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Research Article

FORMULATION AND EVALUATION OF BIOADHESIVE GEL INCORPORATED METRONIDAZOLE LOADED MICROSPHERES FOR PERIODONTAL THERAPY

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ABSTRACT

Gingivitis and periodontitis are the two most common infectious periodontal diseases. Gingivitis occurs in almost all individuals. Bacteria that are associated with periodontal health include primary orearly colonizers such as *S.sanguis, S.mitis, Gemellaspp, Atopobiumspp, Fusobacteriumnucleatum,* and *Capnocytophaga* spp. Species belongingto the genera Veillonella, Streptococcus, and Capnocytophagaarethought to be beneficial to the host. The rationale use of metronidazole in the treatment of periodontal diseases It is also used in treatment of acute necrotizing ulcerative gingivitis and severe odontogenic infections. Excipients selected for the formulation of microspheres on the basis of their properties in which gelatin have Gelling ability, gelatin gels independently of pH and without the need for other reactive agents, Carbopol polymer is a cross-linked polyacrylate polymer that offer significant functional benefits in applications requiring flexible rheological properties and suspension control. Polysorbate containing 20 units of oxyethylene are hydrophilic nonionic surfactants, Gelatin microspheres loaded with 100 mg metronidazole was added to the drug carbopol solution and mixed well. Sodium metabisulphate (0.1 %w/w)) were then added to microsphere/drug/carbopol mixture, while stirring at 250 rpm, to obtain a homogenous mixture. Stirring was continued until a lump-free suspension was obtained. Thereafter, 0.33 ml of triethanolamine was added to produce a gel. Concentrations of drug were calculated from the standard calibration curve prepared in pH 6.8 phosphate buffers,The release study was carried out by using egg membrane. Prior the permeation experiment, membrane was clamped between the donor and the receptor compartment of the diffusion cell apparatus.

Key words- Periodontitis, Metronidazole, Bioadhesive Gel, Microspheres, Periodontal Therapy.

INTRODUCTION

The buccal cavity provides a diverse milieu for colonization by a wide variety of microorganisms. The early oral dental infections are due to facultative or strict aerobes and at later stages most bacteria in oral infections are anaerobic species (Dumitrescu *et .al.,* 2009). Periodontal disease is a collective term for a number of pathological conditions characterized by degeneration of the gums (gingiva), supporting bone (alveolar bone), periodontal ligament and cementum.

Etiology and pathogenesis of periodontal diseases

Periodontal disease is an infection. Most forms of gingivitis and periodontitis are caused primarily by bacteria that colonize the gingival crevice and attach to intra periodontal pockets (Paju*et*. *al.,* 2007).

The role of dental plaque

Dental plaque is a bacterial biofilm which causes chronic gingivitis and periodontitis. The presence of bacteria in the oral cavity has been known since the time of Antonvon Leeuwenhoek, who described the presence of ‘‘animalcules’’ in dental plaque

Biofilm in oral cavity

The human oral cavity is a highly dynamic environment inhabited by more than 750 microbial species. One cubic millimetre of dental plaque contains about 100 million bacteria and serves as a persistent reservoir for potential pathogens (Chung *et al.,* 2014).

Development of dental plaque as a biofilm

The plaque biofilm may be defined clinically as bacterial deposits which cannot be easily rinsed away. It may form on teeth, mucosa or other solid surfaces.

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Antibiotic treatment of biofilm

Biofilm are mostly water, and solutes the size of antibiotic diffuse readily in to the biofilm matrix. Treatment of biofilm infection requires sensitive and well-penetrating antibiotic to ensure a sufficient concentration of effective antibiotic at the site of biofilm infection (Hasan *et. al.,* 2014).

Periodontal health

Bacteria that are associated with periodontal health include primary orearly colonizers such as *S.sanguis, S.mitis, Gemellaspp, Atopobiumspp, Fusobacteriumnucleatum,* and *Capnocytophaga* spp. Species belongingto the genera Veillonella, Streptococcus, and Capnocytophagaarethoughtto be beneficial to the host. (Donald *et. al.,* 2015).

Periodontal Pockets

A periodontal pocket is a pathologically dependent gingival sulcus and is one of the important clinical features of periodontal disease.

Periodontal Local Drug Delivery for Periodontal Pockets

The systemic use of antibiotics raises a number of issues. A prolonged administration increases the risk of problems such as antibiotic resistant and adverse drug reaction. As a result of these, focusing on the development of localized drug delivery system for the release of antibiotics in the periodontal pockets are becoming more frequent (Herrera *et al.,* 2012).

