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Research Article

NOVEL FORMULATION OF RECOMBINANT HUMAN ERYTHROPOIETIN NANOPARTICLES FOR SAFE AND EFFECTIVE RELEASE

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ABSTRACT

Recombinant Human Erythropoietin (rHu-EPO) are the drugs which stimulates the bone marrow to produce red blood cells in the body. rHu-EPO is used as erythropoietin stimulating agents in the treatment of renal and chemotherapy induced anemia. rHu-EPO and its analogues currently available in the market is in parenteral dosage form which is readymade injection preparation (syringe) which needs to be administered 2-3 times weekly to achieve the therapeutic effect of parenterally administered rHu-EPO. These cumulative doses exceed the levels of endogenous Erythropoetin (EPO) which may cause unspecific binding to non-targeted tissue and may lead to severe side effects. In this study we incorporate rHu-EPO in polymeric nanoparticles which release rHu-EPO in systemic circulation for 14 days after single dose of rHu-EPO nanoparticles. Produced nanoparticles were appeared in the range of 128.2±5.3 nm which was spherical in shape and entrapped ~44% rHu-EPO. *In-vitro* release was ~62% within the first week and~81% up-to two-week period. Biological activity performed on albino mice. Single 150 IU and 300 IU dose of rHu-EPO nanoparticles preparation showed improvement in RBC and Hemoglobin rate up to 2 weeks and only up to 4 days after pure rHu-EPO solution and marketed preparation (Eprex).

Keywords: Recombinant Human Erythropoietin, Nanoparticle, Prolong drug release, Anemia

1. INTRODUCTION

Erythropoietin (EPO) is a glycoprotein cytokine and hormone which consists 165 amino acids having the molecular weight 30-36 kDa approximately. Erythropoietin is the primary regulator of erythropoiesis which stimulates production of red blood cells and controls erythropoiesis thus it is acts as a cytokine for erythrocyte precursors in the bone marrow. EPO stimulate the production of red blood cells in response to hypoxemia. Erythropoietin hormone is produced in the kidney by interstitial fibroblasts in association with proximal convoluted tubule and peritubular capillary. It is also produced in a small amount in perisinusoidal cells in the liver [1]. Erythropoietin stimulates the production of red blood cells in hypoxic condition and thus increases the hemoglobin concentration. Thus EPO is used in the treatment of anemia in patients with chronic kidney diseases and chemotherapy induced anemia EPO raise and maintain the hemoglobin level in the patient with chronic kidney disease. EPO is also used in treatment of cancer, HIV and certain neurological disorder like schizophrenia. Researches and trials have also proven that EPO improves the survival rate of children's suffering from cerebral malaria, which is caused by malaria parasites [2]. The worldwide market of EPO drugs is increasing by increasing number of cancer induced anemic patients, HIV patients and ESRD treatment. Thus it will also increase the commercialization of EPO biosimilar. Currently, rHuEPO is available in the market in only injectable liquid vial (syringe) form. There is a need of frequent administration of EPO injection required due to its short half-life (8.5 h after intravenous injection) which is very inconvenient and reduces the patients' compliance [3]. Frequent administration of EPO injection increases the level of endogenous EPO which may induce severe side effects such as prolonged the circulation times of EPO and thus unspecific bindings to non-targeted tissue. It may increase the chance of growth of tumor which may lead to death also. Encapsulation of rHuEPO in a nanoparticulate delivery system increases the half-life of drug by improving its stability and also prevents the proteolytic degradation [4]. Encapsulation of rHuEPO in a nanoparticulate delivery system may also reduce its side effects [5] such as high blood pressure, platelet activation, polycythemia, stroke or myocardial infarction, and pulmonary emboli [6].

Nanoparticles are the colloidal solid submicron sized drug carrier in the size ranging from 10-1000nm [7]. Small size nanoparticles get high surface area due to this there is dramatic increase in the tissue penetration ability and also increase cellular uptake [8]. The absorption of drugs in gastrointestinal tract cause by various sites depend on size of particle. Particles having 1 µm diameter are absorb by phagocytosis through intestinal macrophages, Particles of size <10 µm in diameter are transported by Peyer's patches and nanoparticles of size <200 nm are absorbed via endocytosis [9]. Encapsulation of potent protein drug in nanoparticle increases half-life of drug and also improve the stability by preventing proteolytic degradation [10]. Nanoparticles must be stable, biodegradable and release drug for longer time with targeted drug delivery thus dose frequency can be minimized with nanoparticle and ultimately improve quality of life of the patients [11]. Study proven experimentally that, for the therapeutic and imaging applications, nanoparticles in size range of 2 to 1000 nm should be applicable for different molecules such as small drugs, proteins, vaccines or nucleic acids [12, 13]

