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Meloxicam loaded hydroxypropyl methylcellulose (HPMC) microparticulate: Fabrication, characterization and *in vivo* pharmacokinetic assessment

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Abstract: Meloxicam (MEL) is an oxicam derivative with low water solubility that is useful in the treatment of colorectal cancer (CRC) as a COX-2 inhibitor. MEL-loaded HPMC micro particles were fabricated using an oil-in-oil (o/o) emulsion solvent evaporation (ESE) method. FTIR, XRD, particle size analysis, DSC, SEM and *in vitro* dissolution investigation were utilized to evaluate the produced micro particles physiochemically. Finally, rabbits were used as animal models in an in vivo pharmacokinetic study to assess the MEL concentration in the plasma of rabbits. Pure MEL, F1 and F2 were given to rabbits by a single dose for *in vivo* pharmacokinetic investigations. The XRD and DSC results confirmed the transformation of MEL from its crystalline nature to the amorphous state in micro particles. The formulations F1 and F2 particle sizes were determined 92.43µm and 163.26µm, respectively. The prepared micro particles had a smooth, non-porous, and spherical surface. In comparison to the pure drug (22.4%), the F1 and F2 cumulative drug release (%) was 86.19% and 79.57%, respectively. Pure MEL, F1 and F2 have estimated C_{max} values of 7.21, 25.41 and 22.38µg/mL, respectively. MEL had a half-life of 19.98 hours, which rose to 22.19 hours and 24.75 hours for F1 and F2, respectively. MEL, F1 and F2 had AUC_0-α values of 116.034, 445.95 and 462.72µg/mL*h, respectively. Considering these aspects, MEL-loaded HPMC micro particles may have the potential to better the delivery and control the release of drug that is not easily dissolved in water which could lead to improved therapeutic efficacy and limited side effects.

Keywords: Micro particles, HPMC, HPLC, meloxicam, validation, pharmacokinetics study.

INTRODUCTION

CRC is the world's third most prevalent malignancy, with high morbidity and mortality. It is a chronic disease that can develop as a result of other inflammatory disorders in the intestine. Inflammation is a crucial response to tissue injury and infection that can damage tissue and contribute to the development of chronic inflammatory disorders. (Ricciotti and FitzGerald, 2011). The enzyme cyclooxygenase (COX), which converts arachidonic acid to prostaglandins, may play a role in cancer development. The activity of COX-2 increases during inflammatory processes, and according to pharmacological studies, it could be a good therapeutic target. Thus, inhibiting the COX-2 enzyme could be used as a technique to halt the advancement of carcinogenesis (Suzuki *et al*., 2009).

MEL is an oxicam derivative associated with the NSAID group of drugs, that is made up of 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3 carboxamide-1,1-dioxide and belong to the Biopharmaceuticals Classification System II (BCS II) and have low water solubility but high permeability across the cell membrane (Jiang *et al*., 2018). It's having long-acting analgesic, antipyretic and anti-inflammatory characteristics utilized in the treatment of rheumatoid arthritis, osteoarthritis and post-surgical swelling and has several side effects that make it inappropriate for long-term use (Chen *et al*., 2022). It has also been reported to have anti-tumor properties in research on colorectal cancer, owing to COX-2's participation in the progression of this cancer. Because of its high rate of side effects, specialized NSAIDs formulations based on micro particulate systems (micro or nanoparticles) have been developed, which can create a barricade between the drug and its off-target locations and/or regulate the kinetics of drug release (Shi *et al*., 2017).

To overcome the challenges of traditional drug delivery systems, such as GIT side effects, poor stability, low solubility and numerous novel drugs that have a limited therapeutic index, a variety of particulate carrier systems,

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namely micro particles, microcapsules, nanoparticles, nanocrystal and nanocapsules, are employed in the delivery of targeted drugs to the colon. The micro particulate system is one of the greatest techniques to offer an approach of controlled drug delivery to particular areas of inflammation which is recognized as a useful and effective approach for improving treatment compliance and producing enhanced therapeutic efficacy with fewer side effects in the treatment of CRC. Polymeric micro particles refer to tiny, free-flowing particles formed from synthetic or non-synthetic polymers with magnitudes sizing from 1 - 1000 µm (Rashid *et al*., 2016 and Wasay *et al*., 2022).

Polymeric micro particles with the aim of controlled release of drugs can be fabricated using a diversity of techniques. Most generally, solvent evaporation techniques with simple or multiple emulsion approaches, such as oil-in-oil (O/O), oil-in-water (O/W) and water-in-oil-in-water (W/O/W), are utilized, which mainly depending on the drug's solubility profile (Hombreiro-Perez *et al*., 2003). The oil-in-oil (O/O) ESE technique was used in the current study. ESE is recommended because it is simple to make, does not necessitate the use of severe processing conditions and does not affect drug action. However, the oil-in-oil (O/O) ESE technique is mostly applied to microencapsulate drugs that are hydrophobic and can dissolve in the dispersion phase (Maqbool *et al*., 2020).

