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PREPARATION AND EVALUATION OF ADIPIC DIHYDRAZIDE CROSS-LINKED HYALURONIC ACID MICROSPHERES FOR CEPHALEXIN

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ABSTRACT
Hyaluronic acid also called as Hyaluronan, Sodium Hyaluronate (SA), sodium salt form of Hyaluronic acid is a biodegradable, biocompatible, and viscoelastic linear polysaccharide of a wide molecular weight range (1000 to 10,000,000 Da). In this project, described a method for preparing HA microspheres at different pH conditions by adapting a non-toxic and aqueous based crosslinking chemistry for sustained drug delivery of drugs. The derivatization chemistry of HA utilizing adipic dihydrazide has been used to construct hydrogels, applied for microsphere preparation. ADH was coupled efficiently to carbodimide-activated glucoronic acid residues of hyluronans. These ADH modified hyaluronan can be loaded with drug molecules and then cross linked into hydrogel. The drug was present in the bulk of hydrogel droplets which are present in liquid paraffin are precipitated by IPA. Formulating HA microspheres with this method have several advantages. Preliminary studies were conducted to confirm the better ratio of HA and ADH to show maximum entrapment efficiency and drug release. Then microspheres were prepared at different pH conditions and formulations were subjected to evaluation of various parameters like percentage yield, particle size, drug entrapment efficiency, porosity and bulk density, surface morphology, in vitro drug release among which F2B was optimized as best formulation which showed 74.6% entrapment efficiency and above 90% of drug release in 12 hours indicating Hyaluronic acid microspheres can be used as good carriers for sustained drug delivery of drugs.

INTRODUCTION

"Microspheres can be defined as solid, approximately spherical particles with a diameter ranging from 1 to 1000µm, containing dispersed drug in either solution (or) microcrystalline form" [1]. The core material, defined as the specific material to be coated, can be liquid or solid in nature. The coating material should be

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capable of forming a film that is cohesive with the core materials, be chemically compatible and non-reactive with the core material and provide the desired coating properties such as strength, flexibility impermeability, optical properties and stability. Microcapsules should have desirable physical properties like ability to flow, to be compacted or to be suspended and the capsule wall must be capable of resisting the pressure during compression etc.

Hyaluronic acid and Sodium Hyaluronate (SA) sodium salt form of Hyaluronic acid, is a biodegradable, biocompatible, and viscoelastic linear polysaccharide of a wide molecular weight range (1000 to 10,000,000 Da) [2]. It is composed of alternating disaccharide units of d-glucuronic acid and N-acetyl-d-glucosamine with (1→4) inter glycosidic linkage and is distributed throughout the extracellular matrix, connective tissues, and organs of all higher animals [2,3] . Metabolism of HA occurs through the enzymatic hydrolysis by hyaluronidase which is present in various mammalian tissues. The HA molecule is readily soluble in water, producing a gel that behaves like a lubricant. It also absorbs water, lending it hygroscopic and homeostatic properties. The viscosity of the HA gel is dependent upon a number of factors including the length of the chain and by extension and the degree of entanglement, cross-linking, pH and chemical modification while the absolute concentration seems to be much less important. Cephalexin is a semisynthetic cephalosporin antibiotic with antimicrobial activity similar to that of Cephaloridine or Cephalothin, but have shorter biological half-life. It is effective against both gram-positive and gram-negative organisms by binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall [4]. Microspheres were formulated using Hyaluronic acid at different pH conditions. ADH was coupled efficiently to carbodiamide-activated glucoronic acid residues of hyluronans. These ADH modified hyaluronan loaded with drug molecules and then cross linked into hydrogel which has given best results for prompt use of hyaluronic acid microspheres for sustained drug delivery of drugs.

MATERIAL AND METHOD

Cephalexin is obtained as gift sample of Aurobindo labs, Hyaluronic acid, Adipic dihydrazide and EDCI are obtained from Corpuscle research solutions, Light liquid paraffin, Hcl and other solvents are purchased from Merck chemicals, Span 80 is purchased from Loba chemicals.

Melting point determination:

Melting point of Cephalexin was determined by "Thiele's tube apparatus" to know the purity of the sample. Oil was poured into the Thiele's tube, and then the handle was heated by a small flame. Sample in a sealed capillary, attached to a thermometer with a rubber band, was immersed in the tube. Heating was continued, and the temperature ranges at which the sample melted was observed and noted.

Preliminary studies for selection of polymer concentration:

Three formulations were prepared by using different ratios of hyaluronan and adipic dihyrazide keeping the drug concentration constant at constant pH 2.5 using 0.1N HCl

Evaluation parameters like entrapment efficiency, Invitro drug release for the formulated microspheres were done and F2 was selected for further study.

