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FORMULATION AND CHARACTERIZATION OF MICROSPHERES OF NITAZOXANIDE BY CHEMICAL CROSSLINKING METHOD

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ABSTRACT

The work investigated the design and evaluation of microspheres of Nitazoxanide by Ionotropic gelation technique method. 3^2 Factorial designs were used and concentration of polymer carbopol-934 (X1) and Ethyl cellulose (X2) were selected as the independent variables. The surface morphology study by SEM indicated that microspheres were spherical with smooth surface. There was no interaction between the drug and polymers, as studied by FTIR study. The prepared microspheres were characterized by entrapment efficiency, particle size micromeritic properties. It was observed that on increasing polymer concentration of formulations, % yield, the entrapment efficiency and particle size were increased whereas % drug release decreased. The In Vitro release study was done using U.S.P. dissolution rate basket type apparatus in phosphate buffer pH 7.4 for 10 hr. It shows that on increasing polymer concentration the drug release of all formulations was gradually decreased. In Vitro mucoadhesion study depicts that as the polymer concentration increased, mucoadhesive nature of the formulation was also increased. The microspheres of NTZ (formulation F_9) showed best results due to highest drug entrapment efficiency (85.50%), and percentage drug release after 10.0 hr. was 50.25%. The rate of release followed First order kinetics. The microspheres exhibits good mucoadhesive properties in in- vitro wash-off test at pH 7.4 (Intestinal pH) than pH 1.2 (gastric pH),because the drug was completely absorbed in Gastrointestinal tract, Therefore, it can be concluded that Nitazoxanide Loaded algino-carbopol-934 microspheres can be formulated for sustained drug delivery of Nitazoxanide used in Chronic Hipatitis-C.

Keywords: Mucoadhesive microspheres, Nitazoxanide, Carbopol-934, Ethyl cellulose, Sodium Alginate, Factorial design.

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INTRODUCTION

Microspheres are small spherical particles, with diameters in the micrometer range (typically 1 µm to 1000 µm), manufactured from natural or synthetic polymers. Microspheres have numerous applications depending on what material they are constructed of and what size they are. Microsphere play numerous applications in biomedical sciences from diagnostic to drug delivery microsphere had reported for chemoembolisation (endovascular therapy)' radio imaging topical delivery, vaccine delivery and delivery of Monoclonal antibodies mediated microspheres targeting.[1-10]

Nitazoxanide (NTZ) chemically [2-[(5-nitro-1, 3-thiazol-2-yl) carbamoyl] phenyl] acetate, which is a newly approved antiprotozoal drug used in the treatment of cryptosporidiosis in immune comprised patients including those with AIDS or HIV infection[7-10]. NTZ is rapidly absorbed and converted to active metabolite tizoxanide, which inhibit Pyruvate ferredoxin oxidoreductase pathway. Nitazoxanide appears to have activity against metronidazole (MTZ) resistant protozoal strains and well tolerated.

It is indicated for amoebiasis, helminthiasis giardiasis, fascioliasis, trichomoniasis and cryptosporidiosis[10-14]. The anti-protozoal activity of nitazoxanide is believed to

be due to interference with the pyruvate:ferredoxin oxidoreductase (PFOR) enzyme dependent electron transfer reaction which is essential to anaerobic energy metabolism.[15-20] It has also been shown to have activity against influenza A virus. A survey of literature reveals that very few method & solvents were available for the estimation of Nitazoxanide.

MATERIALS AND METHODS

Materials

Nitazoxanide was obtained from Alembic Pharmaceutical Ltd. Vadodara India. Carbopol-934 was obtained from Manish Pharma, Baddi, India. Sodium alginate, ethyl cellulose, calcium chloride, di sodium hydrogen phosphate, and methanol were obtain from S .D. Fine chemicals. All the other chemicals and reagents were of analytical grade. Drug and polymer were evaluated spectometrically for purity, identity.

Method

In brief weight quantity of sodium alginate (3%) and ethyl cellulose were dissolved separately in distilled water (100mL) and ethanol (5mL). Then solution of ethanol was mixed in previously prepared sodium alginate solution. In separate beaker weight quantity of drug and Corbopol- 934 were dissolved in methanol and added to above solution with continuous stirring. The prepared mixture was dropped into $CaCl_2$ (3% w/v) solution using 26 G Syringe needle. Microspheres were obtained, filtered, washed with distilled water, air-dried at room temperature and stored in desiccators.[20-27]

Formulation Design

Table 1: 3^2 full factorial design, 2-factor, 3-level.

F.code	Drug (mg)	EC (mg) (X_1)	CP (mg) (X_2)	Sodium Alginate %w/v	Crosslinking agent $CaCl_2$ %w/v
F1	30	60	60	3	3
F2	30	30	60	3	3
F3	30	90	60	3	3
F4	30	60	30	3	3
F5	30	30	30	3	3
F6	30	90	30	3	3
F7	30	60	90	3	3
F8	30	30	90	3	3
F9	30	90	90	3	3

Particle size

Particle size of Nitazoxanide microspheres were measured by optical microscopy. The values obtained were from triplicate experiments and expressed as mean ± standard deviation [28-31].

