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Comparative Study of Chitosan and Carboxy Methyl Chitosan Nanoparticles in Mefenamic Acid Drug Delivery

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ABSTRACT

Nanoparticle based technologies improve the efficiency and speed of already existing processes.The larger size materials and reagents which are in reactive form, can be nanosized to give efficient output. Chitosan and the carboxy methyl chitosan nanoparticles were prepared by using solvent evaporation nanoprecpitation method, loaded with mefenamic acid, (anthranilic acid derivative) a poorly aqueous soluble drug. Mefenamic acid, if used as conventional dosage form, duration of action was 6 hours; when the drug loaded into Chitosan and the carboxy methyl chitosan nanoparticles the drug release profile was up to 26hrs. The particle size, entrapment efficiency, poly dispersity index, drug loading, drug release profiles of mefenamic acid were compared for both nanoparticles and it was observed that carboxy methyl chitosan nanoparticles released mefenamic acid more effectively than chitosan nanoparticles.

Keywords: Nanoparticles, Chitosan, Carboxy methyl chitosan, Mefenamic acid

1.0 INTRODUCTION

1.1 Nanoparticles

International Organization of Standardization (ISO) in 2008, defined nanoparticles as that all the Cartesian dimensions should be less than 100nm. ISO defined nano discs, nanoplates as two dimensional nano objects; nano fibers and nanotubes as one dimensional nano objects. As per the European Union commission in 2011, definition of nanoparticle is 'a natural, manufactured, incidental material containing particles, in an unbound state or as an agglomerate or as an aggregate and where of 50% of or more of the particles in the number size distribution one are more external dimensions in the size range of 1nm-100nm.

1.2 Nanodrug delivery system

Preparation of nanoparticles are generally applicable to the drugs which are poorly aqueous soluble, the solubility can be increased by size reduction process, this can be done by various methods like milling, high pressure homogenization, high temperature evaporation, vacuum deposition[1]. These nanonization methods are useful to increase the bioavailability of drug to the target site[2]. Nanosized molecules can target the drug to the specific site without affecting the normal cell line[3]. Nanoparticles surface is covered with polymers or ligands so that they can identify and conjugate with disease site and release the drug at a constant rate and can cure the disease[4].

Studies have been done on animal models in vivo and systemic cell culture in vitro reveals the toxicity of nanopaticles includes respiratory system, skin, lungs, brain, and reproductive toxicity[5]. Due to very less size of nanoparticles, possess more free energies, thus causing aggregation and agglomeration at targeted site. If these drugs are insoluble in biological origin (they may reach other than targeted site) may affect internal organs during their exposure[6,7]. Negative effects like new toxins and environment pollutants may be produced by nanotechnology process[8].

1.3 Chitosan

Chitin and chitosan have similar structure. Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan is produced commercially by deacetylation of chitin, this process is never complete. Chitin is soluble in glacial acetic acid, hydrochloric acid, nitric acid, perchloric acid, phosphoric and formic acid

after stirring at certain temperature[9,10], Insoluble in water and other organic acids and bases. Unlike chitin, chitosan is hydrophilic polymer. In chitosan reactive amino groups protonated in acids with pKa less than 6.2 makes chitosan soluble. Chitosan is biocompatible, immunoadjuvant, non-immunogenic, nontoxic, inexpensive, water soluble, stable after administration, applicable to a broad category of drugs[11].

Over years liposomes are used as drug carriers for targeted drug delivery. But these liposomes have poor reproducibility, stability and low drug entrapment activity. To overcome this problem polymeric nanoparticles have been prepared to get better stability profiles and reproducibility and are taken as alternative drug carriers[12].

Poor stability is major limiting step in the preparation of chitosan nanoparticles. To maintain stability, temperature, environmental factors can be maintained optimized and with a suitable stabilizer; chitosan structure can be changed with proper ionic or chemical compound[13]. Another limitation of chitosan nanoparticles is the poor solubility[14]. Unmodified chitosan nanoparticles can encapsulate some hydrophilic drugs. Modified chitosan nanoparticles can encapsulate hydrophobic drugs. So poor solubility is major problem for some drugs in preparation of chitosan nanoparticles[13]. In most of the cases, *in vitro* studies show efficient results but failed to show the same *in vivo*[15].

Finally, financial support to the industries as well as to the patients to be taken into consideration, due to expensive protocols to be followed in the preparation of NPs, and cost of nanomedicine in the markets[16].

1.4 Carboxy Methyl Chitosan (CMC)

Carboxy methyl chitosan is derivative of chitosan. Solubility is more when compared with chitosan. Chitosan solubility is very less at pH>6, but for the applications in preparation of pharmaceutical products, solubility plays major role and important factor. Chitosan derivative is prepared to increase the solubility. Chitosan is converted to carboxy methyl chitosan by direct alkylation process as these carboxy methyl moieties will change the properties of chitosan[17].This type of chemical modification by direct alkylation will add carboxy methyl groups to two–OH groups (primary& secondary alchohol) and one amino group[18].The extent of water solubility depends on degree of substitution with carboxymethylation. As chitosan is converted into Carboxymethyl chitosan, it will change its physical and biological properties like moisture retention, chelating, sorption, cell functioning, antioxidant, antibacterial, anti apoptotic etc[19]. Carboxymethyl chitosan is used in different drug delivery systems like in DNA drug delivery as permeation enhancer, pH responsive drug delivery. n-Carboxymethyl chitosan was first prepared and developed by Muzzarelli in 1982, it was tested, used as ingredient in cosmetic and biomedical field[20,21,22].

Figure1.1: Synthesis of carboxytmethyl chitosan

1.5 Mefenamic acid

Mefenamic acid is non-narcotic, non-steroidal anti-inflammatory drug. It is used in the treatment of arthritis, pain, migraine associated with heavy menstruation, pain after surgical operations. It is anthranilic acid derivative.
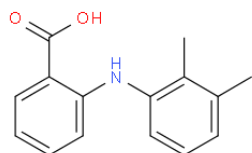

Fig. 1.2: Molecular structure of Mefenamic acid

It is Greyish white odour less, microcrystalline powder; Molar mass-241.285;Water solubility-0.004% at pH-7.1; Formula-$C_{15}H_{15}NO_2$; Chemical Name-N-2,3 xylylanthranilic acid; Melting point230^0-231^{0}C; Metabolism-Hepatic; Mechanism of action is not clearly known, like other NSAIDs, inhibits COX-1&COX-2, potent inhibitor of prostaglandin synthesis, analgesic, antipyretic, anti-inflammatory.

Dose required is 500mg followed by 250mg for every 6 hours, not exceeded for one week.

1.5.1 Pharmacokinetics of Mefenamic Acid

Administered orally, distributed in protein bound form, metabolised in liver, metabolites are excreted mainly in the urine. Onset of Action- 1-2 hrs; Duration of Action- 6 hrs; Half Life -2-4 hours.Side Effects are nausea, vomiting, anorexia, diarrhoea, gastrointestinal bleeding, abdominal distress , constipation, peptic ulcer, dyspepsia, headache, flatulence, insomnia, drowsiness, rash, aplastic anaemia, thrombocytopenia, dizziness; Contra-indicated in patients with hypersensitivity, inflammatory bowel disease, peptic ulcer, pregnancy, breast Feeding**,** below 14 years of age.

Special Precautions**-** Hepatic & Renal impairment, Hypertension, Gastrointestinal diseases, Pre-existing asthma, impairment, Myocardial infarction, Heart failure, Patient on anticoagulant therapy, Stroke

Old Age -Use with caution; Neonates- used only to close the patent ductus arteriosus as IV form only

Indications: Muscular aches, Primary Dysmenorrhoea, Headaches, Acute gout, Dental pain, Patent ductus arteriosus ; **Storage -** Store at room temperature in a tightly closed light resistant container.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Standard chitosan was purchased from Chemica and Biochemica. Mefenamic acid and other chemicals (chloroacetic acid, sodium hydroxide), solvents (2-propanol and methanol), reagents, buffers used were of analytical grade; purchased from standard manufacturers.

