Improved Anticancer Activity of Meloxicam Hydrogels in K562 and HL60 Cell Lines

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ABSTRACT
Objective: The aim of the present study is to prepare poloxamer based formulations of meloxicam to evaluate various parameters like pH stability, drug release and in vitro anticancer activities in cell lines with an intention to formulate injectable sustained biodegradable drug delivery system.

Method: Various strengths of meloxicam formulations were prepared by using poloxamer 407. Prepared formulations were analyzed for drug content and pH stability by using HPLC. Drug release studies were tested by using USP dissolution testing apparatus. Further, we evaluated in vitro anticancer activity among these formulations by using sulphorhodamine-B (SRB) assay in two leukemia cell lines such as HL60 and K-562 cell lines.

Results: It showed that among all formulations, F1 formulation showed stability at pH 6.8, 7.0 and 7.4. It also showed 60% drug release and exhibited good anti cancer activity in HL60 cell line with GL₅₀ <10 µg/ml as similar to adriamycin.

Conclusion: Comparing these results, we concluded that F1 formulation showed good anticancer activity in these cell lines, therefore further studies are necessary to confirm the mechanism of toxicity action studies. Thus these formulations has a potential to be a sustained release, passive targeted deliver system for meloxicam, with reduced side effects associated with the drug.

Key words: Meloxicam, Poloxamer 407, Sulphorhodamine-B, Cyclooxygenase, HL60 and K-562.

INTRODUCTION
Meloxicam is a new nonsteroidal anti-inflammatory drug (NSAID) having cyclooxygenase (COX-2) inhibitory properties and widely used to treat of rheumatoid arthritis and osteoarthritis.¹ It showed good gastrointestinal tolerability compared with other nonsteroidal anti-inflammatory drugs² and has a half-life of approximately 20 hours and suitable for once-daily administration.³ Cyclooxygenases are a class of enzymes plays an important role in the development of cancer. These enzymes catalyze formation of prostaglandins, influence apoptosis, angiogenesis and invasion has involved in production of carcinogens. A high concentration of COX-2 is found in cancer cells.⁴ Literature survey revealed that meloxicam has shown various anticancer activities such as inhibition of HCA-7 colonies, cytotoxic effects on osteosarcoma MG-63 cell line and squamous cell carcinoma of the esophagus.⁵ ⁶ Combinatorial effect of meloxicam with carboplatin and paclitaxel showed improved survival of lung cancer patients, antiproliferative effect against PC3 prostate cancer cell lines, in vitro and in vivo models of urinary bladder cancer.⁷ It enhances the activity of sunsitinib maleate on bladder-cancer cells but activity of meloxicam in K562 and HL60 cells were not reported in earlier studies. Various formulations of meloxicam were available for potential anticancer applications.⁸ ⁹ Currently, in situ gel forming formulations as a novel idea of drug delivery to patients as a liquid dosage form, yet achieve sustained release of drug for the desired period.¹⁰ Different delivery systems based on polymers have been developed, which are able to increase the residence time of the formulation at absorption site of drugs. In recent years, there has been an increasing interest in water soluble polymers that are able to form gels after application to delivery state. These in situ gelling polymers are highly advantageous compared with other polymers, because, in contrast to very strong gels, they can be easily applied in liquid form to the site of drug absorption. At the site of drug absorption, they swell to form a strong gel that is capable of prolonging the residence time of the active substance.¹¹ Poloxamer consists of more than 30 different non ionic surface active agents. These polymers are ABA- type triblock co polymers composed of PEO (A) and PPO units (B).¹² Poloxamer 407 are substances that have recently received remarkable attention in the field of thermo sensitive hydrogels. It is an amphiphilic synthetic copolymer consisting of a hydrophobic poly (Oxypropylene) (POP) block between two hydrophilic poly (Oxyethylene) (POE) blocks.¹³ Due to their amphiphilic nature, poloxamer molecules can readily self- assemble to form micelles depending on the concentration and temperature. These hydrogels are characterized by the ability to carry a significant amount of drug. They are also biodegradable, non toxic and stable, therefore suitable for uses as controlled release agents.¹⁴ In the present study, we have prepared poloxamer based hydrogel formulations of meloxicam and evaluated various parameters like stability at different pH, drug release and in vitro anticancer activity against K562 and HL60 cell lines with an intention to formulate injectable sustained biodegradable drug delivery systems of meloxicam.