Table 1: Preliminary studies for selection of polymer concentration

Formulation code	Drug (mg)	HA and ADH ratio	Span 80 (ml)	Mineral oil (ml)
F1	100	1:1	1	80
F2	100	1:2	1	80
F3	100	1:4	1	80

Preparation of microspheres

Table 2: Formulation table

Formulation code	Drug (mg)	pH	HA and ADH ratio	Span 80 (ml)	Mineral oil (ml)
F2A	100	3	1:2	1	80
F2B	100	4	1:2	1	80
F2C	100	5	1:2	1	80
F2D	100	6	1:2	1	80

HA-Cefalexine microspheres were prepared by a modified emulsion cross linking technique [3,5]. A water-in-oil emulsion was formed by homogenizing 20ml of 0.5%hyaluronan solution with dissolved adipic di hydrazide (ADH), 80ml of mineral oil and 1ml span 80. This solution was mixed for 30min at 1000rpm using a mechanical stirrer fitted with a 0.75cm in diameter impeller. A solution of drug was added, the emulsion was stirred for an additional 20min. Afterwards, the crosslinking agent, Ethyl-3-[3-dimethylamino] propyl carbodimide (EDCI) dissolved in 2ml of distilled water and was slowly added and

mixed for 30min. The addition of Hydrochloric acid (HCl, 0.1N) was subsequently added to maintain required pH and initiated the crosslinking of the HA microspheres. The chemical reaction, at room temperature, was allowed to proceed for 24h. The HA-cefalexine microspheres were precipitated by the addition of 150ml of isopropyl alcohol (IPA) under vigorous agitation. These microspheres were centrifuged at 1500rpm for 5min. The supernatant was discarded and the microspheres were washed three times by centrifugation with IPA (1500rpm for 5min)

After the final wash, the microspheres were resuspended in a reagent mixture containing ADH and EDCI (1:1) dissolved in 100ml of 90% IPA. This mixture was gently stirred using a stir bar and magnetic plate. HCl (0.1N) was subsequently added to maintain required pH to initiate the second crosslinking reaction. After 24h, the microspheres were collected by centrifugation and washed three times with 90%IPA to remove the traces of mineral oil which may affect the drug release from microspheres. They were subjected for drying.

Evaluation of formulated microspheres [4-15]:

Percentage yield [4-8]:

It was calculated using the weight of spheres recovered from each batch in relation to the sum of starting material. Percentage yield of prepared spheres was calculated by using the formula

$$percentage\ yield = \frac{practical\ yield}{theoritical\ yield} \times 100$$

Bulk density and porosity [4]**:**

The bulk density and porosity of the prepared microspheres were determined according the standard procedures mentioned in I.P.

Particle size determination [7,8]***:***

Microspheres size was measured by optical microscopy. The mean diameter was determined by measuring the number of divisions covered by microspheres using ocular micrometer previously calibrated using stage micrometer.

Drug entrapment efficiency [7,8]**:**

5mg spheres of each batch were placed in 10ml phosphate buffer pH 7.4 and mechanically agitated on shaker at 200rpm for 24hrs. The resultant solution was filtered and analyzed at 261nm using UV visible spectrophotometer. The percentage drug entrapment efficiency (%EE) of microspheres was calculated by using the following equation

$$\%EE = {}^{AQ}/_{TQ} \times 100$$

where, AQ is actual drug content and TQ is theoretical drug content.

Surface Morphology [7-15]:

In order to obtain information on the shape and surface structure of the material, SEM analysis was performed.

In Vitro drug release [7-15]**:**

The dissolution studies of the microspheres equivalent to 100mg of cephalexin were performed using USP dissolution apparatus II. The drug release study was carried out in pH 7.4 phosphate buffer up to 12hrs, maintained at 37±2ºC and agitated at 100rpm. Samples were collected periodically and replaced with fresh dissolution media. After filtration through muslin cloth, concentration of cephalexin was determined spectrophotometrically at 261nm.

RESULTS AND DISCUSSION

Melting point determination:

The melting point of obtained drug sample was found to be 196ºC which is within the reported range 196- 198ºC. It complies with the purity of the drug sample.

Standard calibration curve of cephalexin:

The absorbance of standard solutions of cephalexin ranging from 2-10µg/ml in pH 7.4 phosphate buffer was observed and the standard calibration curve for Cephalexin was constructed.