Polymeric nanoparticles can be prepared by various methods such as Solvent evaporation, Nanoprecipitation, Emulsification or solvent diffusion method, salting out method, Dialysis method, Supercritical fluid technology, Reverse micellar methods and Ionotropic Gelation Method [14].

rHu-EPO is a protein drug thus to prevent denaturation of drug, it requires minimum stress condition and minimum and safe use chemical during preparation of nanoparticles. Thus for the preparation of rHu-EPO nanoparticles Ionotropic gelation is best suitable method due to its simplicity, lack of possible toxic reagents and process have to be performed under mild conditions thus degradation of drug can be prevented [15, 16]. Ionotropic gelation is the method in which polyelectrolyte polymer is cross linked with counter ion. Many of the researcher have focused on the preparation of chitosan-tripolyphosphate nanoparticles for various peptides and protein drugs because of the advantage that the process is done under mild condition without any stress to drug [17, 18].

Thus, the present invention is an attempt to overcome the aforesaid problems by developing the nanoparticulate delivery systems of rHu-EPO, which prolong the release of rHu-EPO in a low dose and the side effects related to high EPO levels by parenteral administration can be reduced.

2. MATERIAL AND METHODS

Recombinant Human Erythropoietin was purchased from Sigma Aldrich. Chitosan (Medium molecular weight), Cross linking agent Sodium Tripolyphosphate were also purchased from Sigma Aldrich. Other agent like Sodium Hydroxide, Tween 80, Acetic acid, Potassium Dihydrogen Phosphate, Mannitol, etc. were received from department store.

2.1. Preparation of rHu-EPO nanoparticles by Ionotropic gelation method

Different concentrations of Chitosan solutions (0.25%, 0.5%, 0.75% and 1%) were prepared by dissolving chitosan in 1% acetic acid. 0.5% Tween 80 was added to the solution. The pH of chitosan solution was adjusted to 5.5 by using 5N NaOH. 0.25ml of rHu-EPO solution (2 mg/ml) were added. Different concentrations of Sodium tripolyphosphate (STPP) solution (0.1%, 0.125%, 0.15%, 0.175% and 0.2%) were prepared by dissolving STTP in Milli-Q purified water. Nanoparticles were prepared through ionotropic gelation technique by flush mixing of different concentration of TPP into 5 ml chitosan solution with continuous stirring for 30 minutes at room temperature. Light blueish turbid solution was obtained which indicate that there was cross linking of polymers and formation of nanoparticles. The nanoparticles centrifuged at 4°C at 12000 rpm for 30 min. Lyophilized powder obtained after washing of pallet deposited on bottom with mannitol 2% solution and kept for lyophilization in Freeze dryer [19, 20].

2.1.1. Evaluation of the prepared nanoparticles

2.1.1.1. Average Particle Size and Polydispersity index (PDI)

Particle size and Polydispersity index (PDI) of the nanodispersion were measured by using the Malvern Zetasizer (Version 6.32) by photon correlation spectroscopy. Probe sonicated nanodispersion was used for the measurement of average particle size at ambient temperature with angle of detection 90°. PDI measures size distribution of nanoparticles population [21].

2.1.1.2. Measurement of zeta potential

Particle charge was determined by using Zeta meter system 4.0 [22]

2.1.1.3. Morphology of nanoparticles

Morphology of nanoparticles was examined under Scanning Electron Microscope (Zeiss SEM EVO-18, Carl Zeiss Microscopy). Drop of nanodispersion were spread on a glass slide and allow to dry overnight. Prepared slides were mounted on carbon tape. The particles on the

dried glass slide were subjected to gold sputtering and the slide was attached on SEM sample holder using a double side carbon tape mounted on an aluminum stud. The SEM photomicrographs were captured by operating at an accelerating voltage of 20 kV electron beam at desired magnification [23].