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MATERIALS AND METHODS

Materials and reagents
Meloxicam was obtained as a gift sample from APEX health care, Ltd, India. Poloxamer 407 was purchased from Sigma-Aldrich, India. All the reagents for HPLC grade and preparing pH buffers were purchased from SD Fine Chemicals Limited, Mumbai, India.

HPLC analytical method
Standard stock solution of meloxicam 100 µg/ml was prepared by dissolving 10 mg of meloxicam in 65 ml of HPLC grade acetonitrile and further diluted to 100 ml with triple distilled water. Furthermore, triplicates of calibration concentrations between 100 ng/ml and 20000 ng/ml were prepared and analyzed at 360 nm. The linear regression equation was Area = 50.92 (Concentration in ng/ml) + 4106 with R² value 0.9980 and the relative standard deviation was found to be less than 2% for absorbance measurements.

Preparation of hydrogel formulations
Hydrogels of meloxicam formulations were prepared using poloxamer 407. Poloxamer 407 is thermo responsive polymer which has thermo reversible gelling abilities, promotes solubilization of poorly water soluble drugs and prolongs the release of oral, topical, rectal and injectable formulations. Various strengths of meloxicam F1 and F2 contain 18% w/v, 20% w/v of poloxamer 407 respectively. Each formulation consists of 7.5% w/v of meloxicam. Each formulation was prepared by taking accurately weighed quantity of meloxicam and poloxamer 407 and dispersed in required quantity of triple distilled water. All the formulations were prepared to total volume of 2 ml. The solubility of Poloxamer 407 in water was increased by maintaining temperature at 6ºC for 24 h. Furthermore, the samples were analyzed for drug content and pH dependent drug stability.

Determination of drug content and drug stability
The prepared formulations were further analyzed for drug content and pH dependent stability. Each formulation was accurately weighed to 100 mg and 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. Stability studies of formulations were performed at pH 1.2, 2.5, 4, 5.5, 6.8, 7 and 7.4 respectively. 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 study was performed by taking 100 mg formulation in 2 ml centrifuge tube and diluted to 1 ml with buffer and analyzed at 1, 2, 4 and 24 h respectively. The prepared formulations in triplicate were kept at 25ºC and 40ºC for 3 months and analyzed for drug content.

Drug release from formulations
The formulations of meloxicam were evaluated for drug release using USP Dissolution Testing Apparatus-II and a triplicate of each formulation was analyzed for drug release at pH 1.2, 4, 6.8 and 7.4. The 5 ml sample was withdrawn at 0.5, 1, 1.5, 2, 2.5, 3, 6, 9, 12 and 24 h and replenished with same volume of respective drug free buffer to maintain sink conditions.