2.2 Synthesis of Carboxy Methyl Chitosan (CMC)

2 g of Chitosan suspended in 20ml of 50 % (w/v) sodium hydroxide was left swelling for 1 hour at room temp and kept for alkalization for 12 h at -20 °C, then thawed at room temperature. This alkali chitosan was suspended into 50ml of 2-propanol solution and the mixture was stirred on magnetic stirrer for 30 min and then in water bath shaker at 50°C. 10g of Chloroacetic acid dissolved in 30 ml of isopropanol was added drop wise over a period of 30 min. Reaction mixture was stirred for 12 hours in a water bath at 50°C. Then the liquid fraction was decanted and 100ml of methanol was added to the resulting slurry. The suspension was neutralized using glacial acetic acid. Then the mixture was filtered and washed several times with methanol. The resulting CMC was purified by dissolving in deionized water and filtered to remove undissolved residues. The resultant solution was precipitated in addition of methanol. Finally the pure product was separated by filtration, rinsed with methanol, vacuum freeze dried and stored in desiccators until further use.
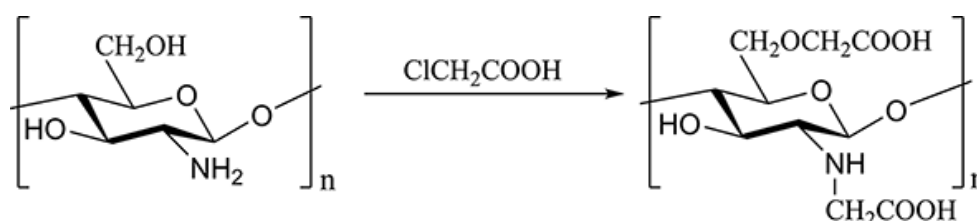

Figure-2.1- N- and O-carboxymethylation of chitosan

2.2.1 Characterization of CMC by degree of substitution

The degree of substitution of CMC was determined by potentiometric titration. CMC was dissolved in distilled water and the solution was adjusted to pH < 2 by addition of hydrochloric acid. Then, the CMC solution was titrated with 0.1M aqueous sodium hydroxide and the pH value of the solution was simultaneously recorded. The amount of aqueous sodium hydroxide was determined by the second order differential method. The degree of substitution (DS) was calculated as follows:

$$DS = \frac{(v2 - v1)DD}{(v3 - v2)}$$

V1=NaOH volume at pH-3, V2=NaOH volume at pH-3.8, V3=NaOH volume at pH-5, DD (degree of deacetylation of chitosan); Where **V**sodium hydroxide and **C**sodium hydroxide are the volume and molarity of aqueous sodium hydroxide, respectively; mCMC is the mass of CMC (g), and 161 and 58 are the molecular weight of glucosamine (chitosan skeleton unit) and a carboxymethyl group, respectively.

2.3 Estimation of Mefenamic acid

UV visible spectroscopic method for analysis of Mefenamic acid was adopted in present work. An accurately weighed quantity of Mefenamic acid (100mg) was dissolved in 100 mL of 7.4 buffers to generate a stock solution having concentration of 10^3 μg /mL. Stock solution (10 mL) was further diluted to 100 mL to produce standard solution having concentration of 10^2μg/mL. The standard solution was serially diluted with 7.4 buffer to get working standard solutions having concentration of 2, 4, 6, 8, 10 μg/mL. The absorbance of the solutions was measured at 285.0 nm using double beam UV visible spectrophotometer against 7.4 buffer as a blank.

2.4 Drug Excipient Compatibility Study

Drug and excipients were mixed and placed at room temperature for 24 hours. The presence of any drug-polymer interaction was studied by FT-IR spectroscopy.

Fourier-transform infrared spectroscopy

To examine possible chemical interactions of MFA-CS NPs and interactions of MFA-CMC NPs, Fourier-Transform Infrared Spectroscopy (FT-IR) was used. The IR spectra of Chitosan, CMC, MFA, MFA-CN NPs, MFA-CMC NPs were recorded using an FT-IR spectrophotometer (FT-IR Nicolet-380, Thermo Fisher Scientific, Madison, USA). Samples were mixed with potassium bromide (spectroscopic grade) and compressed into disks using a hydraulic press before scanning from 4000 to 600 cm^{-1}. Data were analyzed using FT-IR solution software (version 1.10).

2.5 Formulation of MFA-chitosan nanoparticles (MFA-CSNPs) and MFA-CMC nanoparticles(MFA-CMC NPs)

MFA-CSNPs and MFA-CMC NPs were prepared by solvent evaporation and nano-precipitation techniques. The influence of different independent variables as Chitosan concentration (%), tween 80 concentration (%) and sonication time (min) were evaluated on particle size (nm), drug loading (%) and in vitro drug release (%) were investigated.

Chitosan, CMC containing mefenamic acid-NPs were prepared using combined technique of solvent evaporation and nanoprecipitation technique with slight modification[23]. This was a two-step process, in the first step; emulsification of the polymer solution into aqueous phase containing a surfactant was done. Then in the second step evaporation of polymeric solvent was carried out, inducing polymer precipitation of the nanoparticles[24]. The calculated quantities of chitosan/Carboxy methyl chitosan and tween 80 and PVA were varied according to the experimental design. For the preparation of MFA-CNPs concentrations of chitosan (2%,3.5%,5%), tween 80(2%,4.5%,7%), sonication time (3min,5min,7min) were used as low, medium, high levels of independent variables. For the preparation of MFA-CMC nanoparticles concentrations of chitosan (2%,4%,6%), tween 80 (3%,5%,7%), sonication time (2min,5min,7min) were used as low, medium, high levels of independent variables. Chitosan was dissolved in an organic solvent acetone (10 mL) and separately tween 80 dissolved in

double distilled water. The organic solvent was added slowly to the aqueous phase containing tween 80 with a constant stirring on magnetic stirrer at room temperature. The evaporation of the organic solvent was performed at a temperature range of 65-80°C which involves precipitation process lead to formation of nanoparticles. The obtained nanoparticles were ultra sonicated for different time interval (3-7 min.) at 60-80 KHz amplitude) for 1 cycle and allowed to cool at room temperature. The developed MFA-CSNPs and MFA-CMC NPs were lyophilized using the freeze dryer at a chamber pressure (20pa) and cold trap temperature (-120°C) in the entire process. The study was performed for 24 h for freezing, 4 h for primary drying at 0°C, followed by 10°C for 2 h and 15°C for 1.5 h and secondary drying at 25°C for 3 h. Mannitol (3%) was added as a cryoprotectant to avoid lysis of nanoparticles[25].

2.6 Evaluation of Nanoparticles

2.6.1 Particle size and size distribution

The average particle size and size distribution(PDI) of mefenamic acid loaded nanoparticles of Chitosan and CMC were determined by dynamic laser scattering (DLS) technology using a Zeta sizer Nano ZS(Malvern instruments, Malvern, UK). The nano suspensions were suitably diluted with ultrapure water and sonicated for 2 min to form a uniform dispersion before placing the sample in quartz cuvette. The hydrodynamic diameter of particles was measured with a He-Ne laser at a scattering angle of 90°at 25°C. Each sample was determined three times and the obtained results were expressed as the mean size of particle ± SD.

2.6.2 Measurement of surface charge

The zeta potential of the nanoparticles was determined by an electrophoretic light scattering technique using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). A diluted sample of nanoparticles in water was allowed to stabilize at 25 °C and was placed in the clear disposable zeta cells. Zeta potential was obtained based on the electrophoretic mobility between the electrodes. The experiments were performed in triplicate and the results were expressed in millivolts (mV)

2.6.3 Surface morphology

The surface morphology of the nanoparticles was observed by scanning electron microscopy. Sample preparation was done by placing a drop of the nanosuspension on an aluminium stub. The particles were allowed to settle and the excess of the liquid in suspension sample was removed with a capillary. The sample was then coated with 30nm layer of gold using JEOL JFC 1100E sputter coater for 30s and viewed under SEM (JSM 840A, JOEL, Japan).