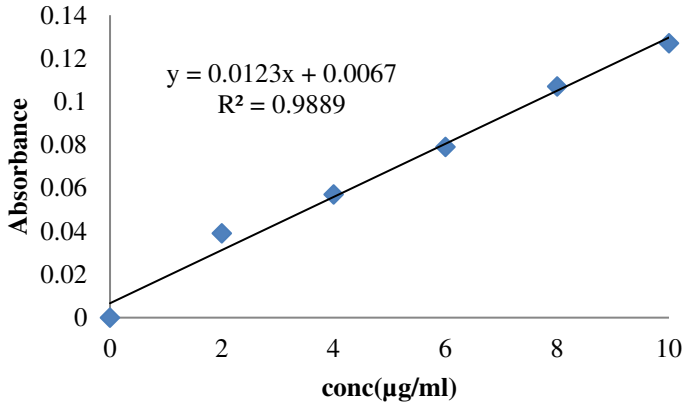

Figure 1: Calibration curve of Cephalexin

Preliminary studies for selection of ratio of HA And ADH ratio for preparation of microspheres:

Based upon the results of preliminary studies, the F2 formulation was selected for further studies as it showed higher entrapment

efficiency of 64.56% and highest In vitro drug release of 54.883% within 10hrs.

Table 3: %EE and %DR of F1, F2, &F3

Formulation	% EE	% DR in 10hrs.
F1	52.78	50.45
F2	64.56	54.883
F3	55.34	49.22

Percentage yield:

Percentage yield of all formulations was calculated and found to be ranging from 27.47 to 73.86%.

Bulk density and Porosity:

Bulk densities of microspheres ranges from 1.73±0.05 to 0.72±0.07g/cc. The bulk density of F2C and F2D microspheres were less as compared with microspheres and F2B. The decrease in bulk density was observed with increase in size and porosity.

Table 4: Evaluation results of microspheres

Formulation code	HA and ADH ratio	% yield	Bulk density (g/cc)	Porosity	Particle size (µm)	%EE
F2A	1:2	44.2	1.73±0.05	14.55	9.46±0.058	64.5623
F2B	1:2	73.86	1.42±0.17	18.35	9.86±0.069	74.6576
F2C	1:2	36.29	0.91±0.27	28.34	10.04±0.068	47.3760
F2D	1:2	27.47	0.78±0.13	32.12	10.65±0.064	20.556

Particle size determination:

The drug loaded microspheres were spherical in shape. The size of microspheres were in the range of 9.46 to 10.65µm. The particle size increased with the increase of pH. This can be attributed to the distention of drug loaded HA gel spheres due to entrapped gas bubbles. The increased pH may be responsible for softening of spheres leading to deformation under force of agitation.

Drug entrapment efficiency:

Entrapment efficiency was more at pH 4. Batch F2D prepared at pH 6 showed lowest drug entrapment than other batches having acidified crosslinking solution. It may be due to decreased crosslinking efficiency at higher pH.

Surface Morphology:

The water-in-oil emulsion of HA, Cefalexin and mineral oil that was cross linked with adipic dihydrazide produced microspheres with spherical conformation and heterogeneous size distribution. The median and mean diameters HA- Cefalexin microspheres were 6 and 10µm respectively, as indicated by particle size analyser.

Drug release studies:

The dissolution study of all formulations of cefalexin was carried out in phosphate buffer pH 7.4. Almost 50% drug released in 6hrs and with in12hrs 90% drug released in all formulations prepared at different pH conditions. Among all pH conditions selected for microspheres preparation, pH 4 used for f2B formulation shown highest drug release.