2.1.1.4. Entrapment efficiency

Nanoparticles were separated from dispersion by centrifugation at 15,000 rpm for 30 min. The supernatant obtained after centrifugation was suitably diluted and analyzed for free erythropoietin by using Bradford protein estimation method. Bradford Kit for protein estimation was used to determine the entrapment efficiency. This Assay is based upon the formation of complexes between Coomassie Brilliant Blue G-250 dye and protein present in sample. When the complexation was occurred, the color of dye was change from brown to blue. There was a shift in the absorption maximum of the dye from 465 nm to 595 nm. The blue color complex was stable for 1 hour. Thus concentration of unknown protein sample was determined by plotting absorbance value on the standard curve. [24]

% Entrapment efficiency = {([Drug] total - [Drug] supernatant)/ [Drug] total} × 100

2.1.1.5. In-vitro release study of rHu-EPO nanoparticles

Nanoparticles with weight equivalent to 300 mcg of rHu-EPO were dispersed in 10ml phosphate buffer solution (pH 7.2). Dispersion was incubated the in a biological shaker at 37°C temperature at 80 rpm speed. One ml of sample was withdrawn after 1, 2, 3, 4, 6, 8, 12, 24, 48 and after each 48 hours up to 2 weeks and sample were replaced with 1 ml of fresh medium at 37°C. Withdrawn samples were centrifuged for 30 min at 12000 rpm at 4°C. The rHu-EPO concentration in each sample from the supernatant was determined by previously mentioned Bradford method [25].

2.1.1.6. Evaluation the biological activity of the EPO-loaded nanoparticles

To determine the *in-vivo* response of prepared erythropoietin nanoparticles, albino mice were used. These mice's will divide in 5 groups. Each group consist 6 animals. Mice were kept at the temperature 25±2°C under 12 hours' dark-light cycle. Following are the group and group wise treatment given [25, 26].

Animal study was done with kind approval (Approval No. IACE/UDPS/2020/48; Dated 04/01/2020) of Animal Ethical Committee of The Department of Pharmaceutical Sciences, R.T.M. Nagpur University Nagpur: Registration No. 92/1999/CPCSEA; Dated: 28/04/1999.

Blood samples were collected by tail vein method after the interval of each day. Blood count analysis (RBC and Hemoglobin) was performed.

Table 1: List of the treatment and dose received by each animal group

Mice Group	Treatment given to mice	Dose
Group 1 (Control)	PBS	0.25 ml
Group 2	Pure EPO Solution	150 IU/0.25 ml
Group 3	Marketed Preparation	150 IU/0.25 ml
Group 4	Suspension of rHu-EPO Nanoparticles	150 IU/0.25 ml
Group 5	Suspension of rHu-EPO Nanoparticles	300 IU/0.25 ml

3. RESULTS AND DISCUSSION

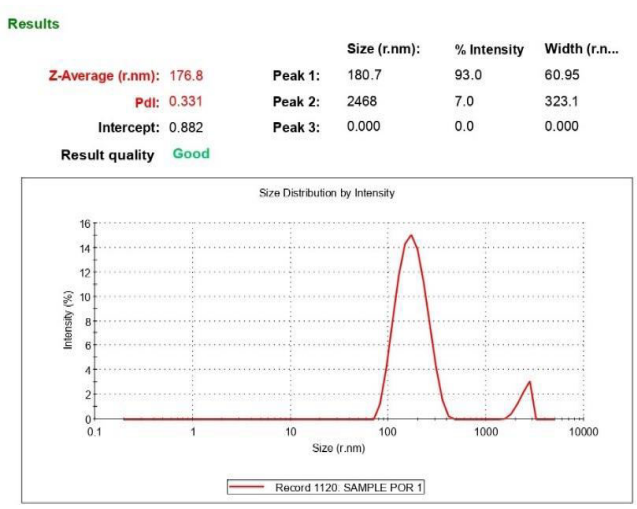
Total 45 batches were prepared using different concentrations of polymer. Eight batches which produce light blue fluorescence shows particle size under 500 nm. The mean particle size diameter for optimized batches were in the range of 120.0 nm to 207 nm with a polydispersity index in the range of 0.224 to 0.416.

The produced rHu-EPO Nanoparticles showed a zeta potential value in the range of 17.4 to 43.2 mV.