Microsphere recovery, drug content and entrapment efficiency

Nitazoxanide Microsphere recovery (%) was calculated by weighing lyophilized Microsphere accurately, and using following formula

$$\text{Microsphere Recovery} = \frac{\text{Wt of microspheres}}{\text{Wt of drug and polymer}} \times 100$$

Drug content study

The drug content of microsphere was determined by spectrophotometrically at 414.4 nm (UV-2201, Systonics). Each determination was made in triplicate. [32-35]

Drug content were calculated by using following formula

Drug Content = Conc. × dilution factor × volume/1000

Morphological Examination (SEM)

A texture of surface for prepared microspheres was done by taking Scanning electron photomicrographs. The sample was spread on stub and coated for 120 Swith a layer of gold using a sputter coater. Afterwards, the stub containing the sample was placed in the scanning electron microscope (ZEISS) chamber at the acceleration voltage of 10 kV, chamber pressure of 0.6 mm Hg. [36-39]

X-ray Powder Diffractometry (XRD)

Diffraction patterns of physical mixtures; drug and polymers were recorded with a PW 3040/60 X' Pert PRO, Netherland. A voltage of 40 KV and a current of 45 mA for the generator were used, with Cu as the tube anode material. The solids were exposed to Cu-Kα radiation (α1=1.54060 Å and α2=1.54439 Å, with a α1/α2 ratio of 0.5), over a range of 2θ angles from 0^0C to 60^0C, at an angular speed of 3^0 (2θ) per minute.

Differential scanning calorimetry (DSC)

DSC provides information about all physical properties of sample as Crystalline or Amorphous nature and demonstrates the possible interaction between Drug and other Polymers.[40]

In-vitro mucoadhesion study

The mucoadhesive property of microspheres was evaluated by an *in vitro* adhesion testing method known as wash-off method by using freshly excised piece of intestinal mucosa (2 x 2 cm) from goat, glass slides (3 x 1 inch) and USP tablet disintegrating test machine [41-43]. In brief microsphere were spread on tissue specimen attached with glass slide and hung it on to the arm of USP apparatus then assembly started. At the end of one

hour no of number of microspheres still adhering to tissue was calculated as following.

$$\%\ mucoadhesion = \frac{weight\ of\ adhered\ microspheres}{weight\ of\ applied\ microspheres} \times 100$$

In-vitro drug distribution study

In-vitro drug distribution study of Nitazoxanide microsphere were calculated spectometrically by using basket dissolution apparatus[44-50].

Stability of Mucoadhessive microspheres

Stability studies were performed according to ICH and WHO guidelines. Optimized microspheres were packed in an aluminum foil and kept in petridish at room temperature (37°C) and in Humidity chamber at 40°C, 75% RH for a period of 28 days[51-55]. At the end of studies, Microspheres were evaluated for physical properties, *in- vitro* drug release and drug content.

RESULTS AND DISCUSSION

Drug Identification Tests

Melting Point Determination (Capillary Method)

Melting point of the drug was determined using capillary method by the melting point apparatus. Drug was filled in the capillary after sealing the capillary from one end and then the sample was placed in the apparatus along with the thermometer and when the drug melted its temperature is recorded. Melting point of the drug sample was found to be 198^{0}C (Ideal m.p.202^0 C).

UV Spectrophotometric Study

The λ_{max} was determined by preparing the 25 ml Actonitrile & water (9:1) solution of 2µg/ml-10µg/ml and further the sample was scanned at the range of 400-200nm. It was observed that the maximum absorbance was seen at 238.3nm, (using UV2201Pharma Spec Systronics) which was regarded as the λ_{max} of the drug Nitazoxanide . The λ_{max} of the drug Nitazoxanide in 50 ml Methanol:water (50:50) mixture was found to be 328 nm as & in pH 7.4 phosphate buffer was found to be 414.4 nm.

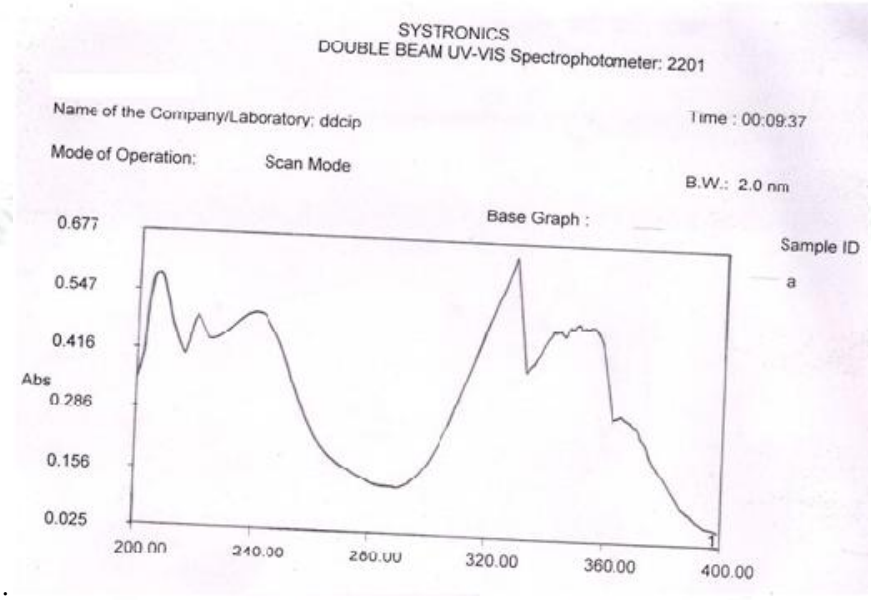


Figure 1: λ_{max} Scan for the drug at 238.3 nm in 25 ml Acetonitrile & water Solution (9:1)