The goal of this research was to prepare MEL-loaded HPMC micro particles by an oil-in oil (O/O) ESE technique, considering the possibilities of improving MEL solubility, bioavailability and lessening its side effects with controlled-release delivery in the colon regions by the use of two different polymeric concentrations. Physiochemically characterization *in vitro* and *in vivo* studies of the MEL, and developed micro particles were performed. Our current contribution takes a step forward in our understanding of biocompatible internal delivery systems for hydrophobic therapeutic agents by providing detailed information on the encapsulation and physicochemical properties of the micro particles that are designed to function as oral DDSs. We already have published one paper in PloS one as first part and this one is the second part.

MATERIALS AND METHODS

Chemicals

Each chemical and reagent mentioned were procured from commercial sources and used as stated by the manufacturer's guidelines. Meloxicam and HPMC were gifted by English pharmaceutical (Pvt) Ltd, Lahore, and Martin Dow Marker Ltd, Quetta, (Pakistan), respectively. Methanol and Acetonitrile (HPLC grade), Ethanol, Dichloromethane, n-Hexane, liquid paraffine (Merck, Darmstadt, Germany), and Acetic acid (VWR Chemicals France).

Method

Preparation of MEL loaded HPMC micro particles

The oil in oil (O/O) / ESE technique was used with slight changes to fabricate micro particles. Two types of micro particles (formulation F1 and F2) were prepared using various drug-to-polymer ratios, such as 1:1 and 1:2.5 which can preserve different drug release profiles (as fast and slow-release). HPMC 200 mg for F1 (fast-release) and 500mg for F2 (slow-release), were dissolved in ethanol-dichloromethane (1:1) solution and stirred at 250 rpm with the help of a magnetic stirrer (Velp Scientifica, Usmate (MB), Italy). Then, in this polymeric solution, thoroughly dispersed 200mg of MEL with magnesium stearate (100 mg) as a stabilizer of droplets, as summarized in table 1. In a 250ml beaker, an external phase was created by adding 50 ml liquid paraffine and 1 % span-80, as a surfactant. Finally, the external phase was carefully introduced to the internal phase and constantly swirled with a tri-blade propeller (Eurostar IKA, WERKE) at 1000 rpm for 4-5 hours or till the organic solvent was evaporated completely. The fabricated micro particles were collected, pour out and filtered using Whatman No.42 filter paper, then washed 4-5 times with n-hexane (35 ml) to remove liquid paraffin and dried for 24 hours at room temperature (Maqbool *et al*., 2020).

Characterization

Fourier Transform Infrared Spectroscopic (FTIR) analysis

A Fourier Transform Infrared spectrophotometer (Bruker, Tensor 27, Germany) was employed in the range of scanning i.e., 4000-400cm^{-1} to test the compatibility of MEL, polymer (HPMC) and MEL loaded HPMC micro particles (formulation F1 and F2). The analyzing sample was located on the disc and the plunger was correctly limited by rotating the arm for sufficient contact with the sample, the scanning of the sample was done in16 seconds (Kashif *et al*., 2017).

Thermal stability analysis

Differential Scanning Calorimetry (DSC), (LAB KITS-100, Hong Kong) was used to measure the temperature of MEL, polymer (HPMC) and MEL loaded HPMC micro particles (formulation F1 and F2). 8±0.1mg of the test sample was deposited on an aluminum pan heated to 30-300°C at a flow rate of 20mL/min. The purging gas was nitrogen, while the standard gases were indium and zinc (Montejo *et al*., 2010).

X-Ray Diffraction (XRD) analysis

X-ray diffractometry (JEOL, JDX-3532, Japan) under 30 mA and 35 kV working conditions was used to analyze the MEL's diffraction patterns, polymer (HPMC), and MEL loaded HPMC micro particles (formulation F1 and

F2). In the range of 5-70, the samples of the test were evaluated at the rate of 2θ /min. In the end, the obtained data were assessed and compared to see the peaks, where they were located and how they were shifting (Abadi *et al*., 2020).

Particle size determination

The particle sizes were determined using an image optical magnifying device (Eclipse E200-LED, Nikon, Tokyo, Japan) and micrometers. The stage micrometer was used to calibrate the eyepiece micrometer. The slide was created by smidgin a small quantity of samples over a glass slide. To determine particle size, the prepared slide was examined with a ×10 lens (Kashif *et al*., 2017). With the assistance of the following mathematical statement, the mean particle size was calculated as:

$$\text{Mean particle size} = \frac{\text{Sum of diameter of observed particles}}{\text{Number of observed particles}}$$

Scanning Electron Microscopic (SEM) analysis

The MEL loaded HPMC micro particle's surface morphology and shape were examined using an SEM (JSM5910, JEOL, Tokyo, Japan). The test sample was taped to metal stubs for SEM measurements with dual-sided adhesive tape. In a vacuum chamber, the sample was dried before being sputter-coated with gold and inspected at various magnifications with a high-resolution scanning electron microscope (Montejo *et al*., 2010).

