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IN-VITRO OBSERVATION OF REPAGLINIDE ENGINEERED POLYMERIC NANOPARTICLES

U. M. DHANA LEKSHMI, G. POOVI[a], P. NEELAKANTA REDDY[*]
Bio Organic Chemistry Laboratory, Central Leather Research Institute, (Council of Scientific and Industrial Research), Adyar, Chennai -600020, India.
[a]*Madras Medical College, Chennai, India.*

Nanoparticles (NP) are tiny materials that have specific physicochemical properties different to bulk materials of same composition and such properties make them very attractive for commercial and medical development. The aim of this work was to produce and characterize Repaglinide (Rg) engineered Ethyl Cellulose NP by the solvent evaporation model, in an attempt to obtain a novel delivery system adequate for the treatment of diabetes. Batches were prepared with different ratios of drug and polymer in order to evaluate the influence of drug on NP properties. The absence of any chemical interaction between drug and polymer was confirmed by Fourier Transform Infra Red spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC) and Thermo Gravimetric Analysis (TGA) techniques. X-Ray Diffraction pattern of formulation confirmed the superimposition of polymer and drug with lower intensity. The average diameter of NP determined by Photon Correlation Spectroscopy (PCS) was about 100nm. NP showed 86.4% Encapsulation Efficiency (EE) and 9.61% drug loading. Finally, the *in-vitro* release profile observed for these NP was characterized by a delayed release phase. Questions about the potential haemolytic activity of formulated nanoparticles i.e., cytotoxicity was proved by our results. Interestingly, these results suggest that nano encapsulation of the drug in biodegradable, biocompatible polymer will improve its pharmacological significance.

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Keywords: Repaglinide, Diabetes, Ethyl Cellulose, Nanoparticles, Polymer, Formulation.
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1. Introduction

Nanotechnology is a powerful tool for creating 'smart' materials. This approach is challenging and is still far from being achieved [1]. Nanotechnology is likely to lead to useful advances in medical diagnosis and treatment, and has considerable commercial potential out with medicine [2]. Nanomaterials have been promoted as a revolutionary technology for cell and tissue engineering, medical device development, and the encapsulation and delivery of drugs, diagnostics, and genes. Advances in nanotechnology have led to the introduction of many nanomaterials in these areas, and the Nanomedicine Initiative of the National Institutes of Health Roadmap for Medical Research initiative predicts that nanomaterials will begin yielding significant medical benefits within the next 10 years [3, 4].

In the controlled drug delivery system, the micro/nanoparticulate drug delivery systems offer numerous advantages over the conventional dosage forms. These embrace improved efficacy, reduced toxicity and improved patient compliance [5, 6, 7, 8] and the controlled release polymeric nanoparticles represent an effective nanocarrier platform for the delivery of hydrophobic and hydrophilic drugs, since the drugs are protected from possible degradation by enzymes [9, 10, 11, 12]. The controlled release (CR) of drugs in slow and sustained manner is one of the major

[*]Corresponding author: dhanamum@yahoo.co.in.

challenges in drug delivery system [13, 14]. Recently, this controlled release has become a very useful tool in pharmaceutical area, offering a wide range of actual and perceived advantages to the chronic diseases [15, 16].

In addition, nanoparticles can be prepared to entrap, encapsulate or bind molecules improving the solubility, stability and absorption of several drugs, as well as avoiding the reticuloendothelial system, thus protecting the drug from premature inactivation during its transport [17]. In fact, it has been shown that nanoparticles have the ability to carry various therapeutic agents including DNA, proteins, peptides, and low molecular weight compounds. Among all of them, liposome and polymer-based nanoparticles are the most widely used nanoparticles as drug delivery systems, as these compounds are generally biodegradable, do not accumulate in the body and they are possibly risk-free [18].

Among the various polymers used for the development of sustained release formulations, ethylcellulose has been reported to be advantageous as it is biodegradable and biocompatible [19, 20, 21, 22]. This is a hydrophobic polymer, widely used in pharmaceutical technology, chemically stable under storage, and characterized by a great tolerability and lack of toxicity for patients [23]. Ethyl cellulose, one of the extensively studied encapsulating materials for the controlled release of pharmaceuticals, was selected as the coating material. Several researchers have investigated the utilization of ethyl cellulose as a polymer to encapsulate a drug by coacervation phase separation technique, emulsion solvent evaporation technique and spherical crystallization technique [24, 25, 26]. Ethyl cellulose polymer is often used to make prolonged release dosage forms [27]. Such prolonged release dosage forms are often utilized to avoid frequent dosing of drugs that show a short biological half life. These systems are available for drugs the plasma concentration of which is critically related to efficacy. That is, such systems can achieve maintenance of drug concentration at the therapeutic range in the body. The concentration of a drug unbound with plasma protein is directly related to its efficacy. In the present study, the antidiabetic agent Repaglinide (Rg), a fast and short acting meglitinide analog [28] with a very short half-life (1h) and low bioavailability (50%) was chosen as the drug to overcome the problem due to the conventional dosage form. Although prolonged release dosage forms orally administered are often useful for sustained action of drugs, they may not be available for maintenance of drug effects when the limited absorption window exists in the gastrointestinal tract. In order to achieve good drug absorption in such case, one function is required to the dosage form. That is, abilities to exhibit prolonged drug release.