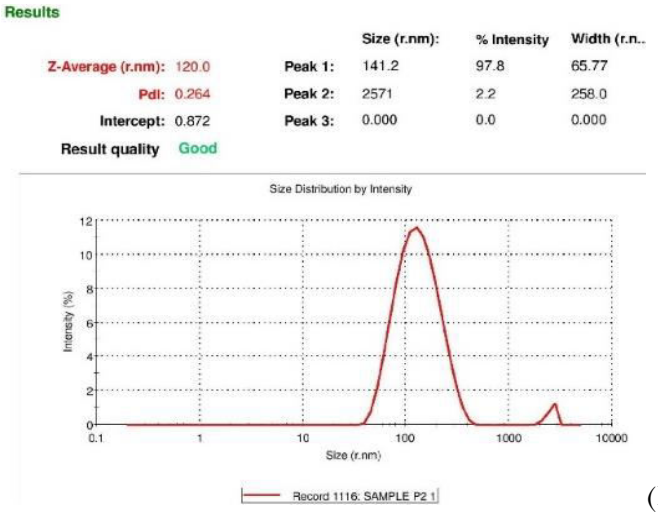
SEM images of rHuEPO nanoparticles presented in fig.5 clear that the nanoparticles were spherical in shape having the smooth and regular surface. There were on aggregation of nanoparticles. The size of nanoparticles found in SEM micrograph was consistent with the particle size obtained by particle size analyzer.

Drug entrapment efficiency of optimized batches were in the range of ~37.1 to 44.6 %. Low entrapment of rHuEPO is may be because of hydrophilic nature of rHu-EPO.

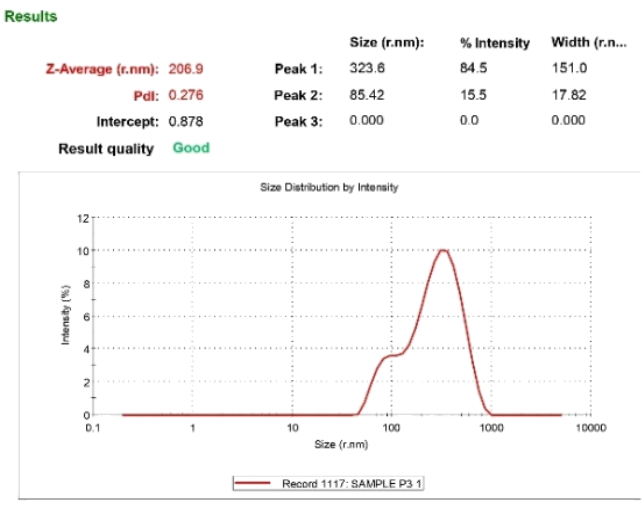
The optimized rHu-EPO nanoparticles released ~26% percent of the encapsulated rHu-EPO in the first 24 hours, ~62% within the first week and~81% within a two-week period. Final optimized formulation having 44% drug entrapment released 62% drug in one week and 81% drug in two-week period. (Bradford Assay).



(A)



(B)



(C)

(D)