In vitro study of drug release

The in vitro release behavior of pure MEL and MEL loaded HPMC micro particles (formulation F1 and F2) was studied using a Type II USP dissolving apparatus (Pharma test Hainburg, Germany) at 50 rpm and 37˚C± 0.5 temperature. A gradual pH adjustment strategy was adopted and dissolution mediums with pH 1.2, 6.8 and 7.4 were utilized for 2, 10 and 12 hours, respectively. Weigh micro particles equivalent to 7.5 mg of MEL and delivered to a dialysis membrane (12-14 KDa) (Medicell Membrane Ltd, UK). It had been steeped in release media for over 12 hours previously. The dialysis membrane's open ends were secured with two clamps and plunged in pH 1.2 simulated gastric medium for 2 hours. After that, the pH 1.2 simulated gastric medium was replaced with two Phosphate buffers pH 6.8 and 7.4 for 10 hours and 12 hours, respectively. 3 ml of dissolving medium was drawn out at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20 and 24 hours intervals, followed by the addition of an identical volume of new dissolution medium to keep the sink conditions acceptable throughout the analysis (Patel, 2017). Each sample was filtered using Minisart (Sartorius Stedim Biotech GmbH, Germany) syringe filters with a pore size of 0.20µm. A UV-Vis spectrophotometer (IRMECO Gmbh, Gaeltacht, Germany) was used to measure at λ_{max} 362 nm the concentration of MEL in micro particle and this was then examined with the DD-Solver software (Microsoft Add-In).

Pharmacokinetic assessment

Equipment and HPLC Conditions

An HPLC technique for MEL assessment was built using reversed-phase High-performance liquid chromatography (RP-HPLC) (Syknm, Germany) with Isocratic mode, a solvent supply system S-1122, a UV/visible detector S-3210, and integrated with the Peak329 controlling software. The separation was performed on the C_{18} column (Phenomenex, RP-$C_{18,}$ Luna 5µm, 250 × 4.6mm of internal diameter). The mobile phase for MEL elution was made up of acetonitrile, water, and acetic acid (55:40:5). The mobile phase was finally passed through a Millipore vacuum filtration system using a membrane filter paper (DURAPORE® MEMBRANE FILTERS-IRELAND), of 0.45 µm. The solvent system was run with a flow rate of 1 ml/min. A sample solution (20-µl) was injected through the sample injector port and detected at a wavelength of 254 nm. The mobile phase was degassed by a sonicator (Elma, Transsonic Digitals, Germany). A digital weighing balance (Shimadzu, Kyoto, Japan), a centrifuge machine (Sigma-Zentrifugen, Osterode, Germany), and a vortex mixer (MS2 minishaker IKA, Germany) were also used in this research work.

Method Validation

Animals

The protocol for this study work was evaluated and approved by the Faculty of Pharmacy and Health Science's Institutional Animal Ethical Committee (IAEC), University of Balochistan (UOB), Quetta, Pakistan (Ref letter NO. FoP & HS/ICE/213/20, dated 20-11-2020). In this experiment, nine rabbits weighing between 2.0 and 2.5kg were used. The animals were housed in a transitory room condition i.e., temperature 23±2°C, humidity 45%, and lighting 12-hour day/night cycle. The rabbits were fed nutritious feed, given water ad libitum, and correctly tagged for identification. They all were kept in cages during the process of dosing and sampling. The rabbits were separated into three groups, each with three animals (n = 3), and orally given a dose comparable to MEL 2mg/Kg body weight after overnight fasting (Patel, 2017), as stated below:

Control group/ Group I: Pure MEL
Test group I/ Group II: MEL loaded HPMC micro particles (F1)

Test group II/ Group III: MEL loaded HPMC micro particles (F2).

Plasma extraction

Blood samples were collected from rabbits via jugular vein puncture. The animals' hair was removed with depilatory lotion before sample collection. Wooden rabbit carriers were used to house the rabbits, and blood samples of 3 ml were obtained in EDTA tubes at specified intervals using disposable sterile syringes.

The collected samples of blood were centrifuged for half an hour at 6,000 rpm. Earlier to testing, the plasma was held at a temperature of -20°C. Before being employed in the dilutions, the subzero plasma was heated to ambient temperature to be converted to a liquid. As a protein precipitating agent, an equivalent amount of acetonitrile (1:1) was inserted at this point. Before centrifugation, the final solution was vortexed for 4–5 minutes (Abadi *et al*., 2020). In a separate, clean Eppendorf tube, the supernatant was collected and labeled.

The samples were filtered using Minisart (Sartorius Stedim Biotech GmbH, Germany) syringe filters with a pore size of 0.20µm, before being injected into the HPLC system. To acquire accurate findings, the system and column were purged with a solvent system for almost half an hour.

MEL quantification and pharmacokinetics profiling

The PK Solver software (Microsoft Excel Add-In) was employed to assess the pharmacokinetic parameters of MEL in the selected groups (n = 3) of rabbits. The time to achieve peak concentration (T_{max}), area under the curve (AUC), the maximum concentration in plasma (C_{max}), mean residence time (MRT), and half-life ($t_{1/2}$), was computed through the log-linear trapezoidal scheme using plasma concentration and time curve.

Validation of Method

A thorough validation technique was designed following ICH guidelines to determine the levels of MEL in plasma (Q8, 2005). The calibration curve was built using the MEL in plasma to examine linearity, precision, accuracy, specificity, the limit of detection (LOD), as well as the limit of quantification (LOQ).

Linearity and the Standard Curve

A calibration curve was created by plotting the mean peak area versus MEL concentration. This approach's linearity was determined by testing it at various concentrations and the peak area was plotted against the estimated concentrations of drugs. The correlation coefficient, slope, and intercept parameters were calculated using the least-squares linear regression technique.