MICROSPHERES

Microspheres are controlled release drug delivery system which comprises of drug-containing microparticles or microspheres, between 10 and 500 microns in size (Badran *et al.,* 2015). Non-biodegradable as well as biodegradable materials are used for the preparation of microspheres. New approaches involve the use of local drug delivery systems based on microparticles made from biocompatible polymers. Such devices enable the introduction of antimicrobial agents or other drugs directly in the periodontal pocket, or inside the root canal, (Alvarez *et al.,* 2011). Bioadhesive gel incorporated drug loaded microspheres-Bioadhesives are natural polymeric materials that act as adhesives or synthetic materials that adheres to the biological tissue.

TABLE 1: MARKATED FORMULATIONS FOR PERIODONTAL PRODUCTS

Product	Antimicrobial agents	Dosage form	Manufacturer
Atrigel	Doxycycline	Gel	Atridox (atrix Lab)
Periochip	Chlorhexidinegluconate	Films	Adrian Pharmaceuticals, LLC
Periochip	Chlorhexidinegluconate	Biodegradable device	DexcelPharmaInc, Jerusalem,
Dentomycine	Minocycline	Biodegrable mix in syringe	Sunstar corp., Tokyo, Japan
Arestin	Minocycline	Microspheres	Oropharmacorp Warminster
Actisite	Tetracycline	Non resorbable fiber	Alza Corp. Palo Alto, CA, USA
Elyzol	Minocyline	Gel	Dumexpharma
OnSite	Antibiotics	fiber	Alza Corp. Palo Alto, CA, USA

Table 2: MARKATED FORMULATIONS OF MTZ FOR PERIODONTAL THERAPY

Product	Dosage form	Manufacturer	References
Gluconate	Inserts	Perioproducts Ltd.	Kaplish*et. al.,*(2013)
OnSite	fiber	Alza Corp. Palo Alto,CA,USA	Sharma *et. al.,* (2015)
Metrogel	gel	Galderma Laboratories, L.P.	Nair *et. al.,* (2012)
Elyzol	Biodegradable gel	Dumex corp.co Denmark	Ramesh *et. al.,*(2016)

MATERIAL & METHODS

Table 3: List of Chemicals and Excipients

S.NO.	CHEMICALS	MANUFACTURERS
1.	Metronidazole	Glenmark pharmaceutical ltd., Mumbai
2.	Gelatin	Merck specialities Pvt. Ltd, Worli,Mumbai
3.	Corbopol 934	Loba chemic pvt.Ltdmumbai
4.	Liquid paraffin	Loba chemic pvt. Ltd, mumbai
5.	Tween 80	Merck India Ltd, India
6.	Acetone	Loba chemic pvt. Ltd, Mumbai
7.	Sodium metablisulphite	Loba chemic pvt. Ltd, Mumbai
8.	Gulteraldehyde	Merck India Ltd, India
9.	Chloroform	Merck life science pvt. Ltd, Mumbai
10.	Span 80	Merck India Ltd, India

Table 4: List of equipments and instruments

S.NO.	EQUIPMENTS	SPECIFICATION/ MODEL NO	MANUFACTURE
1.	Digital Balance	Maximum 4000 RPM	Deniver Instruments
2.	UV Spectrophotometer	Shimadzu 1800	Shimadzu-1800
3.	IR Spectrophotometer	Shimadzu 8400S	Shimadzu8400S FT-IR
4.	pH Meter	700S	Eutech Pvt. Ltd, India
5.	Melting point apparatus	M-565	BEA-54 of Biocraft System(P) Ltd.
6.	Mechanical stirrer	Remi 1 MLH	Remi motors, Mumbai
7.	Scanning Electron Microscope	GOLD coating required, 10mg	Carl Zeiss Ltd, India

DRUG PROFILE (Rowe *et.al.,* 2009), (Hussain *et. al.,* 1980), (Higuchi *et. al.,* 1959)

Name: Metronidazole

Chemical Structure:

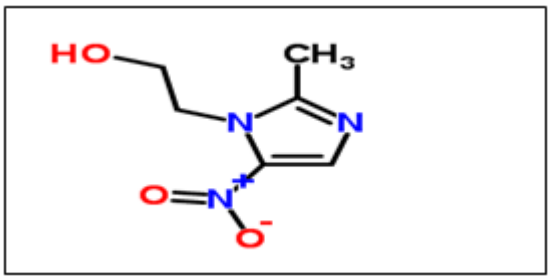

Figure 1:- Chemical structure of metronidazole

Synonyms: 1-(2-hydroxy-1-ethyl)-2-methyl-5-nitroimidazole

IUPAC Name: 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethan-1-ol

Molecular Formula: $C_6H_9N_3O_3$

Molecular Weight: 207.615

Solubility: Sparingly soluble in phosphate buffer pH 6.8, freely soluble in water, ethyl alcohol, chloroform, slightly soluble in ether, dilute acid (IP 2010).