Diabetes mellitus is a major and growing public health problem throughout the world, with estimated world wide prevalence in 2000 of 150 million people, expected to increase to 220 million people by 2010. Recent estimates project that the number of patient's diagnosed with type II diabetes will more than double to 300 million before 2025 [29]. Diabetes mellitus (DM) is defined as a group of metabolic diseases the common feature of which is an elevated blood glucose level (hyperglycaemia). Chronic hyperglycaemia is associated with the long-term consequences of diabetes that include damage and dysfunction of the cardiovascular system, eyes, kidneys, and nerves. The complications of diabetes are often divided into two groups: microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (ischaemic heart disease, stroke, peripheral vascular disease). Together, these make diabetes the seventh most common cause of death in the developed world [30]. So, we have focused the attention on diabetic treatments. Development of novel nanoparticles must proceeds in tandem with assessment of any toxicological and environmental effects. With nanotechnology, we have a unique opportunity of testing hazard and assessing and controlling risk as the technologies themselves develop. Despite the widespread use of nanomaterials, understanding of the toxicity and potential health risks associated with nanomaterial use is extremely limited. In fact, toxicity issues related to nanomaterials used in nanomedicine are often ignored. Thus, along with the development of novel nanoparticles, experts in related scientific fields are calling for a simultaneous assessment of the toxicological and environmental effects of nanoparticles [31, 32].

The aim of this article is modeling of polymeric nanoparticles and observation of the aspects related to the currently used polymer and drug characters during formulation and it will further more point out the most promising strategies in the experiments according to the

biomedical community needs. Other equally important issues, such as *in-vitro* characteristics and toxicity fall within the scope of this article. An ideal drug delivery system possesses two elements: the ability to target and to control the drug release. The reduction or prevention of side effects can also be achieved by controlled release.

2. Experimental

2.1. Materials

Repaglinide (Rg), Ethyl Cellulose (EC) and Poly (vinyl alcohol) (PVA) were received from Sigma Aldrich, Germany. The following materials were obtained from the indicated suppliers and used as received: Acetone, Petroleum ether, Ethanol (Ranbaxy Fine chemicals Ltd, New Delhi). All other chemicals and solvents used were of analytical grade.

2.2. Preparation of Polymeric Nanoparticles

Polymeric nanoparticles were prepared by solvent evaporation method using EC as coating material and Repaglinide (Rg) as core material. Weighed quantity of drug and polymer were dissolved in suitable organic solvent acetone (organic phase). This solution was added drop by drop using syringe fitted with a 24-guage needle to the aqueous phase of PVA and homogenized using homogenizer (IKA T 25 Digital Ultra turrax homogenizer, Germany) at 18,000 rpm for 15 minutes followed by magnetic stirring for 2-3 hrs. The formed Rg-EC nanoparticles were recovered by centrifugation (Sigma centrifuge 3K 30, Germany) at 25,000 rpm for 30 minutes followed by washing thrice with petroleum ether and lyophilized [33, 34] All the nanoparticle formulations with different ratios of drug and polymer were prepared in triplicate to get the reproducibility and reliability.

2.3. Nanoparticle recovery and drug incorporation efficiency

The nanoparticle (NP) recovery, which is also referred to as nanoparticle yield in the literature [34] was calculated using Eq. (1). The individual values were determined.

$$\text{Nanoparticle recovery (\%)} = \frac{\text{Mass of nanoparticles recovered}}{\text{Mass of polymeric nanoparticles, drug and any formulation excipient used in formulation}} \times 100 \quad \text{---- Eq (1)}$$

For incorporation efficiency freeze-dried nanoparticles were dissolved in suitable solvent (50 ml) (a common solvent for polymer and the drug- acetone). The amount of drug in the solution was measured by Ultraviolet spectroscopy at 243nm (Perkin-Elmer Spectrophotometer). Drug Content (% w/w) and Drug Entrapment (%) were represented by Eqs. (2) and (3) respectively. Repaglinide concentration in the sample was determined using a calibration curve.

$$\text{Drug Content (\% w/w)} = \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of nanoparticles recovered}} \times 100 \quad \text{------ Eq (2)}$$

$$\text{Drug Entrapment (\%)} = \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of drug used in formulation}} \times 100 \quad \text{------Eq (3)}$$

2.4. Particle Size Analysis

Particle size was determined using Photon Correlation spectroscopy (PCS) (Malvern S4700 PCS System, Malvern UK). For particle size analysis known amount of Rg-EC

nanoparticles were first suspended in 100 ml of filtered water (0.2µm filter, Ministart, Germany) and subjected to sonication for 30 seconds and vortex mixing for 10 seconds before analysis.