Specificity

The specificity was determined by separating the MEL peak from other undesired peaks such as plasma proteins and other solvent system components. Thus, both blank and spiked plasma selectivity was evaluated. A typical chromatogram was constructed to ensure that further analytes of the sample matrix could be isolated from the parent components. To get the maximum possible analyte resolution, rabbit plasma samples were carefully processed. Consequently, the proposed method produced excellent outcomes (Alvi *et al*., 2021).

Accuracy and Precision

The precision relates to how near the observed values must be in order for a procedure to be considered adequate. The precision is often articulated as a percentage of relative standard deviation (RSD %). The devised approach was more accurate when lowering the RSD value. The two most important precision parameters are intermediate precision and repeatability. Changing either of two variables resulted in intermediate precision, such as changing the day (inter-day) or employing a new instrument, whereas repeatability was obtained by making replicates on the same day (intra-day) with the same equipment. The intra-day variability of the method was studied utilizing repeat analysis of five samples collected on the same day. Similarly, the inter-day variance was also detected via repeat analysis of five samples of validation on five separate days. The degree to which outcomes represent genuine values is referred to as accuracy, and it is frequently mentioned as bias. Accuracy is calculated by comparing the observed mean values to the actual value and it is expressed as a percentage relative standard error (RSE %) (Bae *et al*., 2007).

Limits of Detection (LOD) and Quantification (LOQ)

The term "limit of detection" (LOD) relates to the lowest test sample concentration which can be distinguished by detection, whereas, the term “limit of quantification” (LOQ) refers to the lowest analyte concentration that can be effectively measured using a specific technique. LOQ was calculated using the minimal plasma spiking curve's concentration which gave an adequate level of precision and accuracy (20 percent)(Krier *et al*., 2011). Since LOD is lesser three times than LOQ, it may be determined simply by dividing LOQ by three.

Analysis Time

The total amount of time necessary for an analytical technique, and the number of chemicals or solvents used, are two significant elements in determining the total analysis time, they have an indirect impact on the cost of the analysis process. The run time might vary for each analyte which depends on its nature, but a typical run time from 0 – 15 minutes is for any method. The total run time of the current method has been decreased to 10 minutes. However, reduced run time can jeopardize an analytical process's precision and accuracy. (Alvi *et al*., 2021).

Robustness/Ruggedness

In an analytical procedure, robustness refers to assessing its ability to persist unpretentious by a minute but intentional modifications to method parameters, and it demonstrates its dependability in everyday use. Ruggedness is also recurrently used as a synonym for robustness. Thus, the chromatographic conditions were deliberately modified to investigate the effect on MEL separation. By 0.3 units, the flow rate has been raised. By altering the concentrations of all the solvents

(Acetonitrile, water, and acetic acid), investigate the effect of such changes on the solvent system. The effect of column age was also explored by comparison of responses from fresh columns to answers from columns that had been aged 30, 60 and 90 days, while all other parameters remained constant.

STATISTICAL ANALYSIS

The outcomes were provided as a mean accompanied by a standard deviation (SD). ANOVA (one-way analysis of variance) was performed to ascertain the concentration of MEL in the plasma of the control group and the developed formulations. SPSS Statistics v21.0 was used as a statistical tool. A significant difference was shown by the value of $p < 0.05$.

RESULTS

Characterization

FTIR analysis

As shown in fig. 1, the FTIR analysis was utilized to discover unique absorption peaks in pure MEL, HPMC, and formulations F1 and F2. The pure drug's absorption spectrum (fig. 1A) had typical peaks at 1345.95 cm^{-1} (-S=O), 1524.34 cm^{-1} (aromatic -C-C), 1621.87 cm^{-1} (-N-H-), 2910.05 cm^{-1} (C-H aliphatic) and 3291.43 cm^{-1} (-S-N-) (Shende *et al*., 2015). The HPMC spectrum analysis (fig. 1B) indicated that stretching of the O-H and C-O groups were attributed to the characteristic peaks at 3636.28 cm^{-1} and 1060.31 cm^{-1}, respectively and the bending vibration of the hydroxyl (-OH) group was attributed to the peak at 1384.29 cm^{-1}(Ding *et al*., 2015). Stretching vibration of C-H causes the peak at 2948.47 cm^{-1}. C=C stretching in the aromatic ring is responsible for another peak at 1637.94 cm-1 (Padmavathy *et al*., 2011).

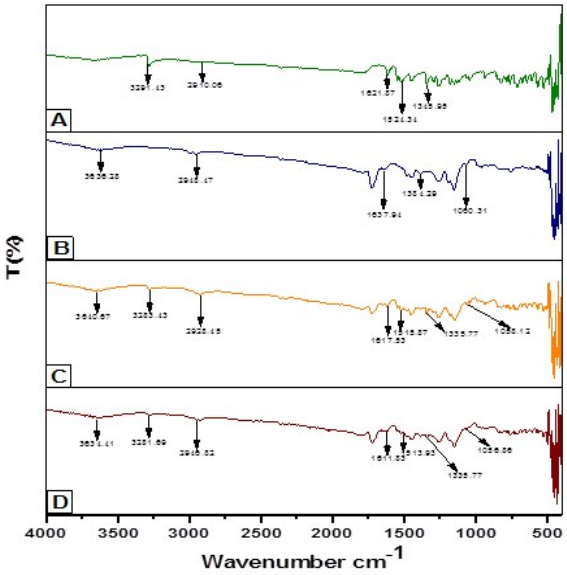

Fig. 1: The FTIR spectra of MEL (A), HPMC (B) and micro particles formulation F1 and F2 (C and D)

