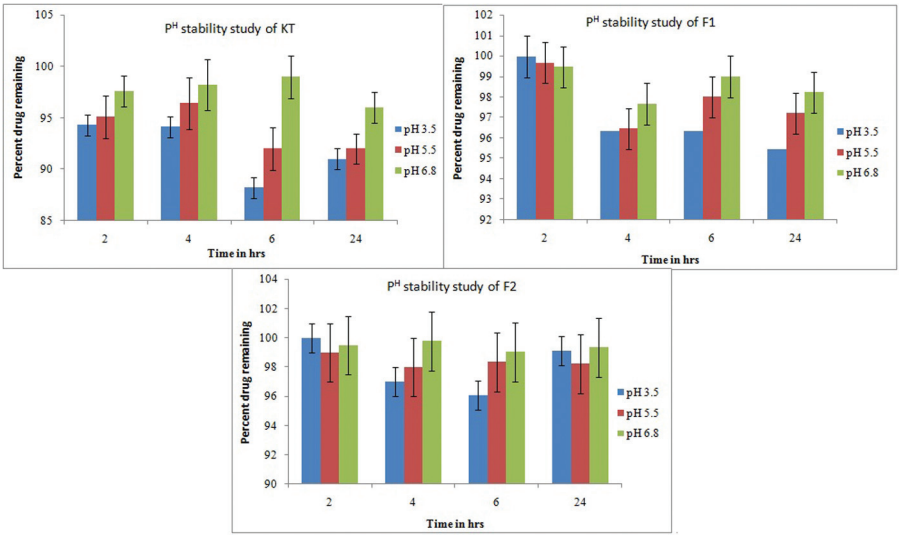
Stability study of ketorolac and nanoparticles at different pH

The stability of KT and formulations were evaluated and determined for drug content at different pH. The formulations of KT were analyzed for drug content using HPLC. pH stability of formulations is very important for drug content maintenance. KT and nanoparticles of KT (F1 and F2) were subjected to pH 3.5, 5.5, and 6.8 at different time internals (2, 4, 6, and 24 h). At pH 3.5, the percentage of drug remaining in F1 was 99.98, 96.35, 96.34, and 95.45. At pH 5.5, the percentage of drug remaining in F1 was 99.67, 96.46, 98.98, and 97.2. At pH 6.8, the percentage of drug remaining in F1 were 99.46, 97.67, 99, and 98.29. Similarly at pH 3.5, the percentage of drug remaining in F2 was 99.98, 97, 96.1, and 99.11. At pH 5.5, the percentage of drug remaining in F2 were 99.0, 98.0, 98.34, and 98.23. At pH 6.8, the percentage of drug remaining in F2 were 99.5, 99.78, 99.03, and 99.34. KT alone has less stability at different pH. Chitosan has showed better stability at 6.8 and extended for 24 h period. Therefore, both formulations have been prepared with chitosan to justify the stability of F1 and F2. Among the two formulations, the overall stability of F1 has been quite higher than F2 (Fig. 4).

In-vitro release of ketorolac from nanoparticles

In-vitro releases of drug from polymer matrix (F1 and F2) were evaluated for the batch-to-batch uniformity of drug product and to observe any change in process parameters. In F1, the percentages of drug release at 0,

Figure 4



Stability study results of ketorolac-loaded chitosan nanoparticle formulations at different pH (n=4, single analysis of variance, P<0.05*).

10, 20, 30, 40, 50, 80, 120, 150, and 180 min were 51.6, 68.19, 81.5, 86.2, 91.5, and 94.32, respectively. Similarly, for F2, the percentages of drug release were 24.5, 48.8, 57.3, 59.2, 61.5, and 69.23, respectively. The drug-release pattern was shown in Fig. 5.