2.5. Scanning Electron Microscopy

The shape and surface morphology of the Rg-EC nanoparticles were examined using Scanning Electron Microscopy (SEM) (JSM –T20. Tokyo, Japan). An appropriate sample of polymeric nanoparticles was mounted on metal stubs, using double-sided adhesive taps. Samples were platinum coated and observed for morphology, at acceleration voltage of 15 KV.

2.6. Fourier Transform Infrared Spectroscopy

Infrared spectroscopy was conducted using a spectrophotometer (Avatar 320-FT IR USA) and the spectrum was recorded in the region of 4000-400 cm^{-1}.The procedure consist of dispersing a sample (drug, polymer and Rg-EC nanoparticle preparation) in potassium bromide and compressing into discs by applying a pressure of 5 tons in a hydraulic press. The pellet was placed in the light path and the spectrum was obtained.

2.7. Thermo Gravimetric Analysis

Thermo gravimetric analysis was conducted to study the thermal stability of polymer, drug and Rg- EC nanoparticles. TGA data were obtained using a thermo gravimetric analyzer (TGA/SDTA851, Mettler, Switzerland). The sample of 5–10 mg was accurately weighed in an aluminum pan. The measurement was conducted at a heating rate of 10°C/ min under a nitrogen purge.

2.8. Differential Scanning Calorimetry

Differential Scanning Calorimetry was performed by using DSC instrument (DSC-60, Switzerland). Approximately 2 mg of samples were accurately weighed into DSC aluminum pans and were crimped followed by heating under nitrogen flow (30ml/min) at a scanning rate of 5°C/min from 25°C to 200°C. Aluminum pan containing same quantity of indium was used as reference. The heat flow as a function of temperature was measured for both the drug and drug – excipients mixture.

2.9. X-Ray Diffraction Analyses

X-ray diffraction analyses were performed on the polymer, drug and Rg-EC nanoparticles. Diffraction powder patterns were obtained with a diffractometer (Siemens D500 diffractometer, Germany) using Copper potassium radiation at 35 kV.

2.10. Haemolytic activity

Measuring haemolytic activity is important as it is an indicator of cytotoxicities. The *in-vitro* haemolysis test has also been employed by many different groups for their toxicological evaluation. It gives a quantitative measure of the haemoglobin release [35, 36]. The test samples were made by preparing stock solution of nanoparticle formulation using phosphate buffer as solvent followed by incubation. Various concentration of the formulation i.e., 20, 40, 60, 80µg in 0.5 ml was used for the study. Haemolytic assay was carried out by adopting the method of Bulmus [37]. Freshly collected rat red blood cells (RBC) were taken and washed three times by 150 mM Sodium Chloride (NaCl) (2500 rpm for 5 minutes). After removing NaCl at the last wash step the cells were suspended in 100 mM Sodium phosphate buffer. The test samples were mixed with 200 µL of RBC solutions and the final reaction mixture volume was made up to 1 ml by adding Sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hr at 37°C. After the incubation time the reaction mixture was centrifuged again at 2500 rpm for 10 minutes. Measure the supernatant absorbance at 541 nm keeping sodium phosphate buffer as blank. Deionised water was used as a positive control. The experiment was done in triplicate and percentage haemolysis was calculated using the following formula.

$$\text{Percentage haemolysis} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of positive control}} \times 100$$

***2.11. In-vitro* Release Study**

The in vitro release of Rg-EC nanoparticles was carried out in stirred dissolution cells at 37.4°C by suspending 2ml of Rg-EC nanoparticle suspension into a beaker containing 100 ml of release media (phosphate buffer saline pH 7.4). The correct in vitro conditions to study the release behavior of a hydrophobic drug were maintained [38]. Drug release was assessed by intermittently sampling the receptor media (5ml) at predetermined time intervals, each time 5ml of fresh phosphate buffer saline pH 7.4 was replaced. The amount of repaglinide released in the buffer solution was quantified by a UV spectrophotometer at 243nm.

2.12. Evaluation of In vitro release kinetics

In order to investigate the mechanism of release the data were analyzed with the following mathematical models: zero-order kinetic (equation 4), first-order kinetic (equation 5) and Higuchi kinetic (equation 6).

$$Q_t = K_0 t \quad (4)$$

$$\ln Q_t = \ln Q_0 - K_1 t \quad (5)$$

$$Q_t = K_h t^{1/2} \quad (6)$$

The following plots were made: Q_t vs. t (zero order kinetic model), ln (Q_0 - Qt) vs. t (first-order kinetic model) and Q_t vs. $t^{1/2}$ (Higuchi model), where Q_t is the percent of drug released at time t, Q_0 is the initial amount of drug present in the microspheres and K_0, K_1 and K_h are the constants of the equations. Further, to confirm the mechanism of drug release, the first 60 % of drug release was fitted in Korsmeyer- Peppas model (equation 7)

$$M_t / M_\alpha = K_p t^n \quad (7)$$

where M_t / M_α are the fraction of the drug release at time t, K_p is the rate constant and n is the release exponent. The n value is used to characterize different release mechanisms and is calculated from the slope of the plot of log of fraction of drug released (M_t / M_α) vs. log of time [39].