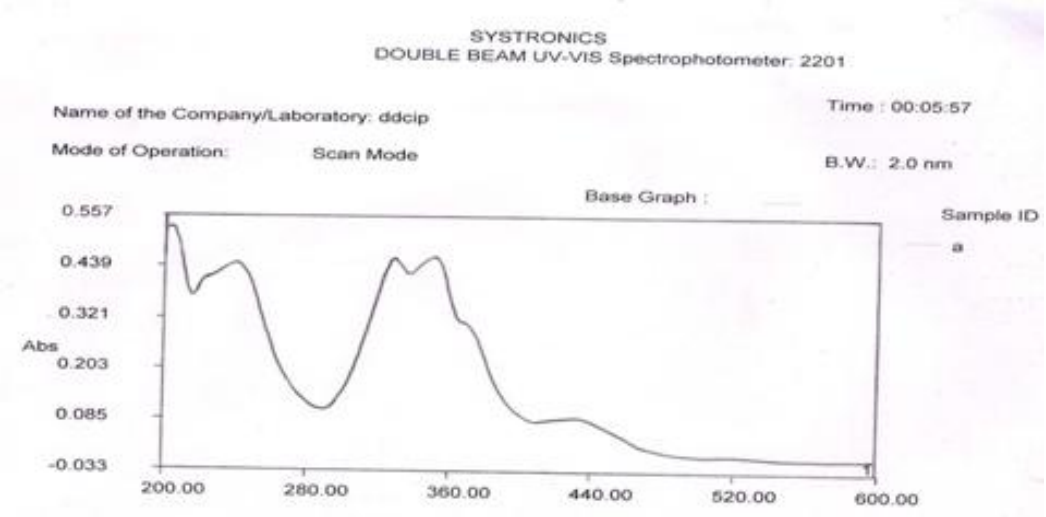


Figure 2: λ_{max} Scan for the drug at 328 nm in methanol:water (50:50) mixture

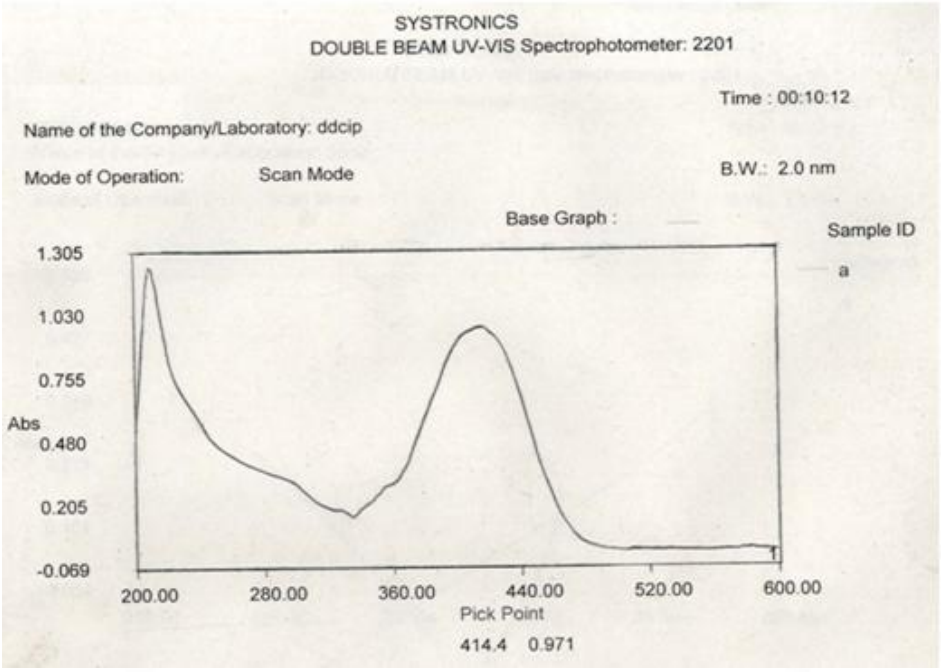


Figure 3: λ_{max} Scan for the drug at 414.4 nm in pH 7.4 phosphate buffer Solution

IR Spectral Analysis

Infrared (IR) spectroscopy was performed using FTIR Spectrophotometer (Shimadzu) the spectrum was recorded in the wavelength region of 4000 to 600 cm^{-1}. Pellets for the spectra were prepared using KBr hydraulic press, by dispersing a sample of drug in KBr and compressed into discs. The pellet was then placed in the FTIR and the spectrum was obtain and its interpretation is shown below.