In vitro cytotoxicity studies
The source of leukemia cancer cell lines (K-562) and HL-60 from NCI (National Cancer Institute, USA). These cells were cultured in complete growth medium (RPMI 1640) supplemented with 10% fetal bovine serum (Sigma, USA), 1% 100 U/ml penicillin and 1% 100 U/ml streptomycin (Sigma, USA) at 37ºC, 5% CO₂ and 98% relative humidity. The human cancer cell lines were routinely grown in 75 cm² canted-neck tissue culture flask and passaged regularly by using trypsin/EDTA when confluence of 90% was reached and then further subcultured. The in vitro cytotoxicity of meloxicam and prepared formulations was performed by using sulforhodamine B (SRB) assay according to the standard protocol. Briefly, 5x10⁴ cells/ well of K-562 and HL-60 cells were seeded in 96 well plates and incubated for next 24 hr's. Different concentrations (10-80 µg/mL) of meloxicam formulations and positive control adriamycin in complete growth medium were added to the wells. The plates were incubated for 48 and 72 hr's and fixed with ice cold tri chloro acetic acid for 1 hr at 4ºC. The plates were washed with distilled water (3 times) and air dried. The SRB dye (0.4%) was added in the plates and kept at room temperature for 30 min's. The plates were washed with 1% (v/v) glacial acetic acid to remove unbound SRB dye. The tris buffer (10 mM, pH 10.4) was added to each well and solubilized by keeping on a shaker. The values were measured by using microplate reader (Biotek Synergy HT) at 540 nm and IC₅₀ were determined by plotting a graph between optical densities versus concentration.

RESULTS AND DISCUSSION

Drug content and drug stability
The prepared formulations of meloxicam were analyzed for drug content using HPLC. The F1 has drug content of 98.62%, F2 has 99.15% respectively. B1 and B2 formulations have not evaluated for stability because of lacking drug content and serves as control. The stability of meloxicam and formulations were evaluated and determined for the drug content at various pH using HPLC method. The areas obtained using HPLC were evaluated with relative standard deviation of less than 4%. Meloxicam powder showed less stability at pH 1.2 and completely degraded in one week but still retained drug content between pH 6.8 and pH 7.4. It is found that pure meloxicam is unstable between the pH 1.2 and pH 5.5 respectively (Figure 1a). F1 and B1 formulations were quite stable than pure meloxicam at pH 5.5. The stability of F1 and B1 at pH 1.2 was above 30%, both formulations retained above 90% drug content at pH 6.8 and maintained till one week (Figure 1b and 1c). The stability study revealed that pure meloxicam, F1 and B1 were stable at pH 6.8, 7.0 and 7.4 respectively. These formulations may be suitable for intramuscular administration. The formulation stability (n=3) was determined at 25ºC and 40ºC and found that there was no change in the drug content at 25ºC but 37% of pure drug and 24% of F1 and B1 were degraded at 40ºC. There was no change in the color of the formulations were observed at 25ºC and 40ºC.

Drug release from formulations
Meloxicam, formulations F1, F2, B1 and B2 were determined for in vitro drug release properties. Meloxicam powder was released 10% at first hour and further showed 60% drug release at pH 1.2. F1 and F2 were released not more than 50% at the end of 24 h. The release of the drug from the formulations was further suppressed as increase in the concentration of poloxamer (Figure 2a). None of the formulations and pure drug was released more than 70% at pH 7.4. The sustained drug release pattern was observed from 9 h. The F1 and F2 have showed 60% and 55% drug release. The sustained drug release of drug at pH 7.4 was similar to the pH 1.2 but varied in the content (Figure 2b).

In vitro cytotoxicity of meloxicam and its formulations
HL-60 and K-562 cell lines were used to evaluate in vitro cytotoxicity of meloxicam and its formulations by using SRB assay After 48 hour’s stimulation in HL-60 cell lines with meloxicam formulations. Following stimulation period, supernatant was removed from the wells and the respective wells were washed with phosphate buffer saline (PBS) and phase contrast images were taken by light microscopy at 40X magnification.
Figure 1: Solution state stability at various pH (a). Meloxicam powder (b). F1 and (c). F2 formulations.

Figure 3: In vitro testing of F1 and B1 formulations anti cancer activity in human leukemia cell line HL-60. Morphological changes observation in (a) F1, (b) B1, (c) Control, (d) Meloxicam and (e). Adriamycin at 40 X magnification.

Figure 2: Drug release at of F1 and F2 formulations at (a). pH 1.2 and (b). pH 7.4.