Protein binding: Less than 20% bound to plasma protein

Partition coefficient: logP 4.02

Physical state: White powder

Odour: Odorless

Melting point: 159-162°C

Packaging and storage: Preserve in tight containers

Drug Category: Antiprotozoal, anti-infective, radiation sensitizing agents

PREPARATION OF MICROSPHERES (Patel *et. al.,* 2013)

Microspheres were prepared by emulsion cross linking method

Solution Aconatin dissolved 1gm gelatine in 10ml distilled water preheated to 60˚C. With stirring at room temp, addition with tween 80(0.1%w/v). Added 1gm metronidazole and thoroughly mixed to obtain a homogeneous solution. Add drop by drop Solution B containing 100ml liquid paraffin containing Span 80 (0.1%w/v) preheated to 60˚C at constant stirring with 3- blade stirrer in order to form w/o emulsion. Glutaraldehyde was added drop wise to the emulsion and stirring and stirred for 1 hr at room temperature to stabilize the microspheresThe mixture was then left to cool at between 5-10 ˚c f 2or 30 min to enhance settling of the microspheres. Microspheres were collected by filtration using Whatman filter paper and washed with 3 times 10 ml of chloroform followed by 2 times 10 ml of 5 %w/v sodium metabisulphite.

Microspheres dried at room temperature and transferred to glass vials.

Table 5: Formula for preparation of microspheres

Formulation	Drug	Polymer (gelatine)	Glutaraldehyde amount (ml)	Span 80	Liquid paraffin
M1	1gm	1gm	0.5	0.1%w/v	100ml
M2	1gm	2gm	0.5	0.1%w/v	100ml
M3	1gm	3gm	0.5	0.1%w/v	100ml

Table 6: Formula for preparation of microspheres by varying gluaraldehyde amount and stirring rate

Formulation	Drug: Polymer Ratio	Gultaraldehyde Amount (ml)	Stirring Rate (rpm)	Continuous phase amount(ml)
M4	1: 3	1.0	500	100
M5	1:3	1.5	500	100
M6	1:3	0.5	1000	100
M7	1:3	1.0	1000	100
M8	1:3	1.5	1000	100
M9	1:3	0.5	1500	100
M10	1:3	1.0	1500	100
M11	1:3	1.5	1500	100

Table 7: Formula for preparation of determine effect of continuous phase amount of microspheres

Formulation	Drug: Polymer Ratio	Gultaraldehyde amount (ml)	Stirring Rate (rpm)	Continuous phase amount(ml)
M12	1:3	1.0	1000	150
M13	1:3	1.0	1000	200

EVALUATION OF METRONIDAZOLE MICROSPHERES: (Patel *et. al.,* 2013), (Gangadharappa *et. al.,* 2011), (Ravi *et. al.,* 2016)

Particle size

Particle size was determined by using a Zetasizernano series 90 (Malvern instruments, UK). The formulation 50mg was dispersed in 50 ml of water in a volumetric flask, mixed thoroughly with stirring, sonicated for 10minutes and light scattering was monitored at 25°C a 90° angle.

Determine of percentage yield

The prepared microspheres were collected and weight. The measured weight was divided by total amount of all non-volatile components, which were used for the preparation of microspheres. The % yield was calculated following formula

% yield = WRec/weight (drug+ polymer) × 100 ……..(1)

Where, WRec= weight of microspheres recovered

Determination of Entrapment Efficiency and drug loading

The entrapment drug concentration was determined by centrifugation of the microspheres. The microspheres were centrifuged for 20 min at 1000 rpm. The supernatant was collected and filtered after centrifugation after suitable dilution and the drug content was measured UV spectrophotometer at a wavelength of 227.72 nm. The drug entrapment efficiency (EE) and drug loading (DL) in the microspheres were calculated from the following equations:

%EE= (W1-W2)/W1X100 ……(2)

W1= the wt. of the drug added to the system

W2= the wt. of the drug in the supernatant

%LC= (W1-W2) / (W1-W2+W3) X 100……(3)

W1= the wt of the drug added to the system

W2= the wt of the drug in the supernatant

W3= the wt of the polymer added to the system

Scanning electron microscopy

The sample the scanning electron microscopy (SEM) analysis was prepared by sprinkling the microspheres on one side of the double adhesive stub. The stub was then coated with gold using Jeol JFC 1100 sputter coater. The SEM analysis of the microspheres was carried out using Jeol JSM 5300, Japan. The microspheres wereviewed at an accelerating voltage of 15-20Kv.