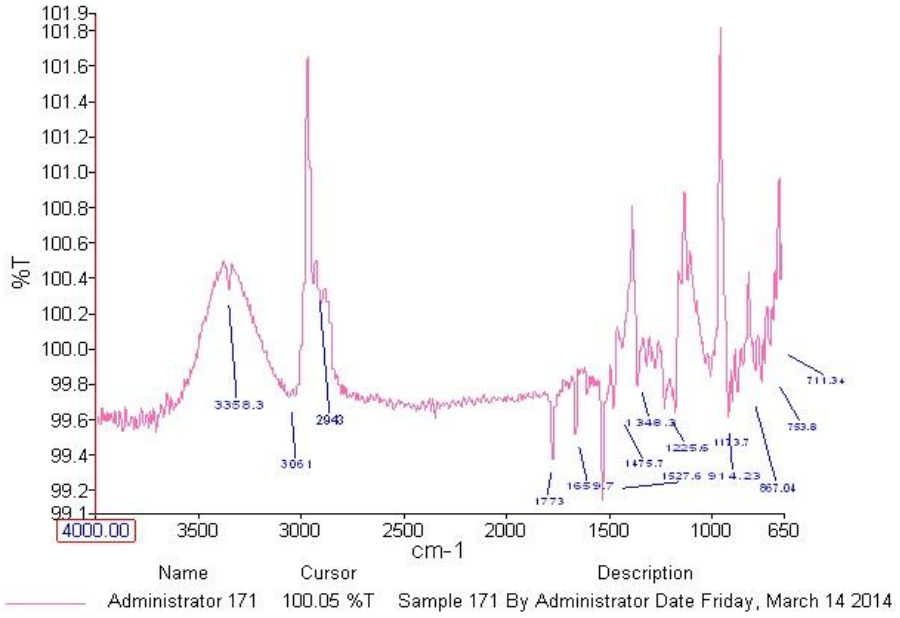


Figure 4: IR spectra of pure drug Nitazoxanide

Table 2: Interpretation of IR spectra of pure drug Nitazoxanide

S.No.	Functional Group	Range (cm^{-1})	Observed Frequency (cm^{-1})
1	Carbonyl group- ester linkage amide Linkage	1690-1760 1700-1680	1773 1659.7
2	Nitro group	1500-1350	1527.69
3	=CH stretch	2960-2850	3061

Compatibility Studies between the Drug and Polymer

For the drug excipients compatibility studies, the sample were kept at 40^0C & 75 % RH for 4 weeks, sample withdrawn, carried out and evaluated. For the result of compatibility studies that there was no change in physical appearance and optimized formulation, no incompatibility in drug alone or with excipients as same peaks were observe. In IR Spectra of physical mixture & optimized formulation are compared to Nitazoxanide drug which shows that there were no interaction between drug and polymer as shown in Figure 5 & Figure 6 .

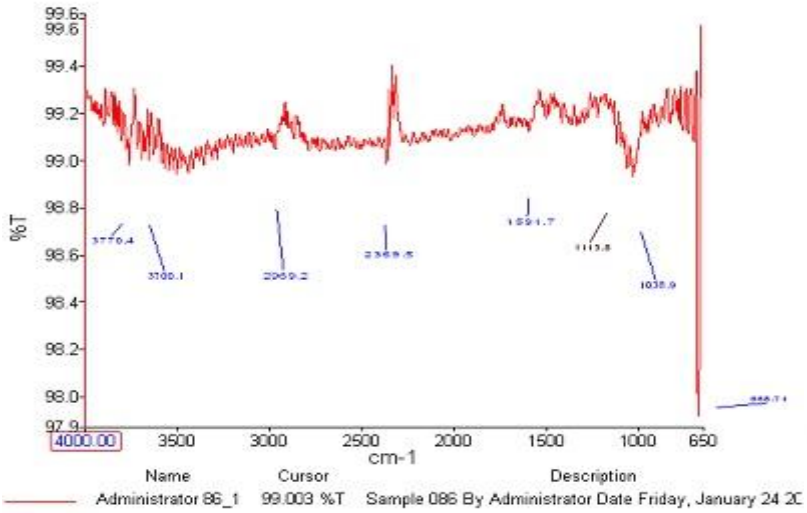


Figure 5:IR spectra of microspheres of Physical mixture of of Drug Nitazoxanide and Polymer carbopol-934

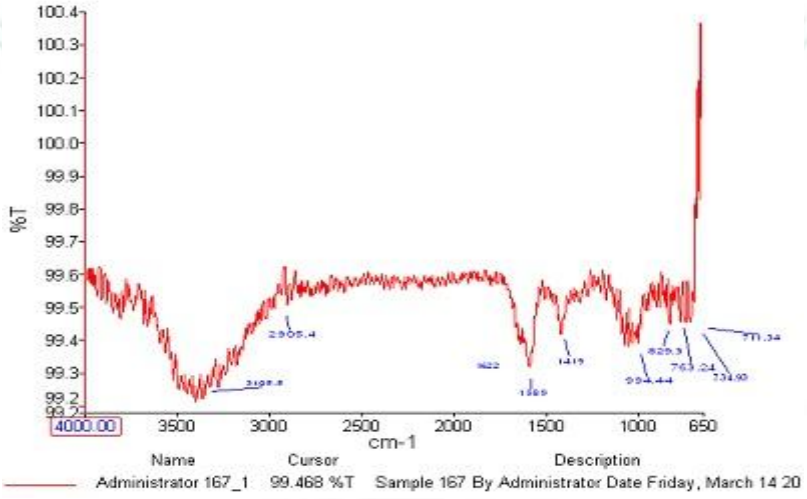


Figure 6 :IR spectra of microspheres of optimized formulation of Nitazoxanide

Table 3: Interpretation of IR spectra of Physical mixture of of Drug Nitazoxanide and IR spectra of microspheres of optimized formulation of Nitazoxanide