2.6.4 Entrapment Efficiency& Drug loading

Entrapment efficiency (EE%) and drug loading (DL%) of the developed MFA-CSNPs and MFA-CMC

NPs were determined by double beam UV Spectroscopy. The prepared nanoparticles were subjected to centrifugation at a speed of 10,000 rpm (Remi centrifuge, Mumbai, India) for 20min and the free drug content in the supernatant was separated and the separated NPs were solubilised in appropriate medium(methanol) and filtered through a 0.22 μm PVDF filter/ultra filter (Pall Life Sciences, Mumbai, India). The supernatant was collected and diluted with an appropriate solvent (methanol) to analyze using UV-spectrophotometer at 285 nm. Consequently, the EE% and DL% were calculated by the following formula:

$$EE\% = \frac{Total\ amount\ of\ drug - amount\ of\ free\ drug}{Total\ amount\ of\ drug} \times 100$$

$$Drug\ loading = \frac{Total\ amount\ of\ drug - amount\ of\ free\ drug}{Total\ weight\ of\ the\ nanoparticle} \times 100$$

2.6.5 Drug release Profile

The drug release study of chitosan and CMC nanoparticles was performed separately with activated dialysis bag technique (molecular weight 12000g/mol, Sigma, MO, USA) with slight modification from suggested methods. A known volume containing nanoparticles in both the formulation was placed in a dialysis bag, and both ends were tied to prevent any leakage. The bag was dipped in 250ml phosphate buffer saline (pH 7.4) as release media at 37 ± 2 °C with continuous stirring at 50 rpm[25].The release samples (1ml) were withdrawn at predetermined time interval and were filtered through 0.22μm PVDF Syringe filter. The amount of drug release was calculated by using UV-spectrophotometer at 285 nm using buffer (pH 7.4) a blank sample.
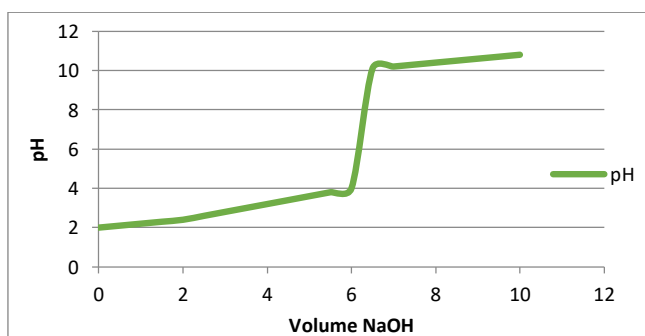
3.0 RESULTS

3.1 Results of synthesis and characterization of CMC

CMC was characterized by potentiometric titration. By this titration one can conclude that the percentage of carboxy methyl groups added to the chitosan.**Figure-3.1-** The potentiometric titration curves of four Carboxy methyl chitosan solutions. Since protonated chitosan was not considered in the study, there is no point after pH 7.0. It was clearly shown that the range of titration jumps were around pH 3.0–5.0 and the inflection point's are located at pH 3.80, thus this point was used as the titration end-point in the solution. The titration end-point pH 3.80 cannot be applied to all CMC samples because of the different origins of samples. In addition, the pH value responded from the composite glass electrode lags behind the pH true value and reagent blank error can affect the result of analysis[26,27].

$$DS = \frac{(V2 - V1) \times DD}{V3 - V2}$$

V1=NaOH volume at pH-3=5, V2=NaOH volume at pH-3.8=5.8, V3=NaOH volume at pH-5=6.8, DD (degree of deacetylation of chitosan)=0.64

Figure-3.1- Degree of Substitution of Carboxy methyl chitosan

### 3.3 Results of Estimation of Mefenamic acid

UV visible spectroscopic method for analysis of Mefenamic acid was adopted in present work. An accurately weighed quantity of Mefenamic acid (100mg) was dissolved in 100 mL of 7.4 buffer to generate a stock solution having concentration of 10^3 μg /mL. Stock solution (10 mL) was further diluted to 100 mL to produce standard solution having concentration of 10^2μg/mL. The standard solution

was serially diluted with 7.4 buffer to get working standard solutions having concentration of 2, 4, 6, 8, 10 µg/mL. The absorbance of the solutions was measured at 285.0 nm using double beam UV visible spectrophotometer **(Figure-3.2)** against 7.4 buffer as a blank. The plot of absorbance v/s concentration (µg/mL) was plotted (**Figure 3.3**) and data was subjected to linear regression analysis in Microsoft excel.

3.3.1 Preparation of standard calibration curve of Mefenamic acid in 7.4 buffer at 285 nm

Calibration curve of Mefenamic acid was developed in 0.1 N HCI at 285.0 nm wave length. Mefenamic acid in 7.4 buffer showed good linearity (r^2=0.990) and intercept 0.010 over the concentration range of 2-10 µg/ml at λmax 285 nm. The data for calibration curve are shown in **Table 3.3** and the calibration curve was shown in **Figure 3.3** .
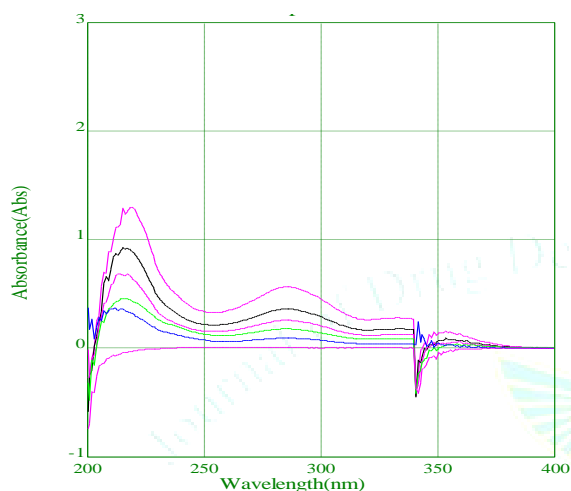
Figure 3.2 Spectra of Mefenamic Acid By UV-Spectroscopy

Table 3.1 Standard curve of Mefenamic Acid in 7.4 Buffer at 285 nm

| S.No.	Concentration(µg/ml)	Absorbance
1	2	0.1177
2	4	0.1764
3	6	0.2688
4	8	0.3684
5	10	0.4717

$y = 0.045x + 0.010$
$R^2 = 0.990$
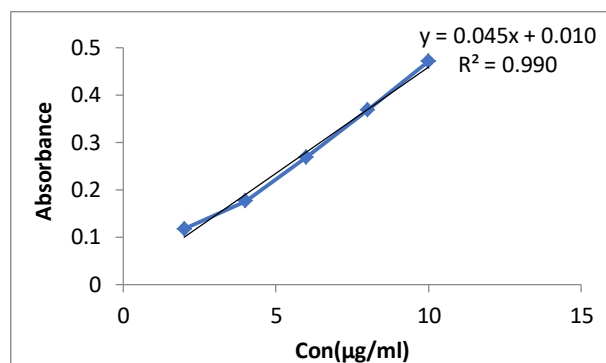
Figure 3.3- Standard curve of Mefenamic Acid in 7.4 Buffer at 285 nm