The spectra of micro particles in formulations F1 and F2 can be seen in figs. 1C and D. In formulations F1 and F2, the meloxicam characteristic C-C and N-H bond shifted to 1515.87 cm^{-1}, 1513.93$cm^{-1,}$ and 1617.53cm^{-1}, 1611.83cm^{-1}, respectively. The -S=O stretching absorption band was seen in the spectra of both formulations at 1335.77 cm^{-1}, while the -S-N- stretching absorption band was observed with slight shifting in the spectra of formulations F1 to 3283.43 cm^{-1} and F2 to 3281.68 cm^{-1}. The peaks in both formulations were detected with a slight shift in wavenumber, indicating that the drug and polymer in the microparticles did not interact. These findings were in line with previously recorded available data (Maqbool *et al*., 2020).

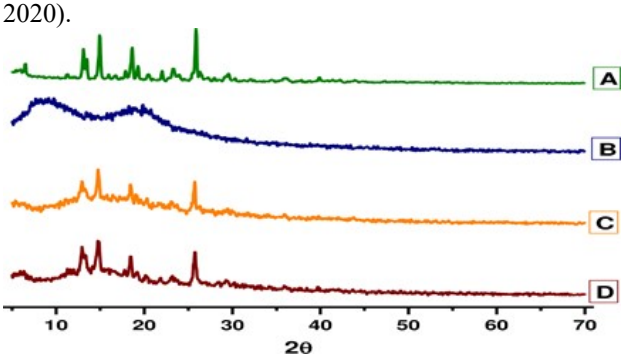

Fig. 2: XRD pattern of MEL (A) HPMC (B) and its micro particles, formulation F1 and F2 (C and D)

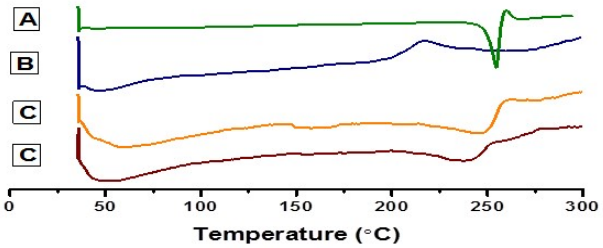

Fig. 3: DSC curves of MEL (A) HPMC (B) and its micro particles formulation F1 and F2 (C and D)

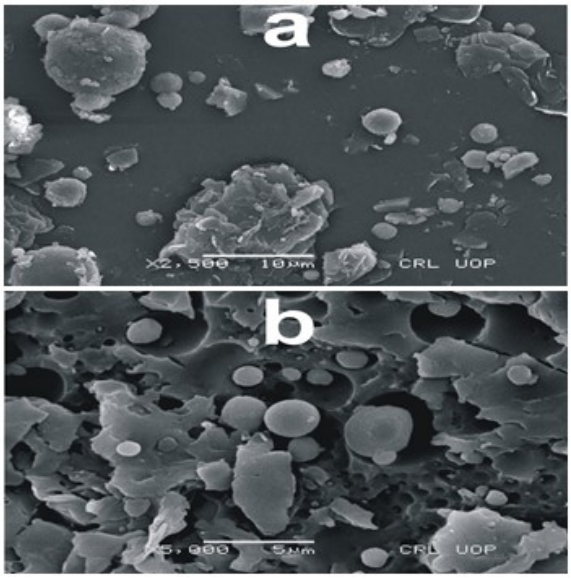

Fig. 4: SEM micrograph of formulation F1 (a) and F2 (b).

Table 1: Summary of microparticles formulations

Formulation code	MEL (mg)	HPMC (mg)	Droplet stabilizer (mg)	Surfactant (%)	Swirling speed (rpm)
F1	200	200	100	1	1000
F2	200	500	100	1	1000

Table 2: The HPLC developed method's intra-day and inter-day precision and accuracy.

Intra-day measured concentration (n = 5)					
S. No	Parameters	Nominal concentration (μg ml^{-1})	Mean concentration (μg ml^{-1}) ± SD	Precision (RSD %)	Accuracy (RSE %)
1	LQC	0.25	0.248 ± 0.015	6.04	99.2
2	MQC	12.00	11.78 ± 0.278	2.36	98.17
3	HQC	20.00	19.79 ± 0.749	3.78	98.95
Inter-day measured concentration (n = 5)					
1	LQC	0.25	0.246 ± 0.017	6.91	98.4
2	MQC	12.00	11.76 ± 0.418	3.55	98.00
3	HQC	20.00	19.71 ± 0.930	4.72	98.55

Table 3: Comparison of MEL, F1, and F2 mean values for several pharmacokinetic parameters

S. No	Pharmacokinetic parameters	Units	Dose (2mg/Kg body weight)		
			Group-I	Group-II	Group-III
			Pure MEL (Control)	Formulation (F1)	Formulation (F2)
1	C_{max}	μg/ml	7.21 ±0.87	25.41 ±1.02	22.38 ±1.39
2	T_{max}	h	6 ± 0.91	8 ±0.76	8 ±0.84
3	$AUC_{0-\alpha}$	μg/ml*h	116.034 ±12.34	445.95 ±9.49	462.72 ±6.55
4	MRT	h	21.61 ±4.72	27.08 ±2.13	29.38 ±6.29
5	$t_{1/2}$	h	19.98 ±0.97	22.91 ±1.70	24.75 ±2.56

* When values were compared with pure drug, a significant variation ($p < 0.05$) was found with formulations.