2.13. Statistical analysis

The statistical package used was Origin 6.0 soft ware.

3. Results and discussion

3.1. Formation of polymeric nanoparticles

The polymeric nanoparticles were prepared by solvent evaporation method in three different ratios of polymer. This method is comparatively easy to prepare than the other techniques. A suspension of polymer and drug in solvent acetone forms the organic phase. This

organic phase was poured into an aqueous phase containing PVA. The organic solvents used in these preparations rapidly partitioned into the external aqueous phase and the polymer precipitated around the drug. The subsequent evaporation of the entrapped solvent led to the formation of polymeric nanoparticles. Specifically the polymer coated or covered around the shaped drug led to spherical shaped polymeric nanoparticles [40]. Any content of the organic solvent dissolve in the aqueous phase can act as a cosolvent, leading to an increased tendency to Ostwald ripening. Immediate lyophilization is required to preserve the particle size.

The Repaglinide engineered ethylcellulose nanoparticles were formulated in three different ratios (1: 2, 1: 3 and 1: 4). Based on the recovery and drug entrapment efficiency of different ratios of formulated nanoparticles, 1:4 ratio was selected as the applicable ratio because 1:3 and 1:4 ratios leads to a low drug entrapment which implies high drug wastage during the formulation process. These polymeric nanoparticles were prepared at three consecutive times for reproducibility and result elicited (Table 1). Ideally the biologically active drug is encapsulated within nanoparticles using ethylcellulose polymer with well defined physical and chemical properties. The characteristics and performance of these fabricated nanoparticles have been extensively described in this work.

Table 1 Percentage of Nanoparticle (NP) recovery, Drug content, entrapment and wastage for different ratios.

Drug–polymer	NP recovery %	Drug content (%)	Drug entrapment (%)	Drug wastage (%)
1:2	53.42± 0.62	13.86± 0.213	58.8± 0.724	41. 2± 0. 218
1:3	82.44± 0.375	10.51± 0.073	78.0± 0.231	22. 0± 0. 371
1:4	92.202± 0.039	9.61± 0.022	86.4± 0.31	14.6± 0.54

All values are mean ± S.D.(n=3)

3.2. Effect of drug content and drug entrapment

Drug loading expresses the percent weight of active ingredient encapsulated to the weight of nanoparticles, drug loading efficiency is the ratio of the experimentally determined percentage of drug content compared with actual, or theoretical mass of drug used for preparation of the nanoparticles. The loading efficiency depends on the polymer–drug combination and the method used. Hydrophobic polymers encapsulate larger amounts of hydrophobic drugs, whereas hydrophilic polymers entrap greater amounts of more hydrophilic drugs. Several formulation parameters, such as emulsifier type, weight ratio of polymer to drug, and organic to aqueous phase ratio, will influence the extent of drug loading [41, 42, 43].

In the present nanoparticle fabrication, the drug and the polymer were dissolved in the same organic phase. Hence there were no chances of diffusion of the drug away from the polymer. The percentage of drug entrapment in the formulations was found to be good at all levels of drug loading. Good entrapment efficiency of 86.4% was showed by 1:4 ratio and hence only 14.6% drug wastage was observed. The high entrapment efficiency of is believed to be due to its poor aqueous solubility otherwise good solubility in the same solvent. The highest entrapment efficiency of 86.4% was achieved by increasing polymer drug ratio. The higher drug loading typically results in changes of encapsulation efficiency due to higher concentration gradients resulting the drug to diffuse out of the polymer/solvent droplets to the external processing media. Among the different polymer drug ratios investigated, 1:4 ratio had the optimum capacity for drug encapsulation. In these experimental modeling the volume of the processing medium and other parameters such as stirring speed, stabilizers concentration was kept constant. The ratio 1:4 was optimum, as drug wastage during nanoparticle preparation was found to be minimum. The ratios 1:2 and 1:3 delivered low yield owing to the high drug wastage and a large quantity of carrier was required to achieve sufficient amount of drug at a target site [34]. Compared to 1:3, 1:4 ratios, the 1:2 ratio showed high drug content and it led to an enhanced drug leakage (Table 1).

Although 1:2 ratio has relatively high drug content than 1:3 and 1:4 ratios, this formulation was not selected for further studies due to low drug entrapment (58%) which implies a high drug wastage during the preparation procedure, and less amount of nanoparticle recovery and increased drug release pattern. The 1:4 ratio showed 9.61% w/w drug content, 86.4% drug entrapment and 92.20% nanoparticle recovery. High nanoparticle recovery is required for reduction of manufacturing cost. The particle size and morphology is important for quality control and bio distribution purposes. The researchers [44] attributed the decreased drug entrapment with increasing theoretical drug loadings to an enhanced drug leakage into the aqueous phase (if drug is water soluble) or into the organic phase (if drug is water insoluble) at high loadings. This would also lead to an enhanced drug loss. Long before one of these compounds can reach the market; it needs to be formulated for the pharmacological activity tests and for the preclinical studies.