***In-vitro* drug release**

Microspheres, equivalent to 10 mg of Metronidazole, were accurately weighed and transferred to 250 ml conical flask containing 100 ml phosphate buffer (pH 6.8). The flask was kept in an incubator at 37 °C, 1 ml samples withdrawn at regular intervals and, after suitable dilution, the amount of drug released was determined using a spectrophotometer at 227.72 nm.

Following each sample withdrawal, 1 ml of phosphate buffer was added to the release medium to replenish it. Five minutes before each sampling, the flasks were gently shaken by manually whirling it clockwise (15 revolutions) to minimize any concentration gradient within the release medium. The microspheres were allowed to settle down and clear supernatant medium withdrawn for drug analysis. The sample was filtered and the microspheres collected were transferred tothe dissolution flask. Similarly, the release of Metronidazole from gelatin microspheres was determined spectrophotometrically at 227.72 nm.

PREPARATION OF BIOADHESIVE GEL (Patel *et, al.,* 2013)

Preparation of the bioadhesive gel was based on a previously reported composition and method. Accurately weighed metronidazole (100 mg) was added to 15 ml of water in a beaker and stirred well to dissolve the drug; 400 mg of carbopol was dissolved in this drug solution. Gelatin microspheres loaded with 100 mg metronidazole was added to the drug carbopol solution and mixed well.Sodium metabisulphate (0.1 %w/w)) were then added to microsphere/drug/carbopol mixture, while stirring at 250 rpm, to obtain a homogenous mixture. Stirring was continued until a lump-free suspension was obtained. Thereafter, 0.33 ml of triethanolamine was added to produce a gel. This was followed by the addition of a sweetening agent (saccharin sodium, 0.1 %w/w) and more water to make up to 20 g of gel.

EVALUATION OF BIOADHESIVE GEL (Patel *et, al.,* 2013)

Surface pH of the gel

An acidic or alkaline formulation is bound to cause irritation on mucosal membrane and hence this parameter assumes significance while developing a bioadhesivegel formulation. A digital glass electrode pH meter was used for this purpose. pH was noted by bringing the electrode near the surface of the formulations and allowing it to equilibrate for 1 min.

Viscosity study

Viscosity of gels was studied on Brookfield viscometer by using spindle number 7 at 4 revolutions per minute at constant temperature.

Estimation of drug content in formulated gels

Formulations containing 1 mg of drug was taken in 10 ml volumetric flask, dissolved in pH 6.8 phosphate buffer made up the volume to 10 ml with pH 6.8

phosphate buffer and then filtered. Absorbance values were measured at respective λmax (227.72 nm) for drug. Concentrations of drug were calculated from the standard calibration curve prepared in pH 6.8 phosphate buffers.

***In-vitro* drug release studies of gels**

In-vitro release study was performed on diffusion cell apparatus. In diffusion cell apparatus, there are donor compartment, receptor compartment and a sampling port. The release study was carried out by using egg membrane. Prior the permeation experiment, membrane was clamped between the donorand the receptor compartment of the diffusion cell apparatus. The receptor compartment was filled with the phosphate buffer pH 6.8. 1g gel containing metronidazole of prepared was applied on the membrane. The receiver compartment was stirred continuously on a magnetic stirrer at 100 rpm with a magnetic bar with maintained temperature at 37°C. About of 1ml sample was withdrawn periodically and replaced with equal volume of the phosphate buffer (pH 6.8) to the receptor medium to maintain the receptor phase volume at the constant level. Samples were diluted with phosphate buffer (pH 6.8) and analysed on UV-visible spectrophotometer (Shimadzu-1800) at λmax 227.72 nm.

RESULT & DISCUSSION:

Determination of Entrapment Efficiency and drug loading

The percentage Entrapment efficiency and drug loading of formulation are shown in table (22). The formulation M3 shows highest Entrapment efficiency and drug loading, which is used for the preparation of microspheres

Table 8: Selection of drug: polymer ratio

Formulation	Drug: polymer	%Drug Entrapment	Particle size (µm)	% Drug loading
M1	1:1	23	28	39.65
M2	1:2	65	50	58
M3	1:3	85	90	83.33

Drug: polymer ratio-In M3 batch highest drug entrapment, particle size, and drug loading obtained. So, Drug: Polymer ratio 1:3 was selected. For reducing particle size of microspheres stirring rate &glutaraldehyde amount optimized.