S.No.	Functional Group	Range (cm^{-1})	Observed frequency (cm^{-1})
1	-OH group	3000-3700	3700.1
2	Carbonyl group	1690-1760	1720
3	Nitro group	1330-1640	1622
4	Amide group	3000-3700	3405.5

Evaluation of Microspheres

Micrometric Properties of microspheres

Particle Size Analysis

The optical microscopy method was used to determine the particle size of prepared microspheres, in this method, the diameter of 100 microspheres was determined and from it the mean diameter was calculated. All readings were taken in triplicate.

The mean particle size was found to be 580.75 ±6.87µm to 729.94 ±10.12µm

Bulk density

The bulk density was found to be 0.320 ±.03 gm/cm^3 to 0.450±.03 gm/cm^3

Tapped density

The tapped density was found to be 0.358 ±0.01gm/cm^3 to 0.520±.02 gm/cm^3

Table 4: Characterization of mucoadhesive microspheres of Nitazoxanide

F.Code	BulK Density	Tapped density	%Carr's index	Hausner's Ratio	Angle of repose ø= tan^{-1}(h/r)
F1	0.398±0.01	0.422±0.02	5.68±0.10	1.060±0.01	14.06 ± 1.20
F2	0.435±0.02	0.464±0.02	6.25±0.15	1.167±0.01	21.88±0.15
F3	0.411±0.01	0.434±0.02	5.30±0.12	1.056±0.01	12.86±0.14
F4	0.510±0.03	0.546±0.03	6.59±0.09	1.071±0.02	14.25±0.15
F5	0.468±0.02	0.524±0.12	10.68±0.07	1.119±0.03	14.35±0.15
F6	0.407±0.02	0.442±0.13	7.92±0.13	1.086±0.02	15.89±0.19
F7	0.528±0.03	0.563±0.12	6.21±0.12	1.066±0.02	25.32±0.11
F8	0.635±0.03	0.658±0.13	3.49±0.13	1.036±0.09	24.02±0.14
F9	0.571±0.02	0.587±.011	2.73±0.11	1.028±0.01	20.45±0.10

*F5 formulation showed the best flow property and flow of all other formulations were excellent this showed that particles were decreases their crystallinity.

Table 5: Evaluation of mucoadhesive microspheres of Nitazoxanide in 10 hr.

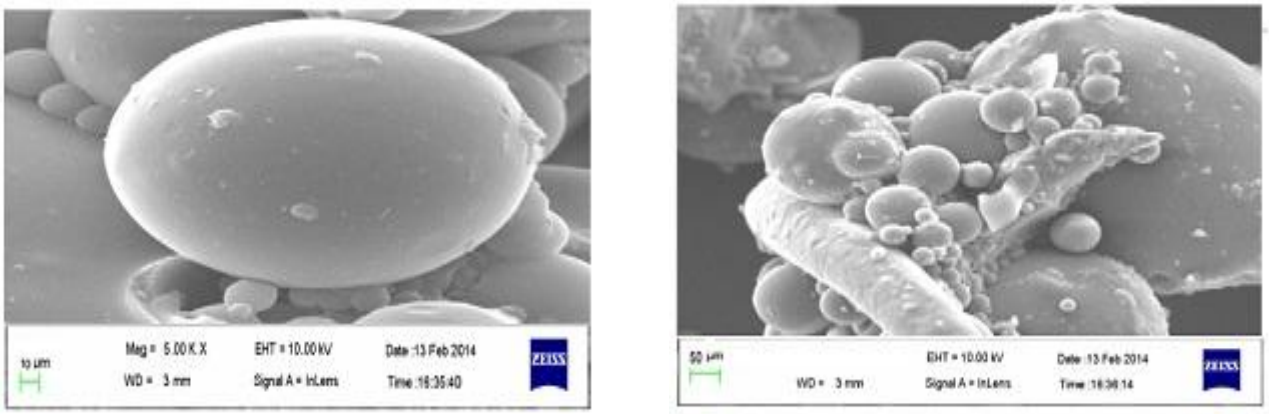
F.Code	%Yield ±S.D.	Theoretical drug content (mg)	Actual drug content (mg) ± S.D.	%Drug entrapment ± S.D.	Average particle size ± S.D.
F1	83.55±1.14	30	22.10±0.71	73.66±1.67	642.65±5.41
F2	80.45 ±2.05	30	21.28±0.74	72.66±2.10	585.45±4.10
F3	87.22±2.01	30	23.15±0.68	77.16±1.96	702.56±5.69
F4	81.61±2.70	30	21.65±0.35	72.16±1.85	642.7±5.13
F5	76.19±1.53	30	20.16±0.25	67.20±1.04	580.75±6.87
F6	85.77±2.93	30	22.69±0.13	75.63±1.25	680.2±6.17
F7	87.09±1.40	30	24.46±0.15	81.53±1.09	648.85±5.51
F8	86.88±1.91	30	22.91±0.08	76.36±1.64	590.62±6.40
F9	**90.22±1.51**	**30**	**25.65±0.10**	**85.50±1.54**	**729.94±10.12**

F9 Formulation showed best result due to high % drug entrapment & high % Yield

Because in F9 formulation higher polymer concentration.