3.3. Morphological characterization of polymeric nanoparticles

Rg-EC preparation has smooth spherical shaped appearance (Fig.1). The surface of formulated nanoparticles depends on two factors (1) A saturated solution of polymer, produced smooth and high yield nanoparticles. The undissolved polymer produced irregular and rod shaped particles. (2) The diffusion rate of solvent is too fast and the solvent may diffuse into the aqueous phase before stable nanoparticles are developed or formed causing the aggregation of nanoparticle preparation. In this preparation the polymer was fully saturated and the diffusion rate of solvent was minimal leading to the formation of smooth, spherical and individually homogeneously distributed particles and has no evidence of collapsed particles. Smooth surface reveals complete removal of solvent from the formulated nanoparticles and is the indication of good quality [42].

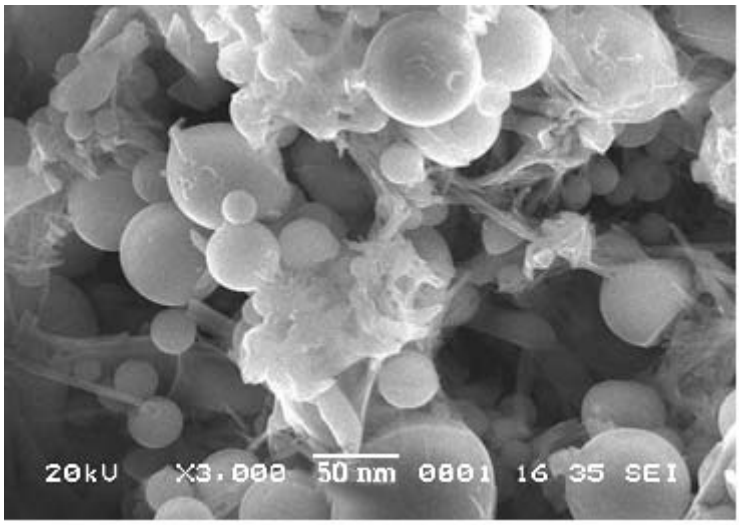

Fig. 1 Scanning electron microscopy photograph of Rg-EC (1:4).

3.4. Particle size and poly dispersity index

The particle sizes of nanoparticles were larger than those obtained by the quantitative analysis of the SEM (Fig. 2). The explanation of this difference of nanoparticles is been given in the literature and can be employed for the charged co-polymer nanoparticles as well [64]. The contrast of the Electron Microscope (EM) pictures allows only the visualization of the nanoparticle core, whereas the hydrodynamic radius of the particles was measured by PCS. Particle size is often used to characterize nanoparticles, because it facilitates the understanding of the dispersion and aggregation [45]. Because of larger surface area and attractive force between the particles, the chance of possible aggregation is high in small sized particles. To overcome such aggregations, which were not solicited, an addition of a surfactant in the preparation was necessary. PVA appeared to be the most suitable surfactant in reducing aggregation between nanoparticles which suspends immediately after formation [45].

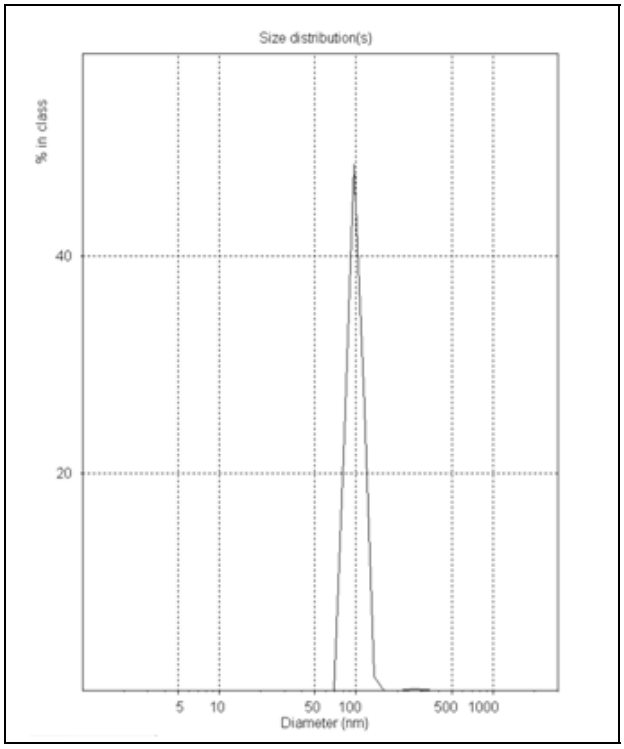

Fig. 2 Size Distribution of Rg-EC (1:4).