Table 9: Selection of glutaraldehydeamount and stirring rate

Formulation	Drug: polymer	Glutaraldehyde amount (ml)	Stirring rate (rpm)	%Drug Entrapment	Particle Size (µm)	%Drug loading
M4	1:3	1.0	500	82.06	299	84.31
M5	1:3	1.5	500	57.39	405	82.64
M6	1:3	0.5	1000	64.90	258	93.31
M7	1:3	1.0	1000	70.50	246.8	91.60
M8	1:3	1.5	1000	84.10	284.6	89.04
M9	1:3	0.5	1500	92.70	232.0	95.23
M10	1:3	1.0	1500	76.90	334.9	90.38
M11	1:3	1.5	1500	86.20	280.2	92.52
M12	1:3	1.0	1500	73.40	232.2	94.02

Glutaraldehyde amount and stirring rate:- From these results, M9 batch in which stirring speed 1000 rpm and glutaraldehydeamt is 1 ml. These microspheres had highest % drug entrapment and lowest avg particle size. So, M9 batch selected.

Table 10: Effect of continuous phase amount on microspheres

Formulation	Continuous phase amount	Avg. Particle size(µm)	%Drug entrapment
M13	150	165	65
M14	200	150	54

Volume of continuous phase: Increase volume of continuous phase, emulsion droplets moved freely in medium, thus reducing collosion induced aggregateswhich could be the reason of high drug extraction in processing medium resulting in lower entrapment efficacy.

Determination of Entrapment Efficiency and drug loading:

The percentage Entrapment efficiency and drug loading of formulation are shown in table (24). The formulation M9 shows highest Entrapment efficiency and drug loading, which is used for the preparation of microspheres. The % drug loading and entrapment efficiency graph are show in Figure (01) and (02) respectively.

Table 11: The percentage drug entrapment and drug loading of formulations

Formulations	Entrapment Efficiency (EE %)	Drug loading (DL%)
M4	82.06	84.31
M5	57.39	82.64
M6	64.9	93.31
M7	70.5	91.60
M8	84.1	89.041
M9	92.7	95.23
M10	76.9	90.38
M11	86.2	92.52
M12	73.4	94.022