3.4 Results of evaluation of mefenamic acid loaded chitosan nanoparticles

3.4.1 FTIR Results of Mefenamic acid, Chitosan, MFA-CSNP

FT-IR spectroscopy was used to analyze the possible interactions between the drug and polymers used to form MFA loaded CSNPs. The FTIR spectra of MFA, Chitosan and CSNPs formulations were represented in **Figure-3.4** There are primarily determinant peaks of Chitosan around 3433 cm−1 (-OH and eNH₂ stretching), 2876 cm−1 (-CH stretching), 1594 cm−1 (-NH₂ stretching), 1396 cm−1 (-CN stretching), 1018 cm−1 (CeOeC stretching), and 615 cm−1 (pyranoside ring stretching vibration). In addition, in preparation of CSNPs, the peak of 3433 cm−1becomes wider, representing that hydrogen binding has been introduced in Chitosan in nanostructure. The peak of 1594 cm−1 for eNH₂ shifted to around 1572 cm−1and a new sharp peak at 1700 cm−1appeared, which can be explained by the interaction of NH₂ of Chitosan. Compared with the spectrum of MFA, in the spectrum of MFA/CSNPs, the absorption peak of about 1632 cm−1 (carboxyl group) shifted and a new shoulder peak of 1572 cm-1 (carboxyl salt) appears. It seems that hydrogen bonds formed between OH groups of the MFA and those of Chitosan and electrostatic interaction taken place between COO- of the drug and NH₃ þ of chitosan. Moreover, the increase in MFA concentration leads to appearance of MFA characteristic absorption peaks at high intensities. On the other hand, using a low MFA weight ratio in the NP (F1) caused the appearance of these characteristic peaks, but at low intensity. The results of FTIR are in agreement with previous studies.
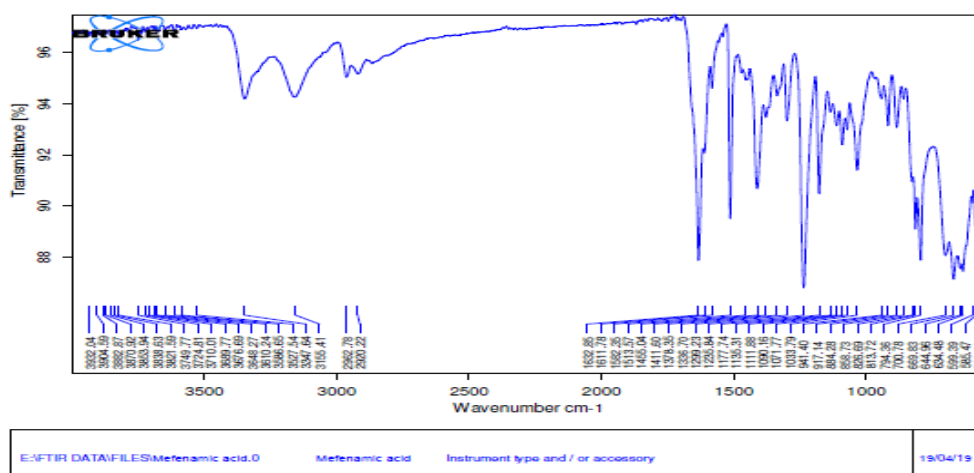
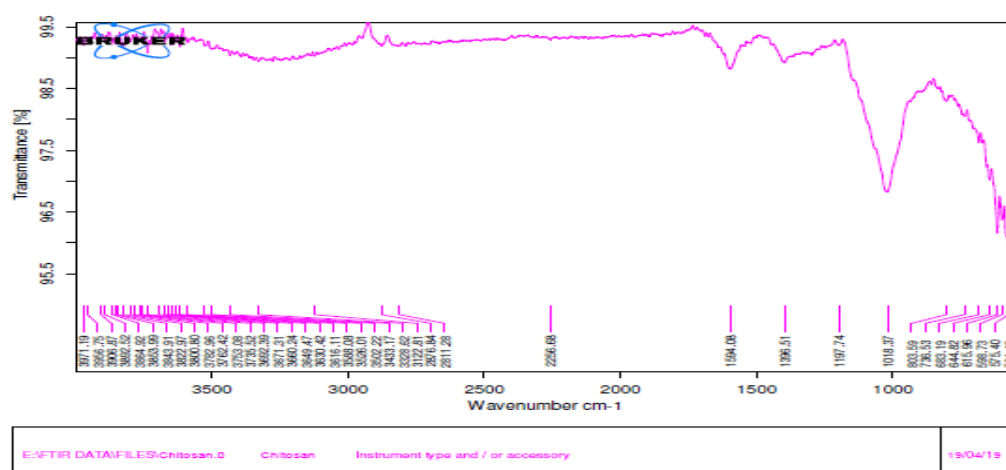**Figure-3.4.1- FTIR of Mefenamic acid**
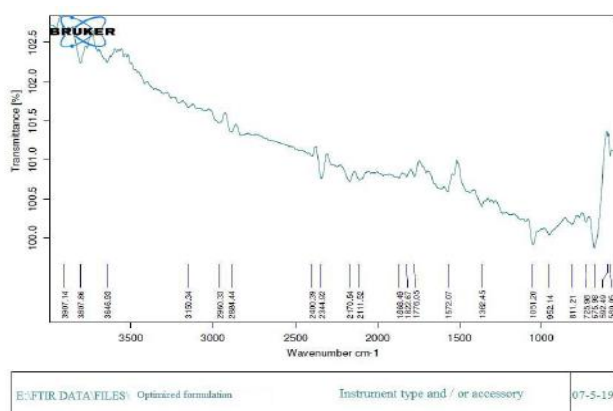
Figure-3.4.2- FTIR of Chitosan

Figure-3.4.3- FTIR of optimized formulation of MFA-CSNP

3.4.2 Effect of concentration and sonication time of chitosan on particle size, PDI, EE% and DL% of formulations of MFA loaded chitosan nanoparticles

The above results depicted the average size of different chitosan loaded nanoparticles and the corresponding polydispersity index (PDI) was shown in **Table 3.2.** The size

of ranged from 150 to 245 nm; Based on the EPR(enhanced permeability and retention) effect, the nanoparticles under 200 nm were the most suitable for penetrating the blood vessels into tumor tissue. All of the three Chitosan based nanoparticles under certain preparing conditions could meet this requirement with favourable PDI. For the nanoparticle stability the zeta potential should be between -25mV to +25mV. Zeta potential of chitosan MFA nanoparticle is found to be -1.8mV (**Figure-3.6**). The cumulative particle size is 1241.5 (**Figure-3.5**).

The results in **Table 3.2**, showed the interaction effect of chitosan concentration and Sonication time on the EE% and DL% of MFA loaded CSNPs. These result revealed that EE% varies from 44.58±3.30% to 82.65±1.16, F1 having high chitosan concentration and longest sonication time showed 82.65% Entrapment Efficiency due to cross linking of chitosan with drug. DL% varied from 3.93% to 8.14% This could be attributed to the binding of hydroxyl groups of MFA to positively charged amino groups on chitosan molecules by electrostatic interaction These results might be attributed to an increase number of interacting units at higher polymer concentrations and to cross-linker levels that lead to the observed increase in particle size and decrease entrapment efficiency

Table 3.2-Particle size of MFA loaded chitosan nanoparticles

Formulation	Chitosan	Sonication time	Particle size(nm)	PDI	EE%	DL%
F1	+1	+1	150 ±7.9	0.23±0.02	82.65±1.16	8.14±0.07
F2	0	+1	210±9.1	0.22±0.03	72.48± 0.45	7.29±0.03
F3	-1	+1	225±8.1	0.33±0.02	58.41± 0.85	5.86±0.08
F4	+1	0	187±8.9	0.24±0.01	72.10±1.21	7.24±0.20
F5	0	0	245±10.1	0.21±0.04	67.50±0.82	6.83±0.03
F6	-1	0	170±6.9	0.22±0.04	77.12±0.49	7.74±0.04
F7	+1	-1	175±7.9	0.24±0.03	63.19±0.83	5.93±1.06
F8	0	-1	164±9.9	0.22±0.01	44.58±3.30	3.93±0.77
F9	-1	-1	200±9.8	0.23±0.02	78.60±0.49	7.89±0.04

N=3   (Mean± SD)

Date	:	17 April 2019 18:41:33
Measurement Type	:	Particle Size
Sample Name	:	Chitosan Nanoparticles-Size
Scattering Angle	:	90
Temperature of the holder	:	25.0 deg. C
T% before meas.	:	21077
Viscosity of the dispersion medium	:	0.895 mPa.s
Form Of Distribution	:	|Standard|
Representation of result	:	Scattering Light Intensity
Count rate	:	1963 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	2495.8 nm	772.7 nm	2100.9 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	2495.8 nm	772.7 nm	2100.9 nm