SEM

The surface morphology of mucoadhesive microspheres was examined by Scanning electron microscopy (SEM) ,the SEM showed that microspheres obtained from optimized formulation was spherical and smooth surface at two different magnifications (10 µm & 50 µm) as shown in Fig.7.



(A): SEM Photograph (10 µm) of formulation (F9 (B): SEM Photograph (50 µm) of formulation (F9)

Figure 7: SEM micrograph of optimized Formulation of Nitazoxanide microspheres (F9 under two different magnifications (A & B 10µm & 50µm).

Powder X-Ray Diffraction Study (PXRD) of Drug and Formulation

The presence of several Large peaks in the PXRD of Pure drug Nitazoxanide at a diffraction angle of 6.50º, 25.02º, 31.50º and 46.25º were obtained, but in Nitazoxanide microspheres formulation small peaks were obtained at diffraction angle 31.53 º, 44.84º&56.85º were obtained revealed that the drug is present as a crystalline form and converted into amorphous form as shown in figure.

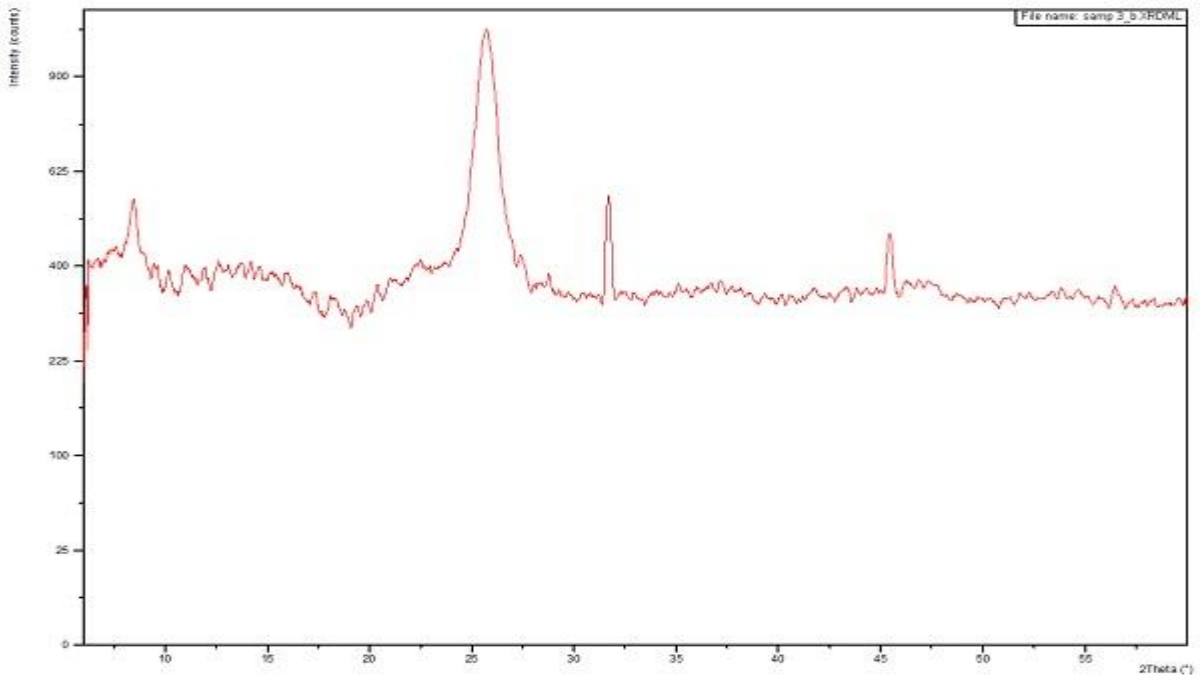


Figure 8: Sample- XRD of NTZ pure drug

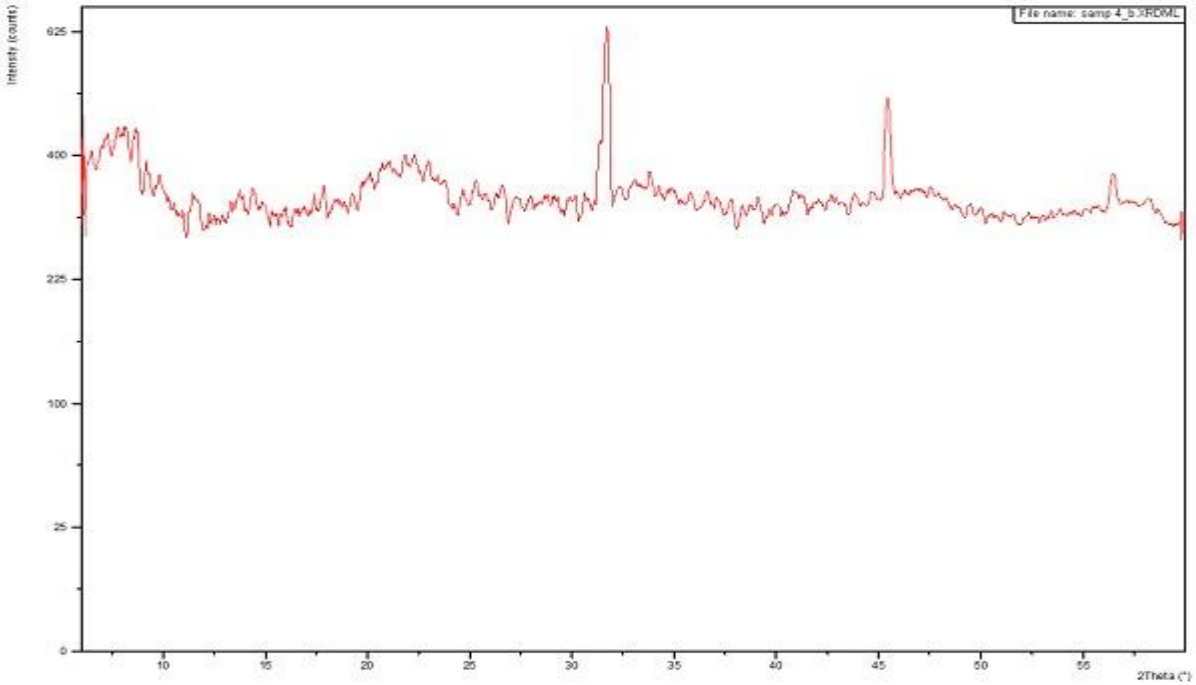


Figure 9: Sample: XRD optimized formulation (F9) of NTZ

Differential scanning colorimetery (DSC)

DSC provides information about all physical properties of sample as Crystalline or Amorphous nature and demonstrates the possible interaction between Drug and other Polymers. The thermal behavior of Nitazoxanide and physical mixture of drug & polymers are shown in (Figure No. 10 and 11), according to thermogram, Nitazoxanide produced sharp Endothermic peak at 197.5^0C which conformed crystalline