Fig. 1: A, B, C, D Particle Size of rHu-EPO Nanoparticles

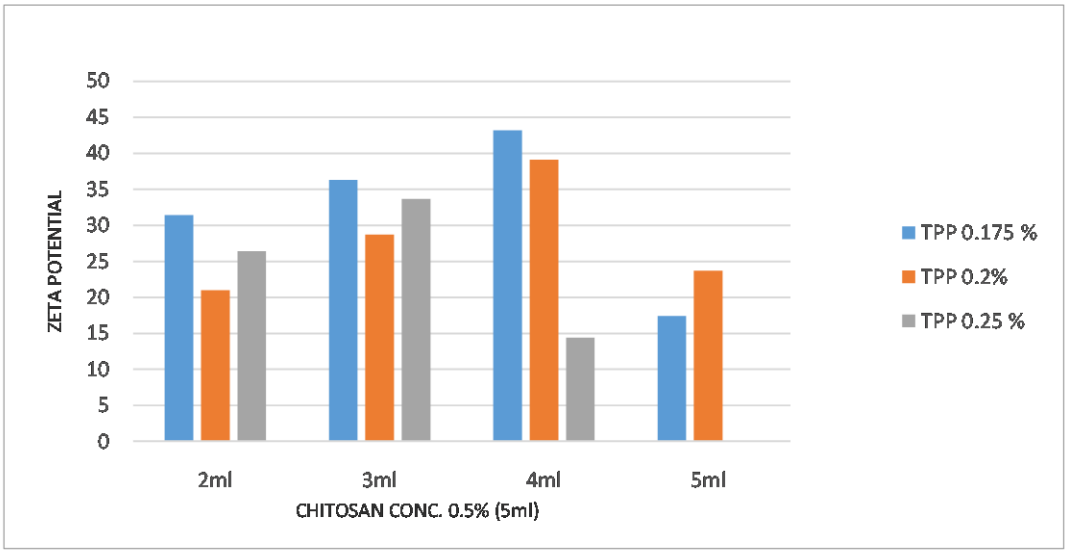


Fig. 2: Zeta potential of rH-uEPO nanoparticles

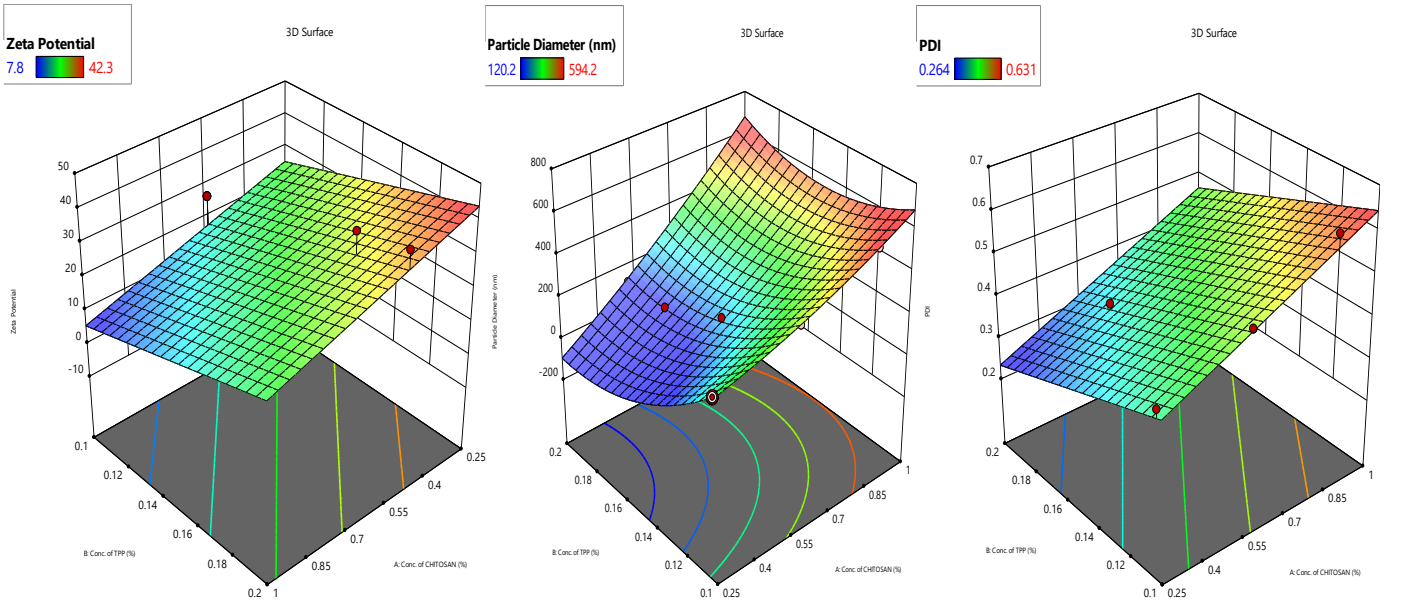


Fig. 3: Response surface 3D models for displaying relationship between concentration of chitosan to concentration of TPP