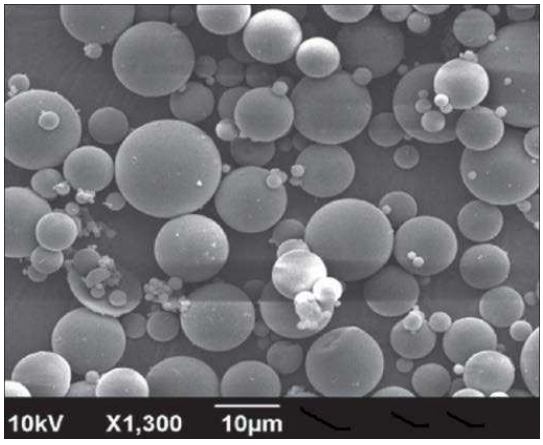

Figure 2: SEM of HA-Cefalexin microspheres of F2B

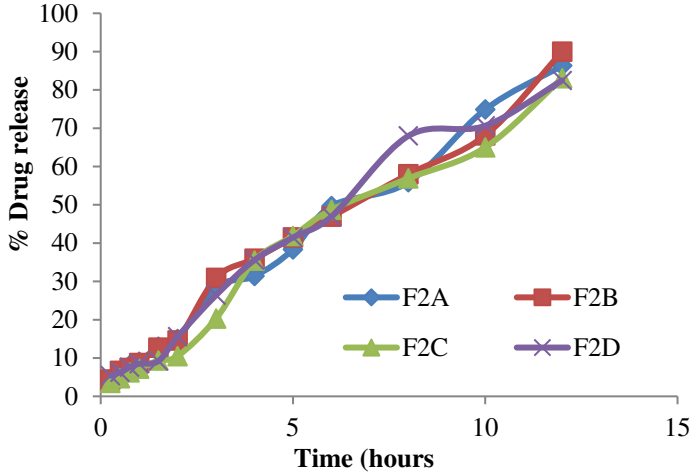

Figure 3: In Vitro dissolution profile

Drug release kinetics:

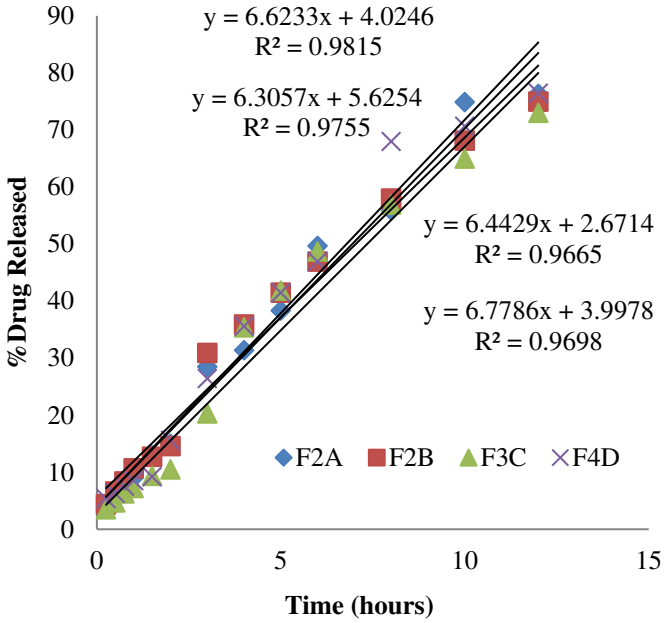

Figure 4: Zero order plot for all formulations

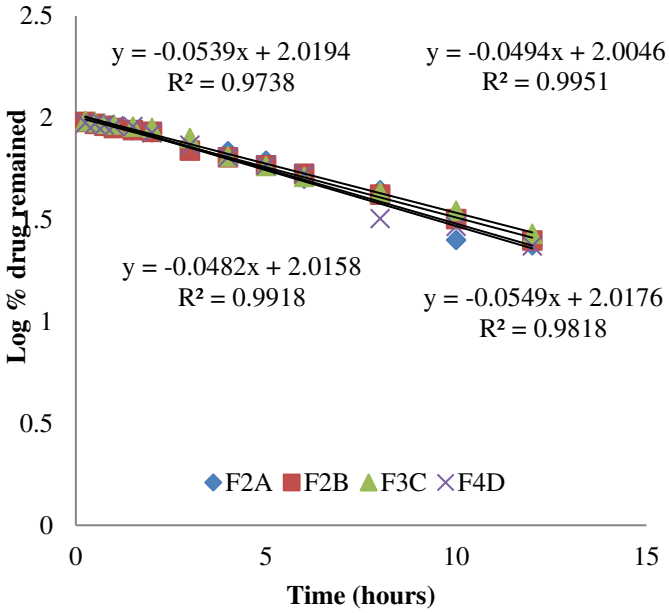

Figure 5: First order plot for all formulations

Table 5: R^2-values of all plots

Formulation code	Zero order R^2	First order R^2	Higuchi R^2	Korsmeyer Pappas	
				R^2	n value
F2A	0.9815	0.9738	0.9709	0.9847	0.8042
F2B	0.9755	0.9951	0.9833	0.9839	0.7842
F2C	0.9655	0.9918	0.968	0.9657	0.8814
F2D	0.9698	0.9818	0.9669	0.9535	0.7999

Table No.6: 'n' value in Korsmeyer Peppas plot

Release exponent (n)	Drug transport mechanism
<0.45	Fickian transport
0.45-0.89	Non fickian transport
>0.89	Case II trans port