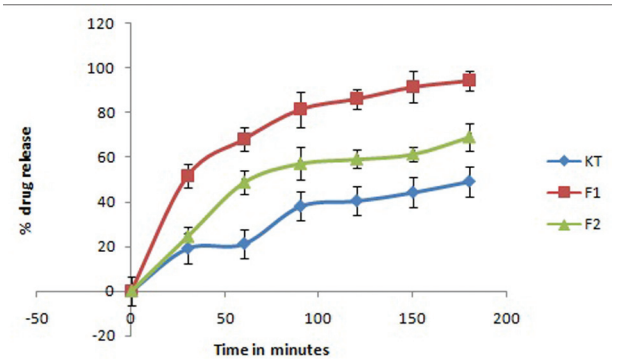
In-vivo pharmacokinetic study

This study was conducted using Wistar rats through oral absorption of both the formulations. The drug contents of F1 found in the plasma samples were 211, 324, 594, 910, 747, 573, and 123 ng/ml at 0.5, 1, 2, 3, 4, 6, and 24 h, respectively. Similarly, the drug contents of F2 found in the plasma samples were 223, 310, 580, 895, 732, 534, and 115 ng/ml at 0.5, 1, 2, 3, 4, 6, and 24 h, respectively. The calculated parameters are shown in Fig. 6 and Table 2. It was found that F1 formulation possessed good swelling ratio, considerable mechanical strength, and better entrapment of drug loading and drug-release properties. The nanoparticle formulations were analyzed by fitting them in various mathematical models such as zero order, first order, Higuchi model, and Korsmeyer model. Further pharmacokinetic study was performed on F1, F2, and KT alone. The plasma concentration and time profile of pure KT, F1, and F2 formulations and their mean pharmacokinetic data of various pharmacokinetic parameters (C_{max}, $t_{1/2}$ and AUC, area under the curve) confirmed that the pharmacokinetic profile of F1 was found to be better in comparison with KT alone. Similarly, the pharmacokinetic profile of F2 was found to be better in comparison with KT alone. Thereafter, when we compared the pharmacokinetic profiles of F1 and F2, F1 showed a slightly better profile than F2. Thus, oral delivery of KT-loaded formulations can be a promising delivery system for local effect in greater extent.

In-vitro cytotoxicity studies

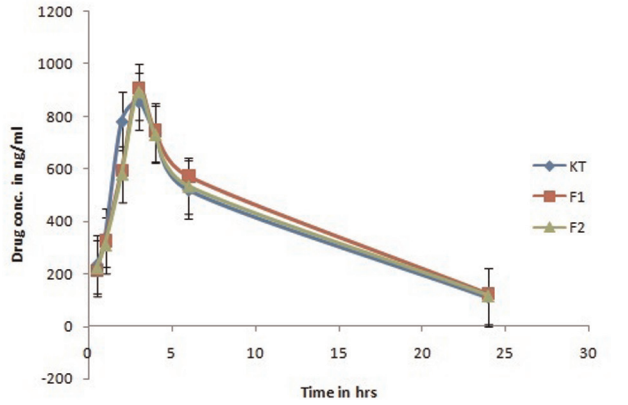
The cytotoxicity of KT and KT-loaded chitosan nanoparticles F1 and F2 was evaluated to determine the effectiveness on SCC-29 cells. SRB assay was used to determine cell viability. The supernatant component was taken out and washed with PBS, and images were taken under 40× (Fig. 7). For the evaluation of cytotoxicity, increased concentrations of chitosan-loaded KT nanoparticles formulations (F1 and F2) were added to SCC-29 cell lines, and variability of cells were measured. These formulations had a remarkable effect on SCC-29 cell lines after 48 h of incubation. After 72 h of incubation period, both of these formulations have reached a comparable level of cytotoxicity. In cells treated with nanoformulations (F1 and F2), F1 induced slightly higher cell death in SCC-29 cell lines, and it is evident from the membrane blebbing and granules formed within the cell.

Figure 5



The in-vitro drug dissolution profile of ketorolac and ketorolac-loaded chitosan nanoparticles (n=4, single analysis of variance, P<0.05*).

Figure 6



The oral absorption profile of ketorolac and ketorolac-loaded chitosan formulations (F1 and F2) in Wistar rats (n=4, single analysis of variance, P<0.05*).