Factor Coding: Actual

Overlay Plot

Particle Diameter

PDI

Zeta Potential

Design Points

X1 = A

X2 = B

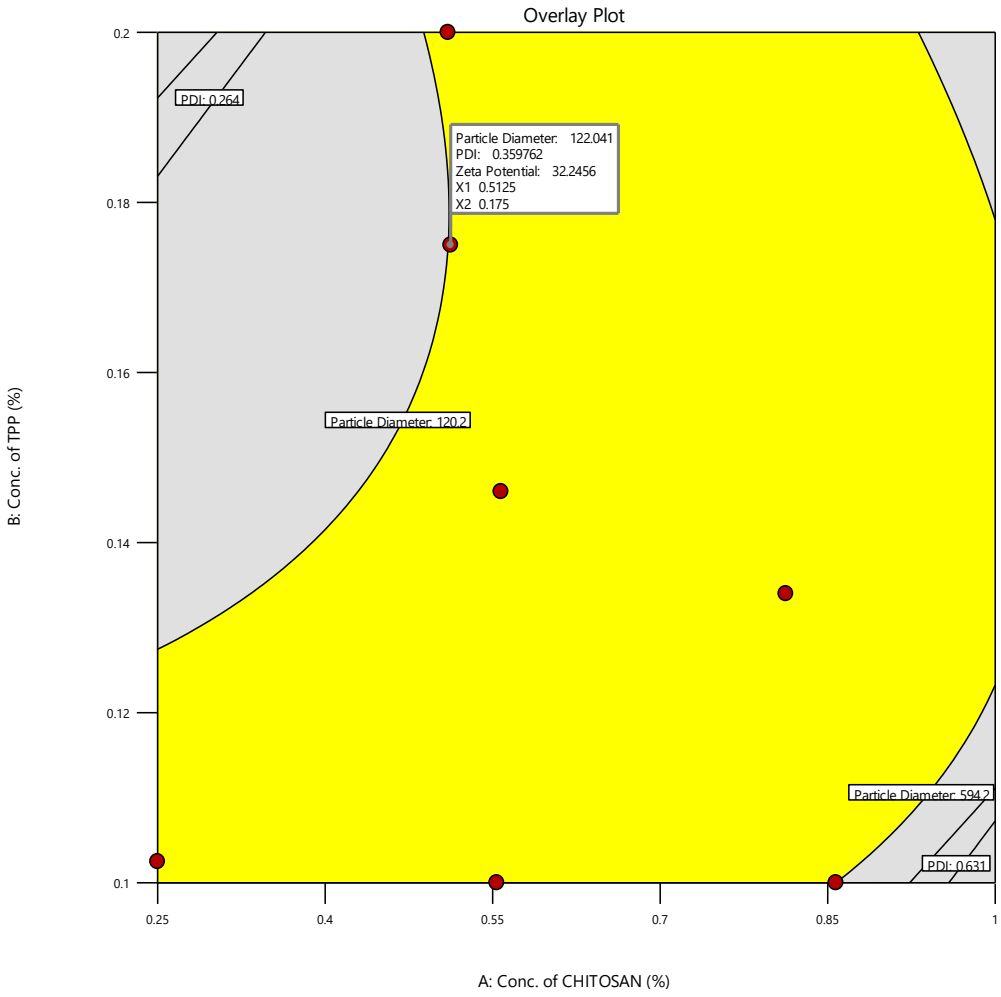


Fig. 4: Overlay plot of Particle Size, PDI and ZP

Animal study shows that there were no significant changes in the group received control (PBS) treatment while pure rHu-EPO solution and marketed preparation shows significant improvement of RBC count up to 6 days but after that it were gradually decrease the RBC count. Fourth and Fifth group which received in different concentration makes improvement in RBC count up to 2 weeks after administration. There was no any significant difference between effect of rHu-EPO NP 150 IU and rHu-EPO NP 300 IU. Hemoglobin content were improved in the group of animal who received EPO or rHu-EPO nanoparticles. rHu-EPO nanoparticles could significantly increase hemoglobin level than EPO solution and marketed preparation.