The particle size data showed that nanoparticles produced were of sub micron size and had low poly dispersity which indicates relatively narrow particle size distribution for Rg-EC preparations. The mean diameter and poly dispersity index (PI) of Rg-EC polymeric nanoparticles was found to be 108.3nm and 0.514PI. Inefficient polymeric synthesis may form polymers with high PI that degrade more rapidly. The particle size and particle size distribution are critical factors in the performance of nanoparticles, as batches with wide particle size distribution show significant variations in drug loading, drug release, bioavailability and efficacy. Formulation of nanoparticle with narrow size distribution will be a challenge. As nanoparticles are internalized into cells by endocytosis, an increase in particle size will decrease uptake and potentially, bioavailability of the drug.

3.5. FT-Infrared Spectroscopy (FTIR)

In FTIR, a vibrational spectrum, characteristics for a given crystal structure is obtained. FTIR spectra of pure repaglinide, polymer and repaglinide loaded polymeric nanoparticle were performed (Fig.3). Infrared spectra of ethyl cellulose sample studied displayed several characteristic vibration properties in the region of between 3750 and 2500 cm^{-1}, a sharp band at 2900 cm^{-1}, associated with a CH stretching vibration [46], and a broader band centered at about 3350 cm^{-1}, which corresponds to OH stretching vibration [46, 47]. FTIR of repaglinide shown peaks at 3320 cm^{-1} (NH stretching), 2947 cm^{-1} (CH stretching), 1728 cm^{-1} (C=O). Similar peaks were seen in repaglinide loaded EC nanoparticle preparation. Significant changes not observed during the study suggest that no interaction that could interfere in polymer and drug structures and has good chemical stability.

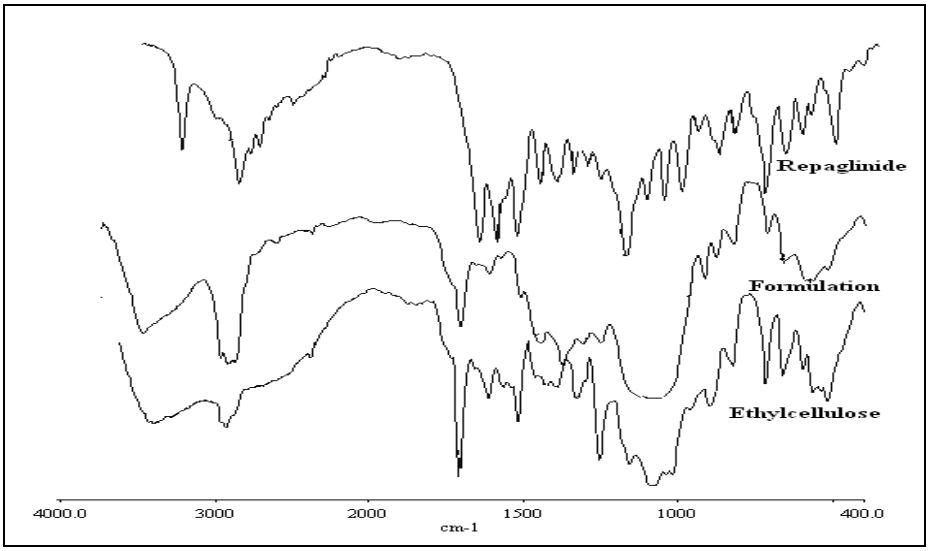

Fig. 3 FTIR of drug, polymer and Rg –EC nanoparticle.

3.6. Thermo Gravimetric Analysis

The TG curves were analyzed from the curve of mass loss versus temperature (Fig. 4). The TG curves of drug, polymer and drug loaded polymeric nanoparticle revealed two thermal decomposition stages. The decomposition of drug proceeds at 234.61ºC and ends at 375.28º C, whereas polymer proceeds at 280.86ºC and ends at 381.0ºC. The formulated nanoparticle showed decomposition starting from 254.06ºC itself and ends at 392.16ºC. Here the starting decomposition temperature shifted when compared to pure polymer but the complete weight loss of formulated nanoparticle occurs only at 392.16ºC. The shift in temperature is visualized from the data, but it is only 20º C, considering the interaction of the nanoparticles, we suspect that the peak shifts might be due to the hydrophobic interaction between the drug and polymer. The percent weight loss at 250 – 300º C from TGA technique observed. For pure drug the percentage weight loss at 234.61ºC is 98.13 and at 375.28ºC only 1.5% remained, where as in case of formulated nanoparticle some changes were observed. The value relates to the amount of incorporated guest. The formulated nanoparticle showed good thermal stability up to 392.16º C, and since all the materials analyzed did not suffered appreciable decomposition in the analytical conditions used, it can be suggested that they can be safely processed using some thermal treatment. In conclusion, no strong chemical interaction that alters the chemical structure and drug structural integrity between drug and polymer was observed.