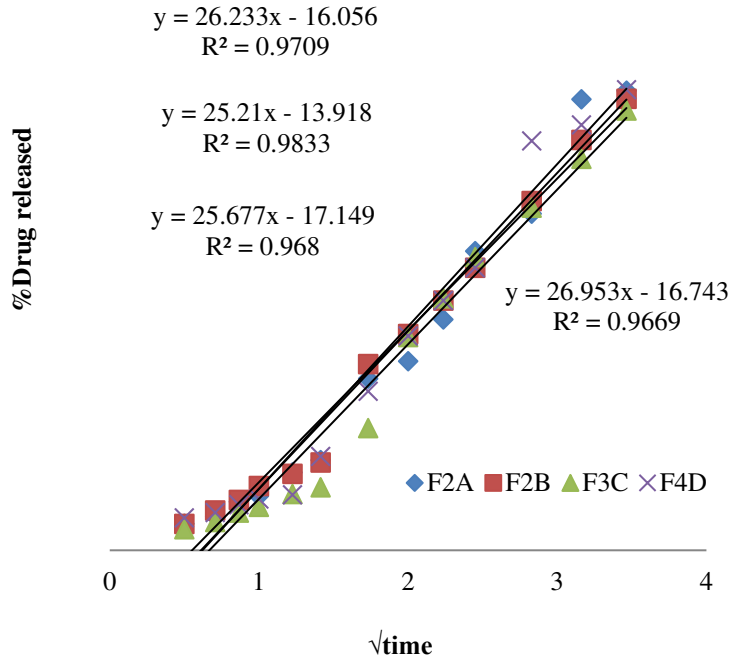

Figure 6: Higuchi plot for all formulations

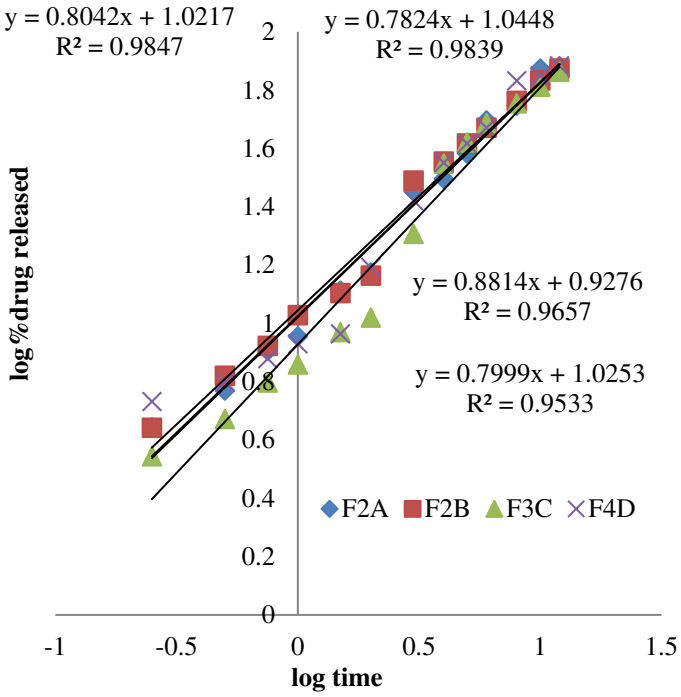

Figure 7: Korsmeyer Peppas plot for all formulations

Based on the r^2 values of the zero order and first order drug release plots and as the 'n' value in of the all formulations is in between 0.45 -0.89, it can be concluded that the optimized formulation is showing first order drug release and following non fickian transport of release mechanism i.e., diffusion with swelling and relaxation of polymer [16].

CONCLUSION

An attempt was made to prepare HA microspheres by adapting a non-toxic and aqueous based cross linking chemistry for sustained drug delivery of drugs. The selected drug Cephalexin was successfully loaded in HA microspheres and the encapsulation efficiency was found to be nearly 75% depending on the ratio of HA and ADH for microspheres. It was also observed that change in pH condition during preparation effected %EE but not the % drug release due to proper washings. Observing the all evaluation tests results, among all pH conditions selected for microspheres preparation, pH 4 used for f2B formulation was found to be acceptable.

All formulations prepared were showing sustained release of drug according to USP up to 12hrs. From the optimized formulation the drug release was following first order and non fickian transport of release mechanism as the 'n' value in the korsmeyer peppas model is 0.7842. So it can be concluded that nontoxic and aqueous based crosslinking method can be adaptable for Hyaluronic acid microspheres preparation and these microspheres can be used as perfect vehicle for sustained delivery of drugs.

FINANCIAL ASSISTANCE

Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Budumuru Padmasri was involved in the design of the work, experimental part of the wok, in literature survey and manuscript preparation. Rambilli Kalyani assisted in the experimentation and involved in analysis of data and observations. Vagada Anilkumar contributed thoughts and efforts for the publication of the work done. Damarasingu Prasanth supported in experimental studies and manuscript preparation. Majji Indu assisted in literature survey.

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