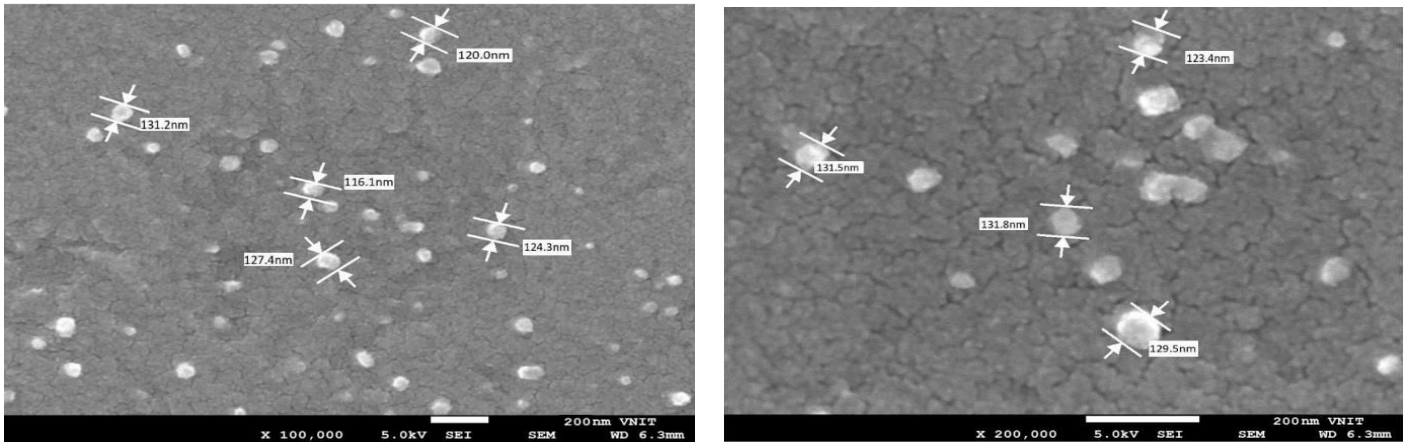


Fig. 5: SEM images of rHuEPO nanoparticles

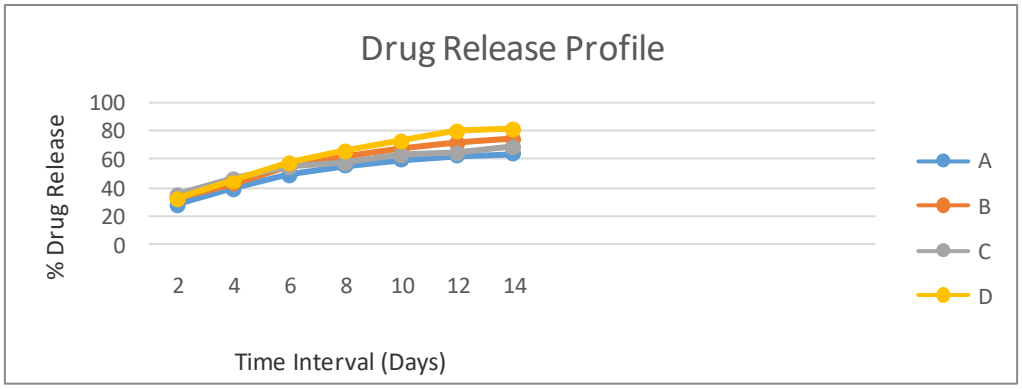


Fig. 6: Cumulative Drug Release Profile of Hu-EPO Nanoparticles

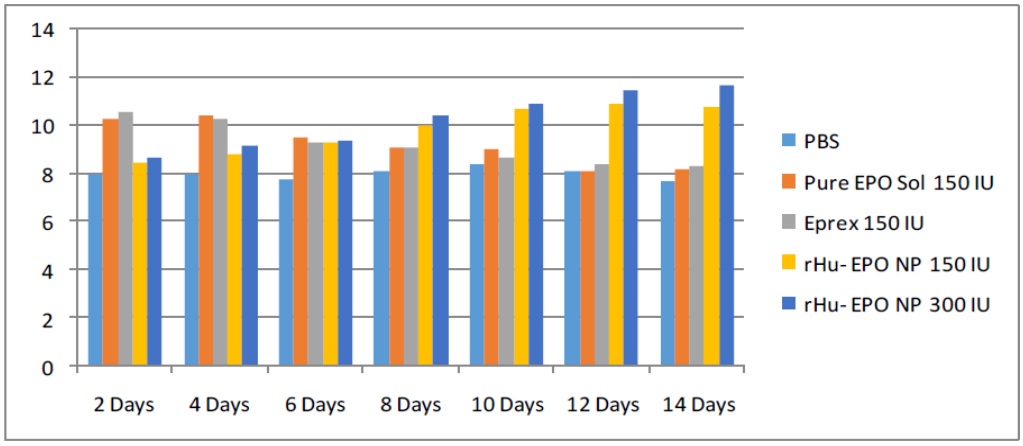


Fig. 7: RBC count of albino mice

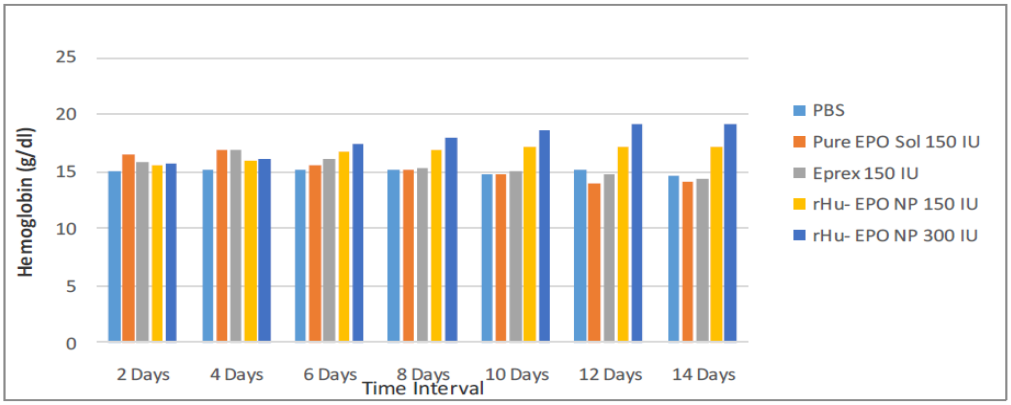


Fig. 8: Hemoglobin count of albino mice

4. CONCLUSION

The nanoparticles prepared by ionotropic gelation method were in the range of 120nm-207 nm with spherical shape. Method was simple with minimum stress condition. Thus drug degradation was too less. Nanoparticles can be only prepared in certain fixed CS: TPP ratio. As the concentration of Chitosan increased beyond the ratio, the aggregation was formed. Accurate response surface models were plotted by using design expert 13 for the nanoparticles characteristics. Encapsulation of rHu-EPO in nanoparticles successfully alters the *in-vitro* as well as *in-vivo* release of rHu-EPO. rHu-EPO release up to 14 days after single administration. Thus, it may be very promising drug delivery for the administration of rHu-EPO in compare to conventional parenteral syringes available in the market.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

1. Wang Z, Goldberg M, Scadden D. *Exp Hematol*, 1993; **21**:683
2. Mateja C, Barbara P, Porekar V, Gaberc S, Mateja N, Spela J, Radovan K, Simon C. *https//www.google.com/patent/ 2015;032981? cl= en &utm_source=gb-gplus-sharePatent WO2015032981A1*
3. Yeh MK, Chen JL, Chiang CH, Chang ZY. *Journal of Microencapsulation*, 2007; **24**:82-93
4. Qi C, Chen Y, Jing QZ, Wang XG. *International Journal of Molecular Sciences*, 2011; **12:**4282-4293.
5. Wissing S., Kayser O., Muller R. *Adv. Drug Deliv.,* 2004; **56**:1257-1272.
6. Boogaerts M. *Current Medical Research and Opinion,* 2006; **22**:S15-S22
7. Lohcharoenkal, W, Wang, L, Chen, YC, Rojanasakul Y. *BioMed Research International*, 2014; **2014**:1-12.