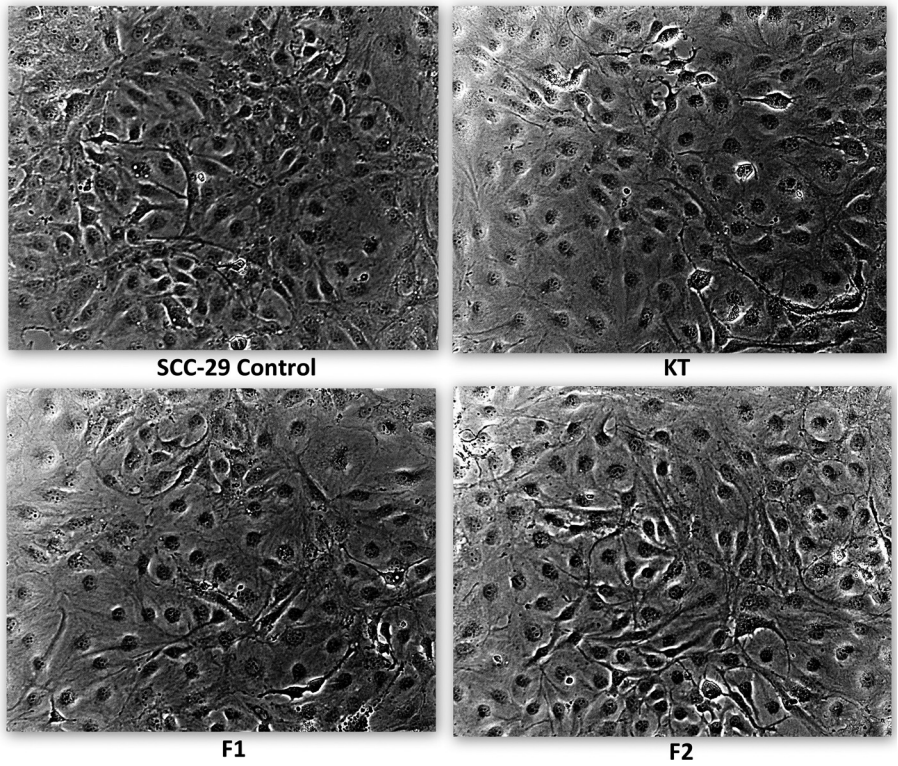
Table 2 Pharmacokinetic parameters of ketorolac, F1, and F2 after oral administration

Pharmacokinetic parameter	Ketorolac	F1	F2
AUC_{0-t} (ng h/ml)	687	864.3	874.3
$t_{1/2}$ (h)	3	3	3
C_{max} (ng/ml)	857	910	897

AUC, area under the curve.

Cells treated with KT alone showed cell wall shrinkage and chromatin fragmentation. In the current study, cytotoxicity is due to polymer degradation, which may be responsible for interfering with cellular function and cellular transportation of various substances. The growth curve was plotted by concentration versus control growth. From the growth curve (Fig. 8), it was found that upon increasing the concentration of KT, cell viability is decreased, and it is may be due to formulation adhesion. From the graph, it was observed that KT and its nanoparticle formulations showed good activity in inhibition of SCC-29 cell lines, and among the two formulations, F1 at less

Figure 7



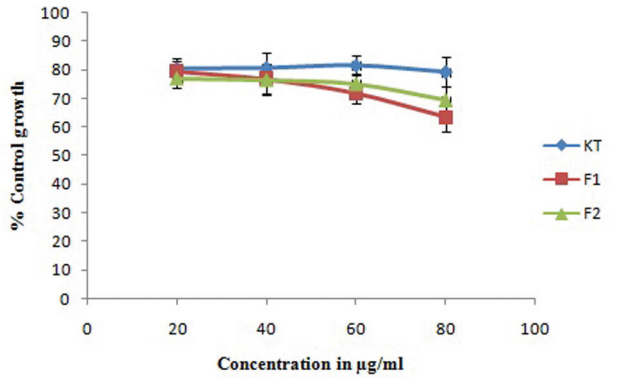
The SCC-29 colon cancer cell lines treated with ketorolac and ketorolac-loaded chitosan formulations (F1–F2).

than 10 µg/ml showed good activity in inhibition of SCC-29 cell lines.

Discussion

The HPLC analysis of KT was conducted to determine the percentage of drug release for the prepared solutions of all the formulations. The percentage of drug release at different time interval has been calculated from the calibration graph of KT, which shows satisfactory values. The characterization of all formulations using dynamic light scattering technique reveals the size distribution was proper. The loading capacity and efficiency of both formulations indicated that with the increasing drug loading, loading efficiency decreased and loading capacity increased. Stability study of nanoparticles of both formulations at different pH was conducted and reveals that the formulation F1 showed highest stability compared with F2 formulations. The pH stability [8] 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. Chitosan was showed better stability at pH 6.8 and extended for 24-h period. Both formulations showed different drug release patterns. For the pharmacokinetic study, the blood samples were analyzed using a validated HPLC method. Both formulations showed higher drug release in compared with KT alone. The peak plasma concentrations were reached at 3 h, and further the concentration of KT was started decreasing drastically. KT alone did not release effectively throughout the absorption phase when compared with both formulations. In-vitro cytotoxicity study was performed [15], and it was found that the SCC-29 cells treated with formulation F1 have showed faintly induced cell death in SCC-29 cell lines.