XRD analysis

X-ray diffraction analysis is a popular method for assessing a material's crystallinity. The XRD conduct of pure MEL, HPMC, and the produced micro particles (F1 and F2) are shown in fig. 2. XRD analysis indicated the pure drug's crystalline structure (fig. 2A), with unique peak values that are sharper and less diffuse at 13.17°, 15.06°, 18.49° and 26.07°, which are quite analogous to the previously reported peaks of MEL by Kifayat Ullah Khan and fellow workers (Khan *et al*., 2021). HPMC had a broad unique peaks at 2θ of 5-20° on the XRD diffractogram (fig. 2B), which corresponded to its amorphous state (Jafari *et al*., 2021). The reduction in peaks intensities of MEL in the formulations (fig. 2C and D), in comparison to that of pure drug, indicated that the MEL was effectively encapsulated in the amorphous system of micro particles. This was due to the amorphous structure of produced micro particles containing hydrophilic polymers, such as HPMC, which endowed drug with its properties. The production of micro particles that lower the crystallinity of drug could aid in its enhancement of solubility and dissolution.

DSC analysis

DSC analyses were carried out to better understand the drugs, polymers and prepared micro particles' thermal behavior. MEL, HPMC, and MEL loaded HPMC micro particles (formulation F1 and F2) thermograms are revealed in fig. 3. According to DSC analysis, the pure MEL was found in a crystalline state with a prominent endothermic peak at 259°C, which is linked to his melting point, as revealed in fig. 3A, The HPMC thermograph showed both endothermic besides exothermic peaks at 45°C and 218°C, respectively, which related to the melting point and decomposition temperatures of the polymer, as shown in fig. 3B. The prominent endothermic peak of the pure MEL vanished in the formulations, indicating the production of a homogenous and uniform mixture with perfect miscibility of the micro particles constituents (Issa *et al*., 2013). However, figs. 3C and D indicate that there was no interaction between the components of the formulation due to the lack of any prominent peaks appearance in the formulations (Sheikh *et al*., 2021).

Particle size

The particle size in F1 was the smallest (92.43 μm), while in F2 was the biggest (163.26 μm). When the polymer concentration was increased from 200 to 500 mg, the mean particle size of HPMC-loaded microparticles increased significantly ($P<0.05$). Because the system's viscosity has grown, dispersion has become more difficult, resulting in larger emulsion droplets and particle size (Kashif *et al*., 2017).

Surface morphology

The SEM technique was employed to evaluate the micro particle's morphology. Fig. 4 a and b, shows SEM images of micro particles. Its findings revealed that MEL-loaded HPMC micro particles have a smooth shape with distinct and chiseled porosity. The micro particle's amorphous nature with a micro-sized diameter resulted in its smooth surface.

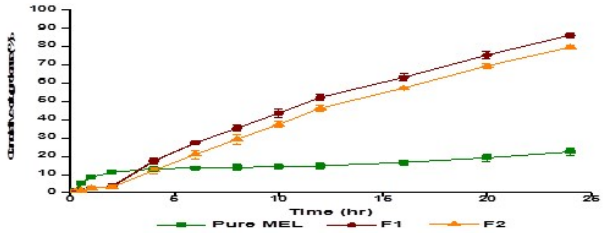

Fig. 5: MEL, formulation F1 and F2 in vitro dissolution experiments in buffers pH 1.2, 6.8, 7.4 at 37 ˚C ±0.5.