Histogram Operations

Size (Median)	:	2335.9 nm
Mode	:	2100.9 nm
% Cumulative (1)	:	10.0 (%) - 1636.1 (nm)
% Cumulative (2)	:	50.0 (%) - 2335.9 (nm)
% Cumulative (3)	:	90.0 (%) - 3595.1 (nm)
% Cumulative (4)	:	30.0 (%) - 1987.7 (nm)
% Cumulative (5)	:	40.0 (%) - 2153.4 (nm)
% Cumulative (6)	:	50.0 (%) - 2335.9 (nm)
% Cumulative (7)	:	20.0 (%) - 1820.1 (nm)
% Cumulative (8)	:	70.0 (%) - 2786.5 (nm)
% Cumulative (9)	:	95.0 (%) - 4025.0 (nm)
% Cumulative (10)	:	100.0 (%) - 8510.6 (nm)

Cumulant Operations

Z-Average	:	1241.5 nm
PI	:	3.266
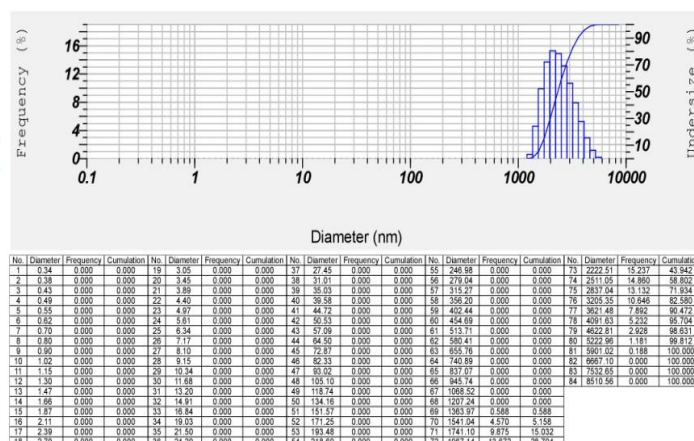
Figure-3.5- cumulative particle size of MFA loaded CSNPs

Date	:	17 April 2019 16:16:09
Measurement Type	:	Zeta Potential
Sample Name	:	Chitosan Nanoparticles-Zeta
Temperature of the holder	:	25.1 deg. C
Viscosity of the dispersion medium	:	0.893 mPa.s
Conductivity	:	0.147 mS/cm
Electrode Voltage	:	3.4 V

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-1.8 mV	-0.000014 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

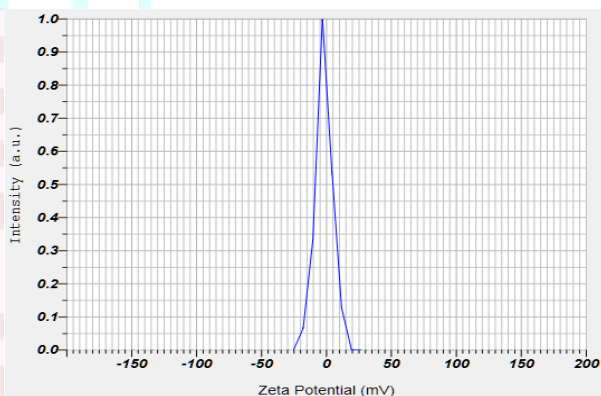|		
Zeta Potential (Mean)	:	-1.8 mV
Electrophoretic Mobility mean	:	-0.000014 cm2/Vs

Figure-3.6- Zeta Report of MFA loaded CSNPs

3.4.3 Morphological studies of MFA loaded chitosan nanoparticles

The morphological characteristics of Mefenamic loaded CSNPs were observed by SEM. The nanoparticles were found to be a roughly spherical in shape with a smooth surface (**Fig. 3.7**). The part of an aggregation of the CSNPs was probably because that the hydrogen bonding gradually becomes dominant in the drying process. It also was noticed that these CSNPs have a deeper colour in the core and the surface (white arrows), indicating that these regions have higher electron density distribution. The difference size of CSNPs in DLS and SEM might be attributed to that CSNPs swell in aqueous media. DLS gives a hydrodynamic diameter of nanoparticles, while SEM gives an actual diameter of nanoparticles in the dry state.
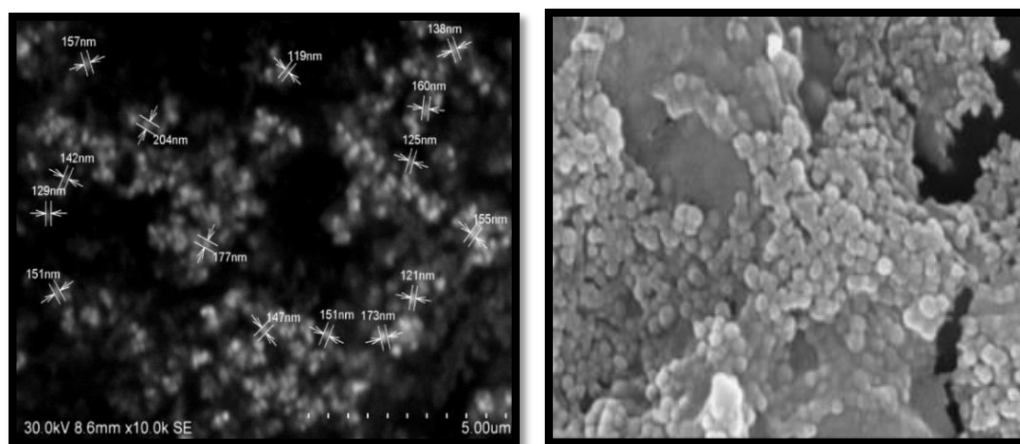
Figure: 3.7- SEM (Scanning Electron micrograph) of Mefenamic acid loaded chitosan nanoparticles

3.4.4   Drug release profile of MFA loaded chitosan nanoparticles (F1-F9 formulations)

The release profiles of MFA from the CS-NPs formulations compared to drug solution as a control were illustrated in **Figure-3.8.2** and **Figure-3.8.3.** In control 35.4% of MFA was released rapidly from the dialysis bag within the first 8 hour. Total drug 99.87% is released within 14hours. On the other hand, MFA loaded CSNPs showed a biphasic pattern with an initial burst drug release followed by a sustained release. Regarding CSNPs, MFA was rapidly released within the first 8 h, followed by a slow release from 8 hr up to 26 hr. The rapid MFA releasing was mostly due to the nanoparticles surface drugs, which could simply diffuse in the first 8 h. The cumulative percentage release of MFA from the CSNPs in the first 8 h were about 21%, 28%, 12%, 43%, 27%, 46%, 28%, 33% and 28% for F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10. The release profile of F1 is showing biphasic release having linear release kinetics after 8hr up to 26 hrs.
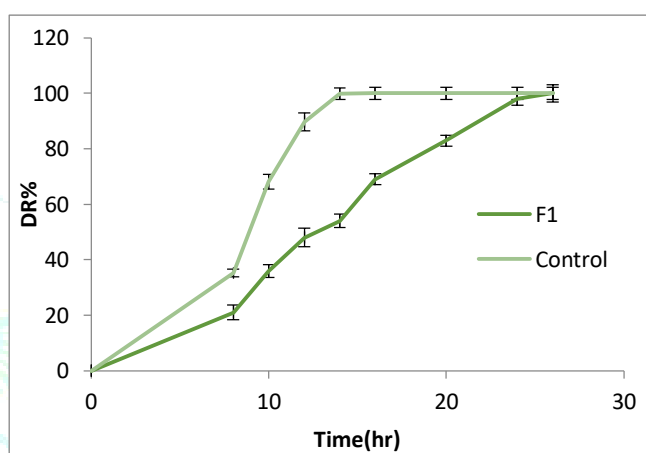