Figure 8



Growth curve of ketorolac and two ketorolac-loaded chitosan formulations (F1–F2) on HT29 colon cancer cell lines (n=4, single analysis of variance, $P<0.05$*).

F1 showed a slightly better cytotoxicity profile over F2. The cells were relieved membrane blebbing and granules.

Conclusion

The study confirmed that the KT-loaded chitosan nanoparticle formulations show better bioavailability and pH stability. Among the two formulations, F1 has shown marginally better cytotoxicity over F2 formulation when compared with KT alone. Hence, KT-loaded chitosan nanoparticles can be considered to be a promising system for the delivery of KT. These nanoparticle formulations are effective in the treatment of squamous cell carcinoma compared with other chemotherapeutic agents leading to fewer adverse effects. Further trials were required to evaluate the in-vivo formulation parameters and improved cytotoxicity with other polymers.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Chemically ketorolac. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/ketorolac. [Accessed 2007 Jun 10].

2 Rooks WH, Maloney PJ, Shott LD, Schuler ME, Sevelius H, Strosberg AM, *et al.* The analgesic and anti-inflammatory profile of ketorolac and its tromethamine salt. Drugs Exp Clin Res 1985; 11:479–492.

3 Rooks WH. The pharmacologic activity of ketorolac tromethamine. Pharmacotherapy 1990; 10:30–32.

4 Jung D, Mroszczak E, Bynum L. Pharmacokinetics of ketorolac tromethamine in humans after intravenous, intramuscular and oral administration. Eur J Clin Pharmacol 1988; 35:423–425.

5 Mroszczak EJ, Jung D, Yee J, Bynum L, Sevelius H, Massey I. Ketorolac tromethamine pharmacokinetics and metabolism after intravenous, intramuscular, and oral administration in humans and animals. Pharmacotherapy 1990; 10:33–39.

6 Gaynes BI, Onyekwuluje A. Topical ophthalmic NSAIDs: a discussion with focus on nepafenac ophthalmic suspension. Clin Ophthalmol 2008; 2:355–368.

7 Samal SK, Routray S, Veeramachaneni GK, Dash R, Botlagunta M. Ketorolac salt is a newly discovered DDX3 inhibitor to treat oral cancer. Sci Rep 2015; 5:9982.

8 Khaggeswar B, Manish Kumar T, Sagar KI, Prathyusha T, Swathi A, Kranthi R, *et al.* In vitro anti-cancer activity of rosuvastatin and ketorolac nanoformulations against DDX3. J Young Pharm 2017; 9:537–544.

9 Guo Y, Kenney SR, Cook L, Adams SF, Rutledge T, Romero E, *et al.* A novel pharmacologic activity of ketorolac for therapeutic benefit in ovarian cancer patients. Clin Cancer Res 2015; 21:22.

10 Zhang Y, Yang Y, Tang K, Hu X, Zou G. Physicochemical characterization and antioxidant activity of quercetin-loaded chitosan nanoparticles. J Appl Polym Sci 2008; 107:891–897.

11 Calvo P, Remuñan-López C, Vila-Jato JL, Alonso MJ. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm Res 1997; 14: 1431–1436.

12 De Campos AM, Sanchez A, Alonso MJ. Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. Int J Pharm 2001; 224: 159–168.

13 Sunil G, Jambulingam M, Ananda S, Thangadurai D, Kamalakannan R, Sundaraganapathy C, Jothimanivannan XX. Simultaneous estimation of atorvastatin calcium, aspirin, ramipril and metoprolol tartrate in bulk and in its capsule formulation by first order derivative spectrophotometry. Arabian J Chem 2017; 10:928–935.

14 Studzinski GP. Cell growth, differentiation and senescence: a practical approach. New York, NY: Oxford University Press; 1999. pp. 41–42.

15 Tuba Sengal-Turk C, Hascicek C, Lale Dogan A, Esendagli G, Guc D, Gontil N. Preparation and *in vitro* evaluation of meloxicam loaded PLGA nano particles on HT-29 Humnan colon adenocarcinoma cells. Drug Dev Ind Pharm 2012; 38:1107–1116.