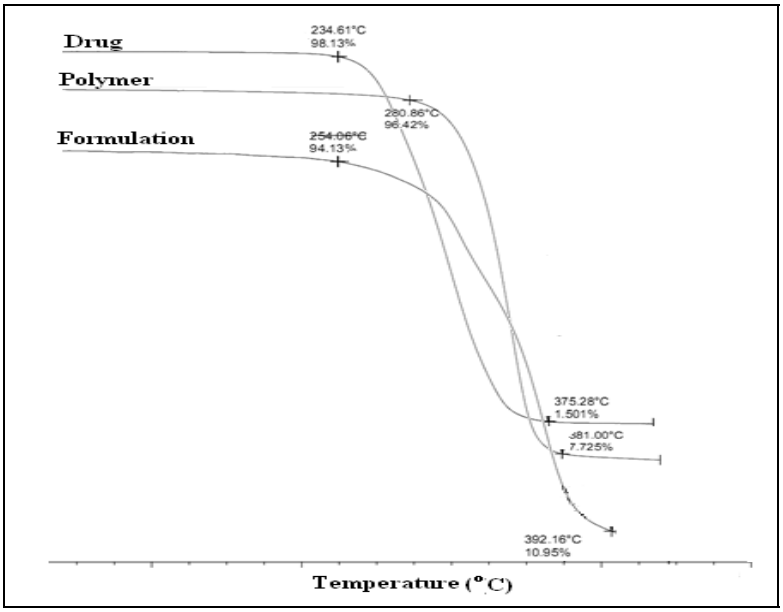

Fig. 4 TGA of Rg– EC nanoparticle, polymer and drug.

3.7. Differential Scanning Calorimetry

DSC detects phase transition such as glass transition (exothermic) crystallization and (endothermic) melting: the nanoparticle sample is heated and changes in the heat flow, compared to reference, are registered [48]. DSC thermograms were obtained to define the physical state of the drug and polymer in the nanoparticles and to detect any drug polymer interactions with in the polymeric network of the nanoparticles [49]. Typical thermograms of drug loaded polymeric nanoparticles and its raw components are displayed (Fig.5). Within the selected temperature thermal event occurred. The purpose of these analyses in our experiment was to detect possible modification in the structure and nature of drug due to their organization in the form of nanoparticle. The DSC curve of repaglinide showed a single endothermic peak at 134.78ºC. The DSC profile of formulated drug shows broad but low intensity peak at 131.5ºC accompanied by endothermic peaks is due to the Tg relaxation, enthalpy of polymer. These results, taken together, suggest that the encapsulation process produce a marked decrease in crystallinity of drug and /or confers to this drug a nearly amorphous state. However it should be taken into account that quantification of low content of amorphous/crystalline phase by DSC [50]. The changes in the melting enthalpy for pure drug is 99.7J/g, whereas in formulation the change in the melting enthalpy at the same temperature of 131.50°C is only 10.1J/g. Pure polymer showed three endothermic peaks at 51.20ºC, 188.01ºC and 230.08ºC. In the thermal analysis of formulation the peak in the area 185.01ºC is progressively reduced as increased intensity peak occurred at 225.78ºC. The peaks were progressively reduced in the area 185.21ºC where as increased peak occurred at 225.78ºC in the formulation. The changes in the intensity and temperature may be due to the electrostatic interaction between the basic drugs and the carboxyl group at the polymer terminal. After formulation, drug could be in an amorphous or disordered crystalline phase of a molecular dispersion or a solid solution state in the polymer matrix [49]. During the nanoparticle formation process, a rapid diffusion of solvent from the globules of the emulsion carries molecules into the aqueous phase, forming local regions of super saturation, from which new globules or polymer aggregates [51].

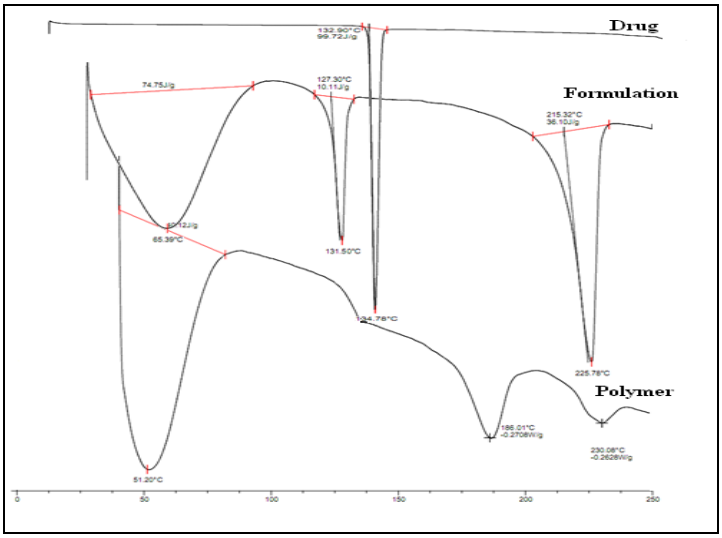

Fig. 5 DSC of Rg –EC nanoparticle, polymer and drug.