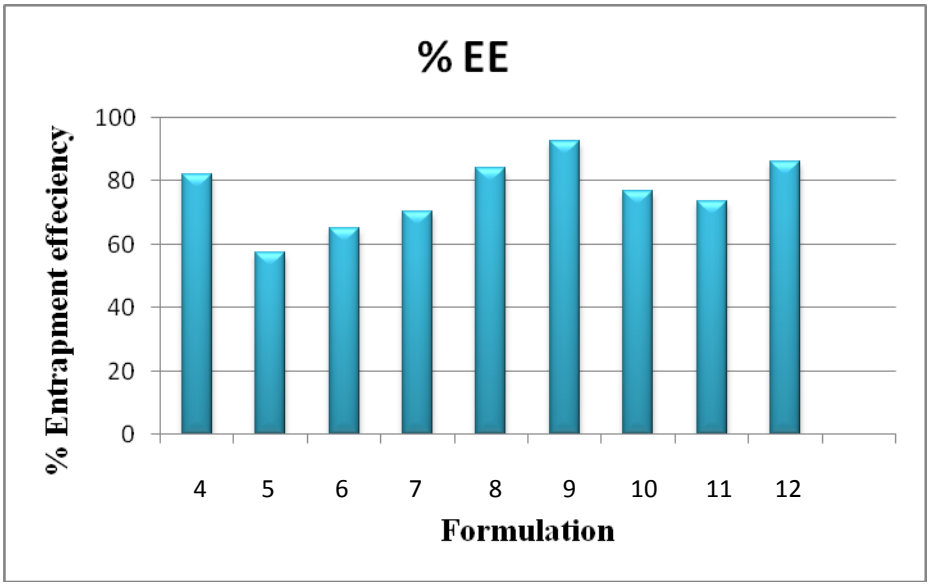

Figure 2:- Entrapment efficiency of microspheres

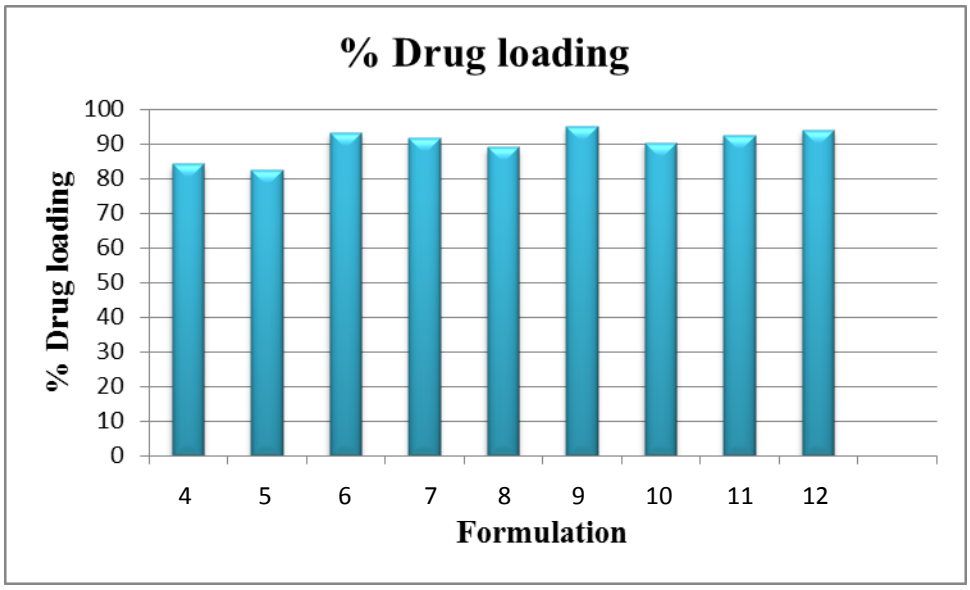

Figure 3:- Percentage Drug loading of microspheres

***In-vitro* drug release studies**

The amount of drug released the formulation M4 of the microspheres was determined by using UV spectroscopy. There was about 89.49 % of drug release observed in 2hours observations. So, it was found suitable for sustain release delivery system. In-vitro drug release of microspheres formulation is shown in table (05) and figure (03).

Table 12: In vitro drug release study of various microspheres formulations

S.no	Formulation code	**Time (min)**		**Phosphate buffer 6.8**					
		5	10	15	30	45	1hrs	1.30hrs	2hrs
1	M4	8.58	17.78	37.83	47.60	70.12	74.05	81.07	89.49
2	M5	8.11	16.83	36.60	46.78	64.13	72.06	76.51	87.29
3	M6	7.68	16.09	35.79	45.73	59.15	71.26	74.78	86.07
4	M7	7.48	15.78	34.39	44.79	55.09	69.55	73.65	81.63
5	M8	6.41	15.34	32.87	40.48	54.07	68.79	71.53	79.41
6	M9	5.48	14.49	28.65	40.65	53.13	67.65	70.83	78.90
7	M10	5.01	13.78	27.48	38.60	52.79	60.27	68.39	74.38
8	M11	4.41	13.22	23.66	38.18	50.13	61.52	69.62	73.29
9	M12	4.30	12.67	24.78	37.29	47.69	62.13	66.38	74.59