Figure-3.8.1-Drug Release Profile of Control (mefenamic acid) and F1-MFA-CSNPs

Table 3.3- Drug Release profile of MFA loaded Chitosan nanoparticles

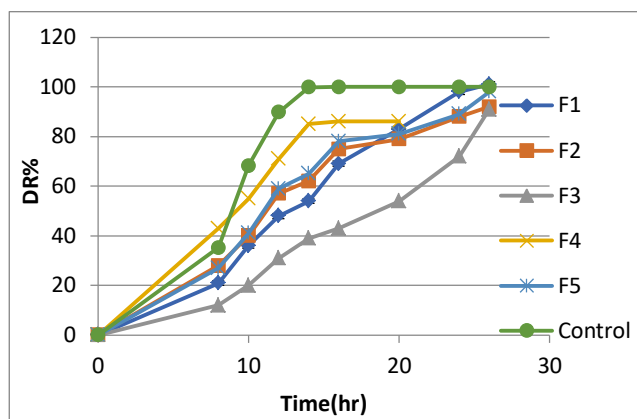| Time (h)	In-vitro Release %									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	Control
0	0	0	0	0	0	0	0	0	0	0
8	21	28	12	43	27	46	28	33	28	35.24
10	36	40	20	55	41	65	40	59	40	68.19
12	48	57	31	71	59	79	57	78	57	89.77
14	54	62	39	79	65	83	62	83	62	99.87
16	69	75	43	89	78	91	75	89	69	
20	83	79	54	99.13	81	100	79	96	73	
24	98	88	72		89		88	100	88	
26	101	99	91		98		99		91	

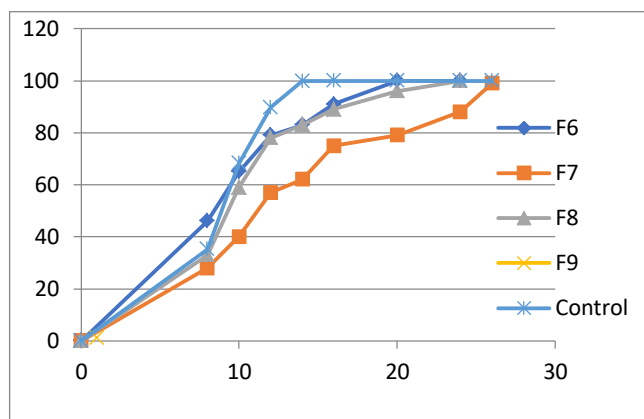**Figure-3.8.2- drug release profile of Control, F1-F5 MFA loaded CSNPs**

Figure-3.8.3- drug release profile of Control,F6-F9 MFA loaded CSNPs

3.5 Results of evaluation of MFA loaded CMC nanoparticles

3.5.1 FTIR results of MFA loaded CMC nanoparticles

Figure-3.9 showed the representative FT-IR spectrum of CMC NPs In the infrared spectra, an interesting characterization peak was in the range of 2700 – 3000 cm-1, indicating the hydrogen bonding. The hydrogen bonding in CMCS polymer was at 2893cm-1 and 2800cm-1 respectively, and they shifted to 2989 and 2764 cm-1 after MA was encapsulated, The vibration peaks of 1598 – 1410 cm-1, corresponding to amide I and II bond, had no obvious shift in all formulations. The vibration peak at 1404 cm-1 in CMCS could be assigned to the symmetric stretching.
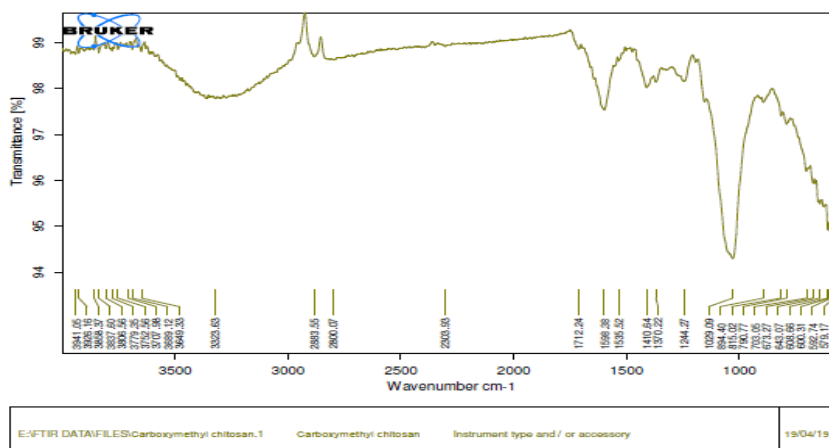

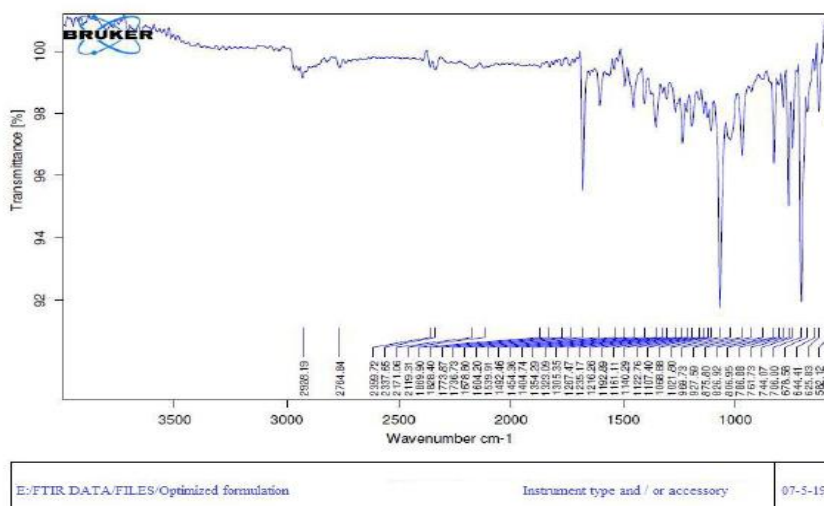**Figurre 3.9.1 FTIR of Carboxy methyl chitosan**

Figure-3.9.2 FTIR of MFA loaded CMC nanoparticles

3.5.2 Effect of concentration and sonication time of CMC on particle size, PDI, EE% and DL% of formulations of MFA loaded CMC nanoparticles

The effects of CMC concentration and sonication time on particle size and polydispersity index (PDI) of MFA-loaded CMC nanoparticles were summarized in **Table 3.4.** The particle size increased linearly from 154 to 201 nm with the increase of CMC concentration. These trends were in accordance with previously reported results[28,29].

The results from **Table3.4** showed that the interaction effect of Carboxy methyl chitosan concentration and Sonication time on the EE% and DL% of MFA loaded CMC-NPs. These results revealed that EE% varies from 59.12±3.30% to 86.65±1.16, F8 having high Carboxy methyl chitosan

concentration and longest sonication time showed 86.12% Entrapment Efficiency due to cross linking of chitosan with drug. DL% varied from 10.51% to 19.31%.

This could be attributed to the binding of hydroxyl groups of MFA to positively charged amino groups on Chitosan molecules by electrostatic interaction, these results might be attributed to an increase number of interacting units at higher polymer concentrations and to cross-linker levels that lead to the observed increase in particle size and decrease in entrapment efficiency

For the nanoparticle stability the zeta potential should be between -25mV to +25mV. Zeta potential of chitosan MFA nanoparticle is found to be -2.8mV (**Figure3.11**). The cumulative particle size is 13.9nm (**Figure-3.10**).

Table 3.4- Particle size and PDI of MFA-CMC-NP

Formulation Code	CMC	Sonication time	Particle size	PDI	EE%	DL%
F1	+1	+1	158.7±13.4	0.234±0.0235	59.19±1.38	12.31±1.24
F2	0	+1	170.3±11.2	0.264±0.0245	79.18±2.19	11.21±1.04
F3	-1	+1	168.7±12.4	0.234±0.0335	81.52±1.27	10.51±1.03
F4	+1	0	160.3±11.2	0.264±0.0205	79.52±3.39	11.01±1.64
F5	0	0	154.7±13.4	0.214±0.0335	82.19±1.69	15.31±0.24
F6	-1	0	177.3±11.2	0.134±0.0435	80.16±1.44	12.31±1.94
F7	+1	-1	201.7±13.4	0.278±0.0425	78.02±1.30	19.31±1.29
F8	0	-1	171.3±10.2	0.134±0.0515	86.12±1.09	18.31±1.38
F9	-1	-1	159.2±12.4	0.264±0.0235	79.02±1.29	11.31±1.44

N=3 (Mean± SD)