Figure 4: In vitro testing of F1 and B1 formulations anti cancer activity in human leukemia cell line K-562. Morphological changes observation in (a) F1, (b) B1, (c) Control, (d) Meloxicam and (e). Adriamycin at 40 X magnification.

Table 1: In vitro anti cancer activity of meloxicam and its formulations using SRB assay

<table>
<thead>
<tr>
<th>Human Leukemia Cell line</th>
<th>Meloxicam</th>
<th>K-562</th>
<th>LC_{50} (µM)</th>
<th>TGI (µM)</th>
<th>GI_{50} (µM)</th>
<th>LC_{50} (µM)</th>
<th>TGI (µM)</th>
<th>GI_{50} (µM)</th>
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<td>&gt;80</td>
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<td>NE</td>
<td>NE</td>
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<tr>
<td>F2</td>
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<td>&gt;80</td>
<td>&gt;80</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td></td>
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</tr>
<tr>
<td>B1</td>
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<td>&gt;80</td>
<td>&gt;80</td>
<td>NE</td>
<td>NE</td>
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<tr>
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<td>&gt;80</td>
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<td>&gt;80</td>
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<tr>
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<td>&lt;10</td>
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LC_{50} = Concentration of drug causing 50% cell kill, GI_{50} = Concentration of drug causing 50% inhibition of cell growth. TGI = Concentration of drug causing total inhibition of cell growth. ADR = Adriamycin, Positive control compound.

The phase contrast micrographs of control cells and formulation treated cells are given in (Figure 3). The cells and nuclei of control cells are normal with clear cut membranes, while nuclei are elliptical. Whereas, the cells treated with nanoformulations among all the F1 formulation slightly induced cell death in HL-60 cell lines, it is evident from membrane blebbing and formation of granules within the cell. Whereas B1 has not showed any substantial difference in relate to control cells. However, enhanced cell to cell attachment was noticed which is evident from reflection of white light under microscope as compared to control cells alone. Whereas cells treated with meloxicam showed cell wall shrinkage and chromatin fragmentation. Whereas, in case of standard drug (adriamycin) treated cells showed cell death, which is evident from lack of nucleus in the cell, cell membrane distortions and difference between nucleus to cytoplasmic ratio was also noticed. Similarly, in case K-562 cell lines treated with meloxicam formulations all the cells are healthy compare to control and adriamycin treated cell lines, it may due to poloxamer 407 formed a protective layer around the cell membrane (Figure 4). After morphological changes observation, we evaluated cell growth inhibition potential of the drug, after 48 hr’s the LC_{50} (concentration of drug causing 50% cell kill), GI_{50} (concentration of drug causing 50% inhibition of cell growth) and TGI (concentration of drug causing total inhibition of cell growth) were calculated. The LC_{50}, GI_{50} and TGI values of meloxicam and its formulations and standard positive drug (adriamycin) values were summarized in Table 1. Results showed that meloxicam...
and its formulations F1, F2, B1 and B2 showed LC_{50}, GI_{50} and TGI values >80 µg/ml, indicating they were inactive on human leukemia cell line K-562. All the mean values of each formulation were represented in the form of standard graph (Figure 4a & 4b). Similarly meloxicam, B1 and B2 formulations does not showed any inhibition on HL-60 cell line (Figure 5). However, F1 formulation GI_{50} <10 µg/ml in HL-60 cell line, which is similar to positive control drug adriamycin GI_{50} <10 µg/ml (Figure 5a & 5b). Comparing these results, we observed F1 formulation showed good activity in inhibition of HL-60 cell line.

CONCLUSION
The F1 formulation was found to be stable between pH 6.8 and 7.4. This formulation integrity was maintained at 25°C for a period of three months. It showed good anticancer activity in HL-60 cell lines and further study is required to analyze injectability and in vivo parameters for sustained drug release.

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CONFLICT OF INTEREST
There are no conflicts of interest.

REFERENCES