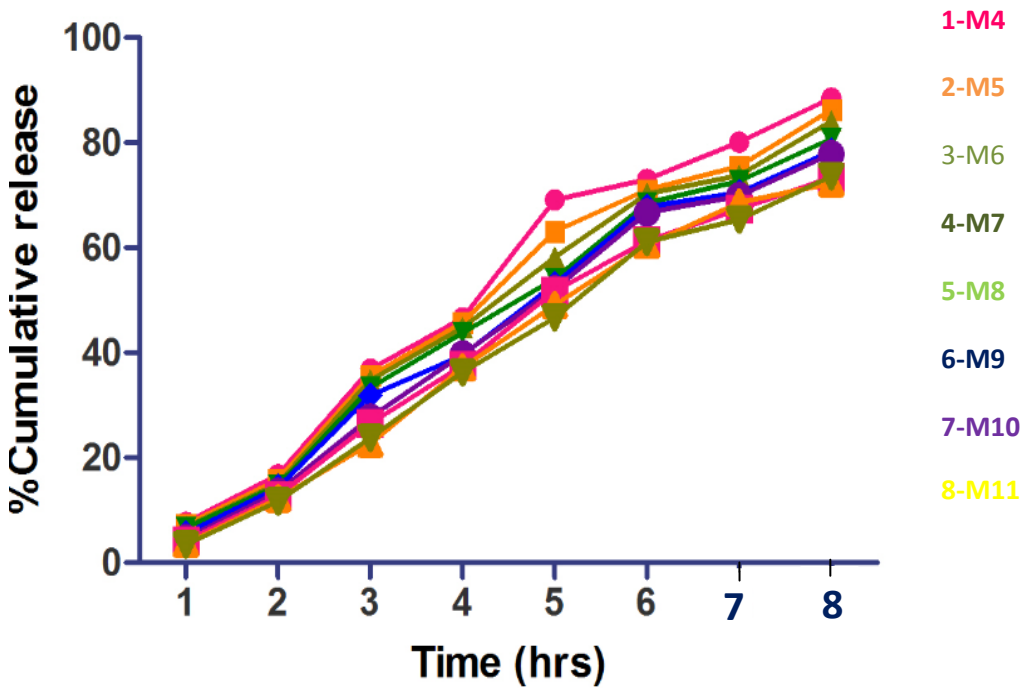

Figure 4: *In-vitro* drug release of microspheres

Formulations prepared by using phosphate buffer 6.8 are analyzed for In-vitro release study and results show that M4 formulations are better than all formulations due to higher release (more than 80%) in 2 hours.

Scanning Elecron Microscopy

The surface morphology of single crosslinked microspheres were selected on the basis of optical microscopy was visualized by scanning electron microscope at 10kv.Scanning electron microscopewas used to evaluate the surface texture shape and sizeof the microspheres.The sample for SEM was prepared by tightly sprinkling the single crosslinked microspheres on a double adhesive tape,which struck to an aluminium stub.They were coated with gold for 10 min.Under vaccum by using SPI sputterM .These samples were scanned and images are as

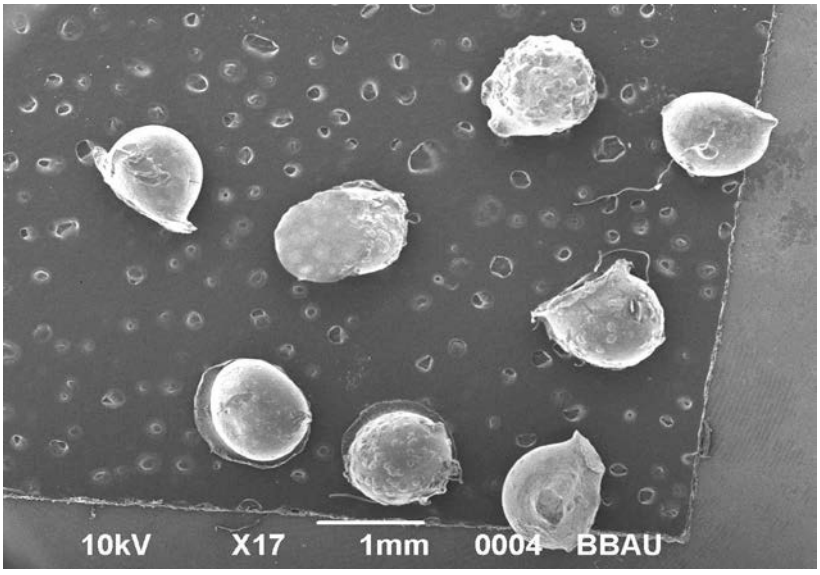

Figure 5:- SEM image of Gelatin microspheres loaded with metronidazole (a)

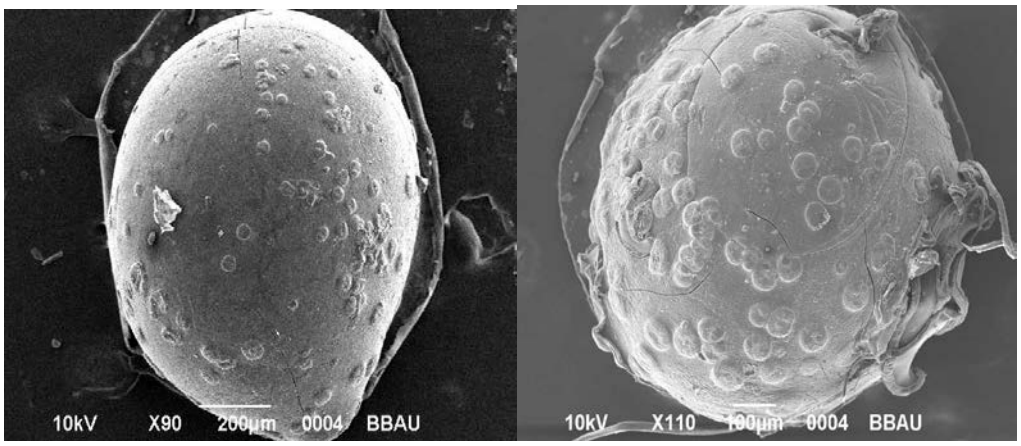

Figure 6: SEM image of Gelatin microspheres loaded with metronidazole (b)

The M9 formulation had smoother surface and spherical in shape when observed in scanning electron micrographs. The gelatin microspheres had 1-30µm size which is smallest and required range.