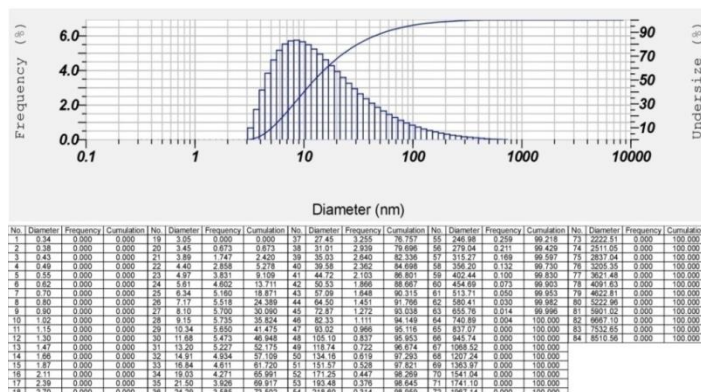|	
Date	: 17 April 2019 18:07:48
Measurement Type	: Particle Size
Sample Name	: CMC Nanoparticles-Size
Scattering Angle	: 90
Temperature of the holder	: 25.1 deg. C
T% before meas.	: 20332
Viscosity of the dispersion medium	: 0.893 mPa.s
Form Of Distribution	: |Standard|
Representation of result	: Scattering Light Intensity
Count rate	: 5059 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	25.9 nm	42.5 nm	8.6 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	25.9 nm	42.5 nm	8.6 nm

Histogram Operations

Size (Median)	: 12.5 nm
Mode	: 8.6 nm
% Cumulative (1)	: 10.0 (%) - 5.1 (nm)
% Cumulative (2)	: 50.0 (%) - 12.5 (nm)
% Cumulative (3)	: 90.0 (%) - 55.8 (nm)
% Cumulative (4)	: 30.0 (%) - 8.1 (nm)
% Cumulative (5)	: 40.0 (%) - 10.0 (nm)
% Cumulative (6)	: 50.0 (%) - 12.5 (nm)
% Cumulative (7)	: 20.0 (%) - 6.5 (nm)
% Cumulative (8)	: 70.0 (%) - 21.6 (nm)
% Cumulative (9)	: 95.0 (%) - 91.7 (nm)
% Cumulative (10)	: 100.0 (%) - 8510.6 (nm)

Cumulant Operations

Z-Average	: 13.9 nm
PI	: 0.757

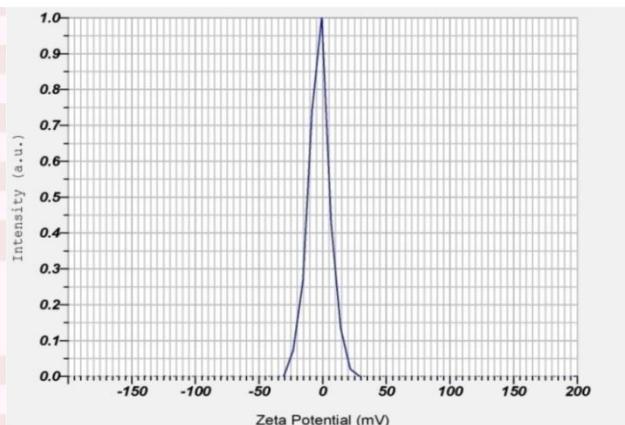**Figure-3.10-Cumulative Particle size graph of MFA-CMC-NP**

Figure-3.11-Zeta report of MFA-CMC-NP

3.5.3   Morphological Results of MFA loaded CMC nanoparticles

MFA-CMC-NPs, prepared in the optimal condition were observed using SEM (**Figure-3.12**) All nanoparticleswere spherical or ellipsoidal in shape with a smooth surface and well dispersed without aggregation. Spherical particles with uniform particle size in the nanoscale formed, ranging from 154 to 201 nm. The aggregates, usually having a rod shape, as observed in the SEM photos were probably formed during the drying process. The particle size of nanoparticles obtained after cast drying was in good agreement with that measured in an acidic aqueous system presented.
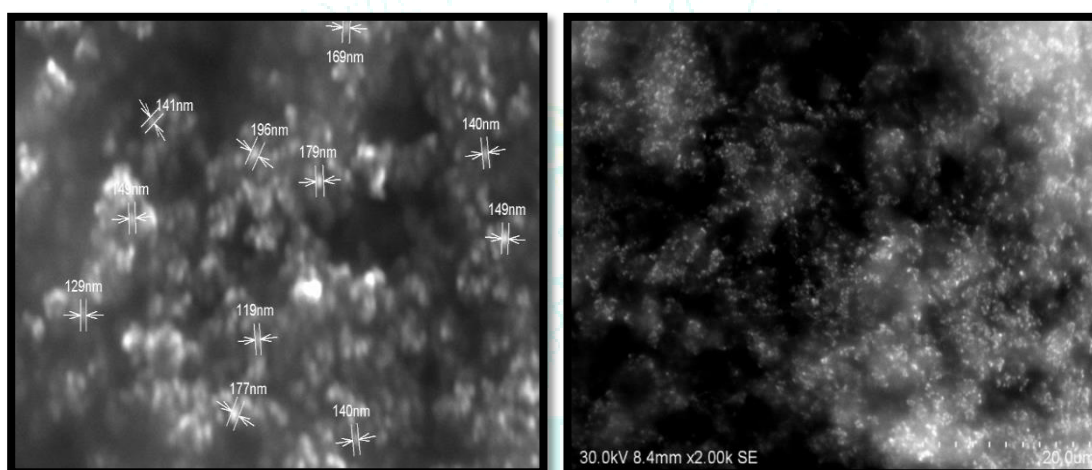

Figure-3.12-Scanning electron microscopy   results of MFA-CMC-NP (Mefenamic acid loaded carboxy methyl chitosan nanoparticles)

3.5.4   Drug release profile of MFA loaded CMC nanoparticles (F1-F9 formulations)

The release profiles of MFA from the CMC-NPs formulations compared to drug solution as a control were illustrated in **Figure-3.13.1** and **Figure-3.13.2.** 72.33% of MFA in control was released rapidly from the dialysis bag within the first 8 hour into solution.Total drug 100.1% is released within 12h. On the other hand, MFA loaded CMC-NPs showed a biphasic pattern with an initial burst drug release followed by a sustained release. Regarding CMC-NPs, MFA was rapidly released within the first 8 h, followed by a slow release from 8 hr up to 26 hr. The rapid MFA releasing was mostly due to the nanoparticles surface drugs, which could simply diffuse in the first 8 h. The percentage release of MFA from the CMC-NPs in the first 8h were about 44.02%, 31.72%, 34.22%, 37.01% ,53%,36.01%,38.33%,61% and 28% for F1, F2, F3, F4,F5,F6,F7,F8,F9and F10,The release   profile of F8 is showing biphasic release having linear release kinetics after 8hr upto 26 hr(**Figure-3.13.3**).

Table: 3.5- Drug Release Profile of MFA-CMC-NP

| Time (hr)	In-vitro Release %									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	Control
0	0	0	0	0	0	0	0	0	0	0
8	44.02	31.77	34.22	37.01	53	36.01	38.33	61	28	72.33
10	59.21	48.43	68.03	58.27	61.37	55.01	52.11	63	40	96.32
12	69.43	62.02	70.11	69.43	65.04	79.12	62.01	71	57	100.1
14	82.33	75.03	74.19	82.33	71.34	83.12	72.51	72	62	100.5
16	93.12	72.04	75.01	93.12	73.13	93.12	75	81	69	100.9
20	98.08	99.04	96.06	98.08	78.05	100	81.09	94	73	100.9
24	100	100	100	100	92.02	100	88.43	96	88	100.9
26	100	100		100	100		99	100	91	100.9
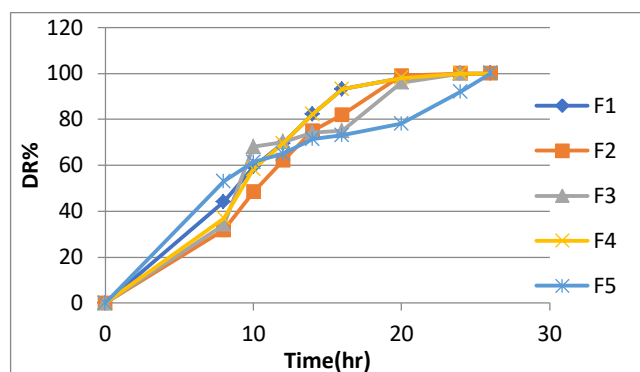
Figure-3.13.1-DR% of F1 to F5 MFA-CMC- NP
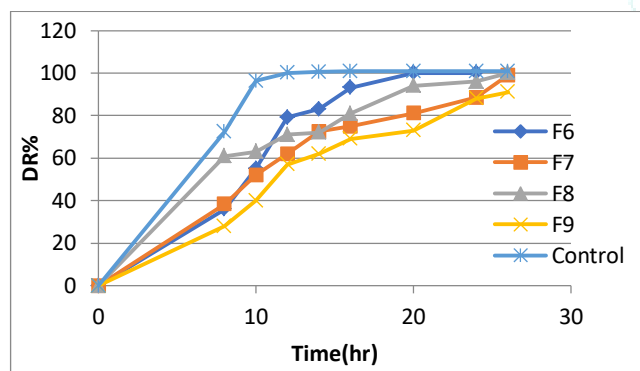