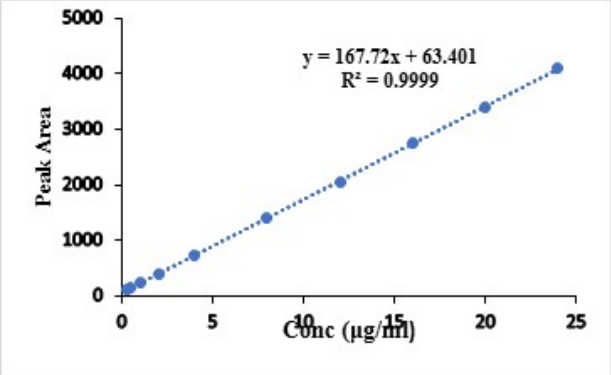

Fig. 6: MEL standard curve in the varied concentration of rabbit blank plasma

In vitro drug release study

An in vitro controlled release study of MEL loaded HPMC micro particles was carried out in a sequential pH shift technique using dissolving mediums pH 1.2, 6.8 and 7.4 for 2, 10 and 12 hours, respectively, and maintained at 37 °C ± 0.5, to explore the influence of MEL release from HPMC micro particles. Pure MEL had a cumulative drug release rate of about 22.4%, due to its hydrophobic nature (Avdeef, 2012). Micro particle development was used to get around this problem. The cumulative drug release (%) of micro particles was significantly more than that of pure drugs, which could be attributed to the drug's crystalline nature being converted to an amorphous state, improved surface area, and particle size (Katona *et al*., 2020). In any of the formulations, MEL release behavior reveals no burst effect, indicating uniform drug dispersion. In fig. 5, the cumulative release of pure MEL and formulations (F1 and F2) prepared with various ratios of HPMC was compared, and it was discovered that the cumulative release decreased as the polymer content increased. Formulation F1 has a higher drug release rate (86.19%) due to its low HPMC ratio (1:1), compared to formulation F2, which has a lower drug release rate (79.57%) and a high HPMC ratio (1:2.5). When the density of a polymer increases with a higher concentration of polymer, it increased the diffusional path length that ensuing in a diminution in the total release of the drug (Sharma *et al*., 2015).

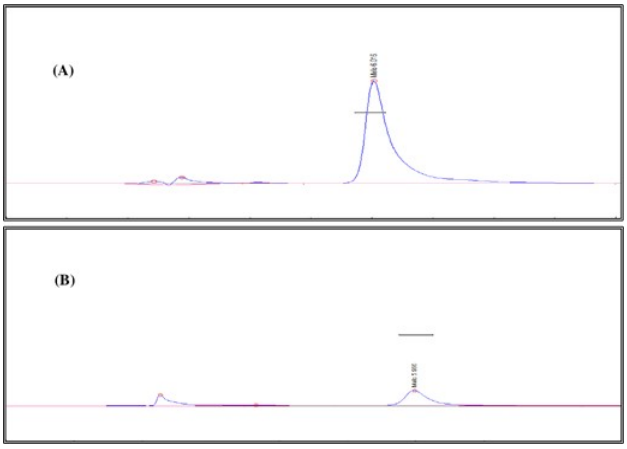

Fig. 7: MEL chromatogram without rabbit plasma (A), and MEL chromatogram with rabbit plasma (B)

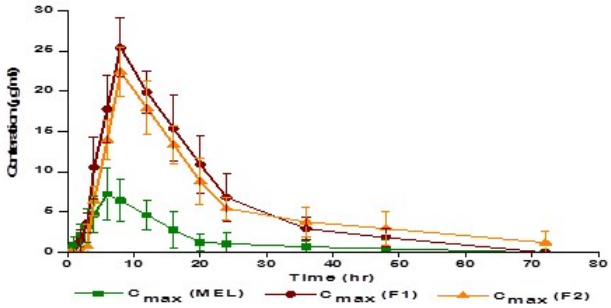

Fig. 8: A comparison of pure MEL, F1 and F2" after oral administration in plasma concentration versus time profile.

In vivo pharmacokinetic analysis

Method Development

The development of a technique is the first step in the development of any chromatographic method. The goal of development is to achieve high levels of sensitivity, accuracy, precision, resolution and repeatability. During the method's development, different C18 columns with particle sizes of 5µm were tried, such as Phenomenex RP-C18, Luna 5µm (100 × 4.6mm), Phenomenex RP-C18, Luna 5µm (150 × 4.6mm) and Phenomenex RP-C18, Luna 5µm (250 × 4.6mm). Moreover, varied ratios of organic and aqueous components with ACN, water and acetic acid were used to test some mobile phases. Following extensive testing, the column Phenomenex RP-C18, Luna 5µm (250 × 4.6mm) was chosen for HPLC analysis. Furthermore, a mobile phase prepared of acetonitrile, water and acetic acid (55:40:5) was shown to be best in terms of retention time, peak form and sensitivity. The method was also tested at a flow rate in a series of 0.5-1.5 ml min^{-1} and then a 1 ml min^{-1} flow rate

was selected because it provided the best and most consistent separation time. Although the overall analysis time was kept at 10 minutes, the MEL retention time was determined to be 5.55-6.2 minutes in this approach (Chaudhari *et al*., 2020). The pressure was set at 110-130 bars, and ambient temperature was chosen throughout the experiment,

Plasma Calibration Curve

In spiking plasma at specified concentrations and dilution levels between 0.25 and 24µg/ml, a MEL calibration curve was created. The concentrations were plotted against the average area of repeated injections. As illustrated in fig. 6, linear regression was employed to construct a straight line. The values 63.401 and 167.72 were found to be the b-intercept and y-intercept, respectively. The linearity coefficient (R^2) was 0.999, showing that the approach performed linearly across the selected range.

DISCUSSION

Working Range

The method's working range is defined as the greatest and smallest concentrations of analyte where the system response remained linear, precise, and accurate. The method was discovered to be linear across the specified concentration series of 0.25 - 24 µg/ml of MEL within the plasma, hence this series has been selected as the working series of MEL within the rabbit's plasma (Alvi *et al*., 2021).

Isolation and Selectivity

In less than 6.3 minutes of total run time, MEL was successfully separated from rabbit plasma. Lacking endogenous plasma peak formation, MEL exhibited a retention time of about 6.2 minutes. At optimal chromatographic conditions, the separation of MEL from plasma was decent. The characteristic chromatograms of MEL without and with plasma were shown in fig. 7.