form of the drug. DSC curves of the drug and Physical mixture of drug & polymers Exhibited an Endothermic peaks at 201.5^0C, which has been attributed to the evaporation of water. The thermogram of the physical mixture of Drug and Polymers showed that there was no interaction between drug and polymers.

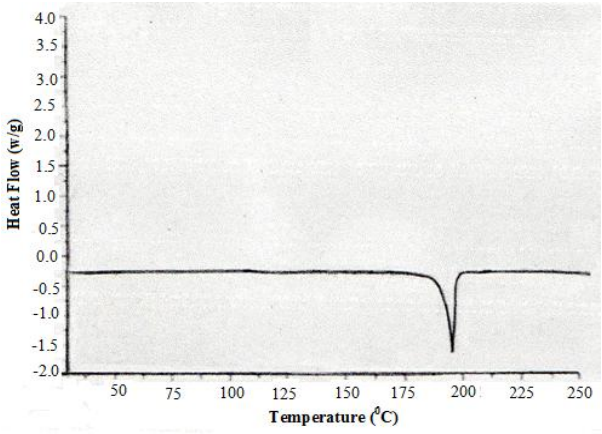


Figure 10: DSC Spectra of pure drug Nitazoxanide

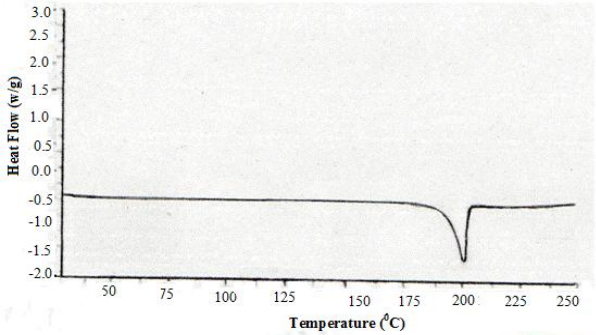


Figure 11: DSC Spectra for physical mixture of pure drug Nitazoxanide +Carbopol 934+Ethyl Cellulose +Sodium Alginate

Mucoadhesion property of optimized formulation of microspheres of Nitazoxanide

The mucoadhesive property of microspheres was evaluated by an in vitro adhesion testing method known as wash-off method in phosphate buffer pH 7.4.

Table 6: % Mucoadhesion of optimized formulation of microspheres of Nitazoxanide

F.code	% mucoadhesion ±S.D.	% Cummulative release ±S.D.
F1	84± 1.45	65.81±1.42
F2	90± 1.24	73.31±1.35
F3	70± 1.35	56.81±1.21
F4	85± 1.65	75.18±1.12
F5	45± 1.75	80.06±1.15
F6	78± 1.85	68.44±1.28
F7	90± 1.65	58.31±1.45
F8	93± 1.75	69.94±1.38
F9	95± 1.23	50.25±1.21

Invitro Studies

The prepared microspheres of Nitazoxanide were placed in each of the six basket dissolution apparatus. The assembly was maintained at a temperature of 37^0C in phosphate buffer, pH 7.4. Samples were withdrawn at definite time intervals and replaced by equal volume of fresh medium. The absorbance of samples was measured from UV spectrophotometer. Concentration and % cumulative release of drug from the formulation was then calculated [56-57].