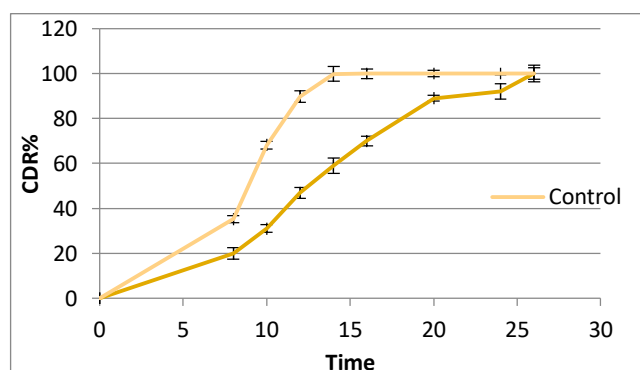**Figure-3.13.2—DR% of Control, F6 to F9 MFA-CMC-NP**

Figure-3.13.3—Drug Release Profile of F8 MFA-CMC-NP and Control

3.5.5 Comparison of Drug release profiles of MFA loaded CMC nanoparticles (Formulation F8), MFA loaded Chitosan nanoparticles (Formulation F1) and control.

The release profiles of MFA from the CMC-NPs formulation F8, Chitosan-NPs formulation F1 compared to drug solution as a control were illustrated in **Figure-3.14.** MFA was released rapidly from the dialysis bag within the first hour by using the drug solution. On the other hand, MFA loaded CS-NPs and CMC-NPs showed a biphasic pattern with an initial burst drug release followed by a sustained release. In both CS-NPs & CMC-NPs, MFA was rapidly released within the first 8 h, followed by a slow release from 8 hr up to 26 hr. The rapid MFA releasing was mostly due to the nanoparticles surface drugs, which could simply diffuse in the first 8 h. In case of CN-MFA-NP formulation 1(F1) is 8, 10, 12, 14, 16, 20, 24, 26 hours 21%, 36, 48, 54, 69, 83, 98, 101% of drug is releasing. In case of CMC-MFA-NP formulation F8 release profiles are- 61%, 63%, 71%, 72%, 81%, 94%, 96%, 100% at 8, 10, 12, 14, 16, 20, 24, 26 hours respectively. The release profile of F8 of CMC-NPs and F1 of CS-NPs are showing biphasic release having linear release kinetics after 8hr up to 26 h (**Figure-3.14**).
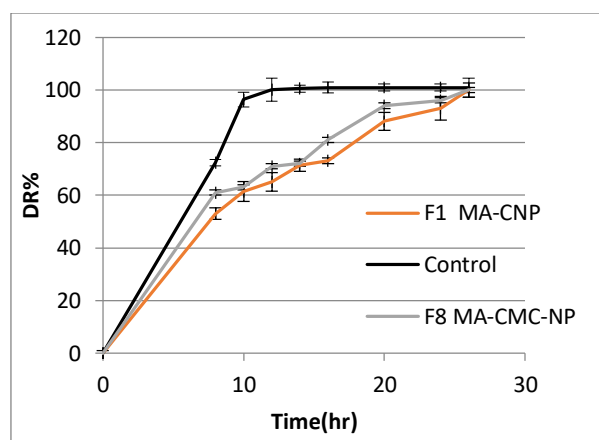

Figure-3.14—Drug Release Profile of Control, F8 of MFA-CMC-NPs and F1 of MFA-CSNPs

4.0 DISCUSSION

Comparative discussion with previous reviews

Chitosan is soluble at pKa less than 6, to increase the solubility; chitosan derivatives were synthesized as Tzeneva[30]. The solubility depends on degree of substitution of carboxy methyl groups, which is useful parameter in many applications. The degree of substitution in CMC was between 0.40-0.45, it was more water soluble as proved by the chen[31]. The degree of substitution was calculated by potentiometric titration in this study as 0.51(DS of CMC was found to be 0.68 by Vaghani[26]., 0.6 as per the findings of Mourya[32], 0.89 as obtained by Ge[33], i.e >0.85, so their preparative method was said to be perfect method). So the prepared CMC was more water soluble, solubility depends on NaOH concentration and useful in drug delivery system, similar results were found in case of Mourya[32], described that 50% NaOH was the optimum alkaline solution which favoured the solubility of CMC. Similar results were found by the other researchers[34,35].

Chitosan-mefenamic acid (40mg) and Carboxy methyl Chitosan- mefenamic acid (40mg) nanoparticles were prepared by solvent evaporation nanoprecipitation method. Chitosan, CMC concentrations, tween 80 and sonication time are taken as variables. The prepared nanoparticle size, Entrapment Efficiency, Poly Dispersity Index, Scanning Electron Microscopy, FTIR, Drug release profile are studied. These Drug release profile obtained for mefenamic acid loaded chitosan nanoparticles (MFA CSNP) were compared with that obtained for mefenamic acid loaded carboxy methyl chitosan nanoparticles (MFA CMC-NP). MFA CSNP entrapment efficiency is ranging from 44.58-82.65%, Drug loading capacity was 3.95-8.14, particle size ranging from 150-245nm. MFA CMC-NP entrapment efficiency was ranging from 59.19-86.12%, Drug loading capacity is 10.51-19.31, particle size ranging from 154-201nm. Due to high entrapment efficiency (interaction of OH groups with the NH2 groups of CMC) the particle size was lesser and drug loading capacity was more than chitosan-MA nanoparticles. Drug release pattern for MFA CSNP and MFA CMC-NP are showing similar release pattern (i.e initial burst release and then sustain release), In case of MFA CSNP formulation 1(F1) is 8,10, 12, 14, 16, 20, 24, 26 hours 21%, 36, 48, 54, 69, 83, 98, 101% of drug was releasing. In case of MFA CMC-NP formulation 7(F7), F8 release profiles were- 38.33, 52.11, 62.01, 72.51, 75, 81.09, 88.43, 99% and 61, 63, 71, 72, 81, 94, 96, 100% respectively at 8,10, 12, 14, 16, 20, 24, 26 hours. This drug release profile was depend on the sonication time and the concentration of drug used. MFA CSNP sustain release was observed with high concentration of drug and high sonication time used (F1), whereas MFA CMC-NP sustain release was observed with high concentration of drug with less sonication time used due to the solubility of CMC than chitosan.

5.0 SUMMARY AND CONCLUSION

Chitosan is biodegradable polymer. Its application is limited because of its limited solubility at higher range of pH. To overcome this chitosan derivative was prepared by carboxy methylation to obtain carboxymethyl chitosan, was more soluble in wide range of pH. CMC was characterized by FTIR, DS (51%). CMC was having more applications in sustained and controlled drug delivery systems. Nanoparticles of mefenamic acid loaded chitosan (150-245nm) and CMC (150-201nm) were prepared. Drug release profiles were compared for both nanoparticles. These CMC nanoparticles showed sustain release profile and can release the drug for 26hours.

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