Limits of Detection (LOD) and Quantification (LOQ)

To establish the LOD and LOQ of MEL, the sensitivity of the sample extraction can be tested using the HPLC method. The LOD of the sample was found to be 0.076 µg, although this amount of MEL could be determined, and it could not be quantified. As LOQ the amount of 0.228 µg was determined, indicating that the lowest amount of MLX could be identified and quantified.

Precision and Accuracy

For accuracy at intra-day fluctuation, five repeats sample of each control concentration, such as LQC (0.25µg/mL), MQC (12µg/mL), and HQC (20µg/mL) were formed. For precision at the inter-day level, Five LQC, MQC and HQC repeat samples were created. The dependability and validity of the developed method are supported by table 2, which shows that biological fluids have greater than 99 percent accuracy. When employed under specified conditions, the results of this method's intra-day and inter-day precision demonstrated that it was repeatable and would yield consistent results. For intra-day levels, RSD (percent) values for LQC, MQC, and HQC were recorded 6.04%, 2.36% and 3.78 %, whereas for inter-day levels were recorded 6.91%, 3.55% and 4.72%, respectively. According to the data, the developed method generated very precise findings. The developed method's accuracy was in the series of 98.00 - 99.2%, showing consistent outcomes. The intra-day and inter-day precision and accuracy are sufficient to assert that the developed method for MEL quantification within the plasma is exact and dependable.

Robustness

The chromatographic settings were deliberately changed to study the impact of MEL separation. The flow rate was raised by 0.3 units. The solvent system's effect was investigated by increasing the volume of all solvents by 2 ml for the first two and 0.3 ml for the third one (ACN, water, and acetic acid), respectively. The column age impact was also investigated by comparing the answers of a new column to those of old columns after 30, 60, and 90 days while keeping all other variables constant.

MEL Pharmacokinetic Profile in Rabbit Plasma

To design and confirm or validate High-pressure liquid chromatography several characterizations were carried out such as isolation and selectivity, precision and accuracy, robustness as well as working range. In order to conduct the pharmacokinetic investigation, the detection and quantification limits were also tested. In three independent rabbit groups (n = 3), the average MEL concentration within plasma was achieved, after oral administration of pure MEL, MEL loaded HPMC micro particles (formulation F1 and F2), as revealed in fig. 8. As the statistical findings of the SPSS test demonstrated, there was a significant (<0.05) increase in the concentration of drugs of F1 and F2, as compared to the control group's plasma. table 3 compares the overall functioning and pharmacokinetic parameters of the pure drug. C_{max} refers to the greatest concentration of drug that arrives in the systemic circulation with its compact natural state after oral delivery. The C_{max} values for MEL, F1, and F2 are 7.21±0.87µg/mL, 25.41±1.02µg/mL and 22.38 ±1.39µg/mL, respectively. Both test formulations had significantly higher C_{max} values, which could contribute to improved pharmaceutical bioavailability. T_{max} is the time it takes for a medication to reach its maximum plasma concentration. T_{max} for MEL, F1 and F2 is 6±0.91 h, 8 ±0.76 and 8±0.84 h, respectively. The cause for this extended T_{max} could be the drug's-controlled release of test formulations as compared to the pure drug. AUC stands for the area under the plasma concentration curve. The curve was described as a zero-moment curve by

statistical moment theory. There are a few other methods, however, the trapezoidal rule is the most commonly used rule, that represented the active medicinal substance's bioavailability. It's also quite beneficial for comparing the relative efficacy of several pharmacological products. The mean (±SD) $AUC_{0-\alpha}$ for MEL, F1, and F2 were 116.034 ±12.34, 445.95±9.49 and 462.72±6.55µg/mL*h, respectively. The value of $AUC_{0-\alpha}$ were arranged in the following sequence F2 > F1 > MEL. The values of $AUC_{0-\alpha}$ for the test formulations were greater than the pure drug, indicating that they had a higher bioavailability. The acronym MRT stands for mean residence time. The MRT for MEL, F1 and F2 was 21.61±4.72, 19.08±2.13 and 29.38±6.29h, respectively. The half-life of drug plasma is a pharmacokinetic parameter that indicates how long it takes for a medication's concentration to fall to half of its initial value. The stability and circulation time of the delivery mechanism in the physiological environment were indicated by this value. MEL, F1 and F2 have half-lives of 19.98±0.97, 22.19±1.70 and 24.75±2.56h, respectively. The recorded half-lives of F1 and F2 were compared to that of MEL estimated values, suggesting that the test formulation will have superior control.

CONCLUSION

In the proposed study, we describe the successful fabrication of MEL loaded HPMC microparticle (formulations F1 and F2) using an oil-in-oil (o/o) ESE technique with a hydrophilic polymer as the matrix component in various ratios. The resultant microparticle was physicochemically characterized, and the mechanism of in vitro drug release was also investigated. The MEL was changed during the preparation process from a crystalline state to an amorphous state, as validated by DSC and XRD findings. Variations in polymer concentration have a substantial impact on particle size and cumulative drug release which retarded the MEL release rate in acidic medium and controlled the MEL release rate in basic media as compared to pure drugs. In addition, healthy rabbits were used in in vivo studies of control MEL, formulations F1 and F2, which revealed acceptable pharmacokinetics features, implying that fabricated microparticles could be good candidates for significantly increasing solubility. As a result, the efficacy of poorly water-soluble drugs has improved with desired controlled release in the colon region.

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