Dissolution profile revealed that after 10 hr. Formulation F1-F9 released 65.81%, 73.31%, 56.81%, 75.18%, 80.06%, 68.44%, 58.31%, 69.94% and 50.25% drug respectively. The reason behind the lesser drug release of F3, F7, F8 and F9 in comparison to F1, F2, F4, F5, and F6 might be the use of high concentration of Carbopol-934, Ethyl cellulose.

Formulations (F9) showed lesser drug release than (F5) because higher the polymer concentration lower the drug release where as in case of F5 formulation the polymer conc. was less so drug release is higher so F9 Formulation showed best result for sustained release.

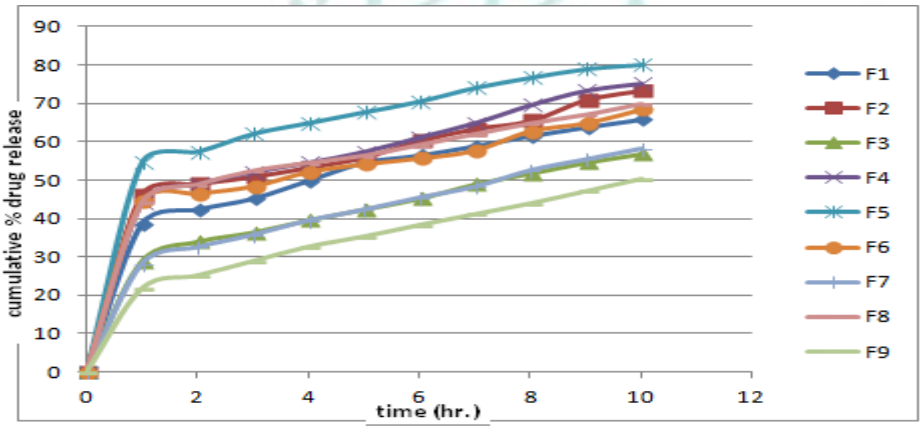


Figure 12: *In vitro* release profile of Microspheres of Nitazoxanide.

Statistical Analysis

Factorial design was used to select the factors displaying the most effects on the microspheres properties. 3^2 full factorial design, 2-factor, 3-level.The two obtained factors were carbopol-934 conc. (X1) & ethyl cellulose conc. (X2) which is in dependent variables & % mucoadhesion, in vitro release, % drug entrapment which is dependent variables.

Table 7:Two- way ANOVA of Microspheres of Nitazoxanide

Source of Variation	SS	Df	MS	F	P value Prob.> F
Model	858.76	3	286.25	285.18	0.0128 **Significant**
A-A	300.31	1	300.31	21.64	0.0288
B-B	729.59	1	729.59	6.43	0.0022
A-B	180.37	1	180.37	6.75	0.0749
Pure error	400.37	9	44.49	17.82	
Cor total	1259.13	12		4.05	

The Model F-value of 6.43 implies the model is significant. There is only a 1.28% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant.

Stability studies

Before storage- Drug Content- 25.65±0.10, % Cummulative release- 50.25±0.12

Table 8: Stability Analysis of Optimized Formulation F9

Date in*- 04/04/2014,* ***Storage Condition****- 40°C, 75% RH,* ***Packaging****- Glass Vials*

Sampling interval	One week	Two week	Three week	Four week
Date / **Evaluation parameters**	10/04/20114	17/04/2014	24/04/2014	01/05/2014
Drug content (mg)	25.09±0.15	24.89±0.11	23.95±0.05	22.98±0.07
%cumulative release	50.02±0.13	49.97± 0.11	48.88 ±0.10	47.99 ±0.12

CONCLUSION

Nitazoxanide loaded Alginate microspheres were successfully prepared by Ionotropic gelation technique. The micromeritic study of microspheres suggests that on formulation of microsphere from pure drug the flow behavior of drug was improved. From the SEM of microspheres it was evident that the microspheres were spherical in shape with smooth surface. The mean particle size of microsphere was found in the range of 580.75µm to 729.94µm. A systemic study using a 3^2 Factorial design was done. The independent variables had significant influence on dependent variables. Concentration of polymer ratio influence drug release profile & entrapment efficiency of microspheres. As the polymer concentration increases, % drug release decreases whereas the entrapment efficiency increases. All the formulation followed first order release kinetics. The Formulation F_9 showed maximum entrapment efficiency of 85.50% and 50.25% of control drug release up to 10hr. The microspheres exhibited good mucoadhesive property in the in vitro wash off test and also showed high percentage drug entrapment efficiency. Formulation F_9 microspheres were selected as best formulation for preparation of sustained drug delivery system. The data obtained thus suggest that mucoadhesive microspheres can be successfully designed for sustained delivery of Nitazoxanide and to improve patient compliance.

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