Table 13: Characterization of bioadhesive gel incorporated metronidazole microspheres

S.No.	Evaluation parameters	Observation
1	Surface pH	6.8
2	Viscosity(centipoises)	3000±0.08
3	Drug content(%)	98.25±0.04

±SD, n=6

In-vitro drug release studies of bioadhesive gel

The amount of drug released the formulation M8 of the bioadhesive gel was determined by using diffusion cell apparatus. There was about 89.6 % of drug release observed in 8hours observations. So, it was found suitable for controlled release delivery system. In-vitro drug release of bioadhesive gel formulation (M8) is shown in table (07) and figure (06).

Table 14: *In-vitro* drug release of bioadhesive gel formulation (M8)

Time(hour)	% Drug release
1	57.32
2	65.73
3	70.41
4	75.19
5	78.2
6	81.3
7	84.5
8	89.6

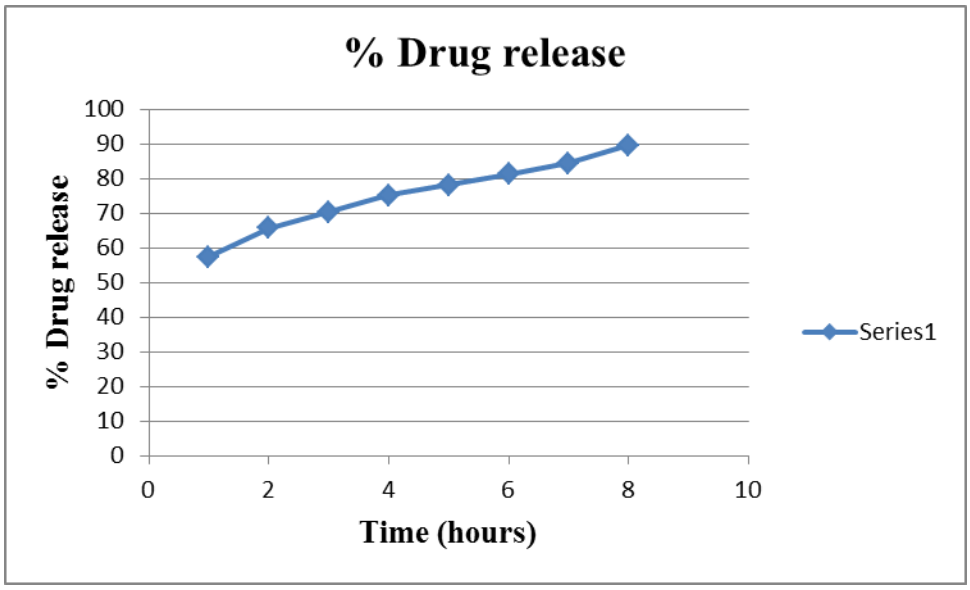

Figure 7: *In-vitro* drug release of bioadhesive gel (M8)

CONCLUSION:

The proposed research work was aimed for the ''Formulation and evaluation of bioadhesive gel incorporated loaded microspheres for periodontal therapy". The drug of choice selected for study was metronidazole which is macrolideanti bacterial drug. Metronidazole is bactericidal against *Actinobacillusactinomycetemcomitans, Porphyromonasgingivalis, Prevotellaintermedia, Bacteroidesforsythus* which are the main causes of periodontitis. The proposed methodology may enable the development of bioadhesive gel for local therapy in periodontal pockets. High percent of drug loading in incorporated loaded microspheres is anticipated to achieve effective therapeutic level of antibiotics. The osteoconductive and ostheophilic property of the incorporated loaded microspheres carriers may fill the pockets to promote tissue regeneration. Further bioadhesivegel provide desirable combination of properties.

In Major project bioadhesive gel formation of incorporated loaded microspheres with metronidazole was done. Total 14 formulations were prepared and optimized by response surface methodology. Among them M9 microspheres formulation was found to be best one with its average particle size 232.20 nm, drug loading 95.23% , entrapment efficiency 92.7%. Due to its small particle size and effective drug release, this system was found to achieve improved therapeutic efficacy and controlled release. The pH, viscosity of bioadhesive gel was 6.8, 3000cps obtained. This work will result in the development of novel formulation ensuring improved safety profile and reduced side effect, improve local and prolonged drug delivery in for periodontitis.

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