8. Panyam J, Labhasetwar V. *Adv Drug Delivery Rev*, 2003; **55**:329-347.
9. Sharma G, Sharma A, Nam J, George P, Doss C, Lee S. *Journal of Nanobiotechnology,* 2015; **13**:74.
10. Patel J, Chauhan S, Seth A. *International Journal of Drug Discovery and Medical Research*, 2012; **2-1**:56-60.
11. Chakraborty C, Pal S, Doss GP, Wen ZH, Lin CS. *Frontiers in Bioscience,* 2013; **18**:1030-1050.
12. Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V. *A mechanistic approach. J Control Release*, 2006; **116**:1-27.
13. Singh R, Lillard JW. *Exp Mol Pathol, 2009;* **86**:215-223.
14. Nagavarma BVN, Hemant KS Yadav, Ayaz A, Vasudha LS, Shivakumar HG. *Asian Journal of Pharmaceutical And Clinical Research,* 2012; **5**:16-23.
15. Bulmer C, Margaritis A, Xenocostas A. *Biochemical Engineering Journal*, 2012; **68:** 61-69.
16. Fernandez-Urrusuno R, Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. *Pharmaceutical Research,* 1999; **16**:1576-1581.
17. Patil JS, Kamalapur MV, Marapur SC, Kadam DV. Journal *of Nanomaterials and Biostructure,* 2010; **5:**241-248.
18. Gupta VK, Karar PK. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2011; **3**: 2
19. Calvo P, Remuñan C, Vila-Jato JL, et al. *Pharmaceutical Research*, 1997; **14**:1431-1436

20. Sobhani Z, Mohammadi Samani S, Montaseri H, Khezri E. *Advanced Pharmaceutical Bulletin*, 2017; **7-3:** 427-432.
21. Mourdikoudis S, Pallares RM, Thanh NTK. *Nanoscale,* 2018; **10:**12871-12934.
22. Haider M, Mehdi M. *International Journal of Scientific & Engineering Research*, 2014; **5:**381-387.
23. Khaira R, Sharma J, Saini V. *Scientific World Journal*, 2014; **2014**:1-6.
24. Zhang X, Wu Y, Sun K, Tan J. *Experimental and Therapeutic Medicine,* 2014; **7:**1659-1662.
25. Fayed BE, Tawfik AF, Yassin AEB. *Journal of Microencapsulation*, 2012; **29(7)**:650-656.
26. Dara T, Vatanara A, Maybodi MN, Vakilinezhad MA, Malvajerd SS, Vakhshiteh F, Mosadegh MH. *Colloids and Surfaces B: Biointerfaces,* 2019; **178**:307-316.