3.8. X-Ray Diffraction Analyses

Crystallinity /amorphicity properties are obtained from XRD analysis when diffraction pattern of the X-ray from the sample is determined as a function of scattering angle [52]. XRD analysis of drug, polymer and drug loaded polymeric nanoparticle were performed and are illustrated (Fig. 6). Repaglinide has shown the characteristic intense peaks at 2θ of 5.4º, 10º, 13.2º, 17.5º, 21º and 25º because of its crystallinity. However, the intensity of these peaks was decreased in repaglinide loaded polymeric nanoparticles. Ethyl cellulose polymer showed intense peaks at 12.07º and 18.54º. There were ill defined peaks observed in the polymer matrix. Generally, XRD peaks depend upon the crystal size, but in the present study, the drug loaded polymeric nanoparticles, the characteristic peak of repaglinide overlapped with the noise of coated polymer. From this, it is evident that XRD signals of encapsulated drug is very difficult to detect, which shows that the drug is dispersed at a molecular level in the polymer matrix and hence no crystals are found separately in the drug loaded matrix [41, 53].

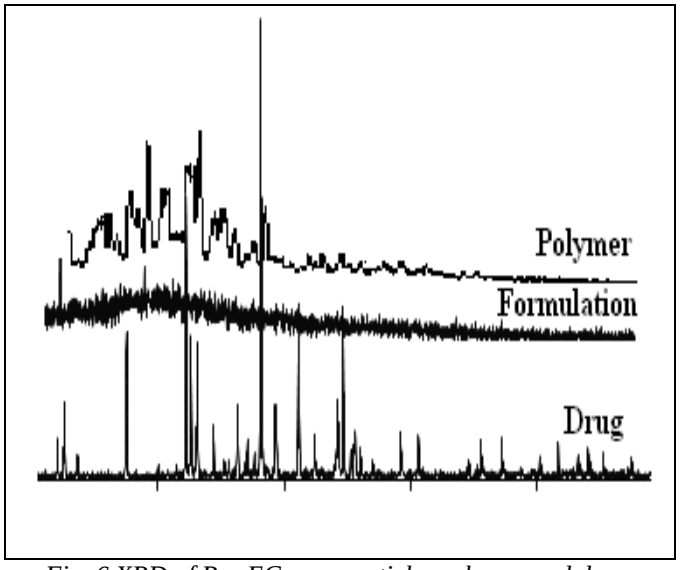

Fig. 6 XRD of Rg- EC nanoparticle, polymer and drug.

3.9. In-vitro Release Study

Drug release process is controlled by dual mechanism; the liquid enters the polymer matrix, dissolves the drug and enable the drug to diffuse out through the liquid located in the polymer matrix. Both these transfers are controlled by diffusion and the diffusivity of the drug which increases with the liquid concentration in the dosage form. As a result, drug delivery in the intestine is effectively controlled. Drug release is governed by polymer structure and properties [54]. Ethyl cellulose is widely used to control the dissolution rate of drugs from sustained release preparations [55, 56]. The result of the dissolution experiment of ethyl cellulose coated nanoparticles performed in media of pH value which is 7.4 (Fig. 7). The correct in vitro conditions required to study the release behavior of a hydrophobic drug were maintained because repaglinide showed two Pka values at 4.16 and 6.01. Solubility of repaglinide increases 3-6 times using phosphate buffer as solvent. In water, solubility of repaglinide is 39.82µg/ml. In phosphate buffer the solubility of repaglinide is 140.86 µg/ml [57]. About 20 % of drug release at pH 7.4 after the specified period. This release is attributed to the physical and chemical properties particularly on the pKa and solubility profile of the drug. For polymer like EC that possess plastic and hydrophobic property, drug particles present in the surface of martrix is initially released into the surrounding media generating many pores and cracks which facilitate further release of drug and EC did not change its drug retaining activity due to the change of pH. The fact can be substantiated by the fact that release profile of drug molecules, irrespective of their chemical nature was almost linear with time. But when married with the principles it is clear that polymer forms a more compact wall around the drug.

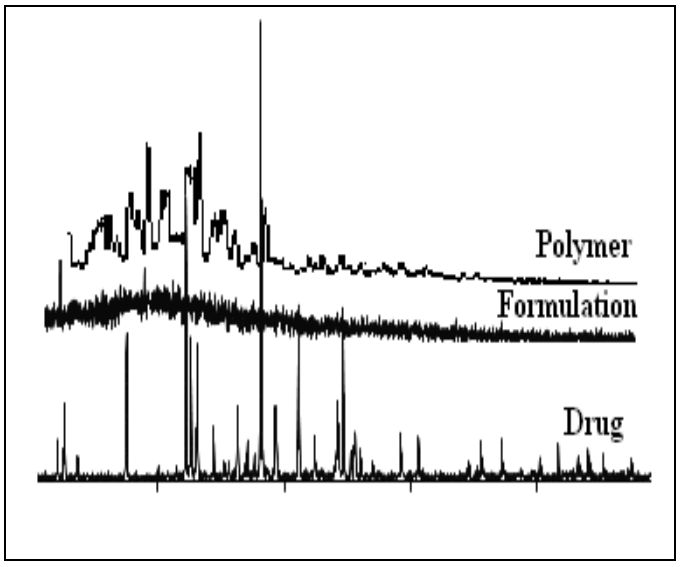

Fig. 6 XRD of Rg- EC nanoparticle, polymer and drug.

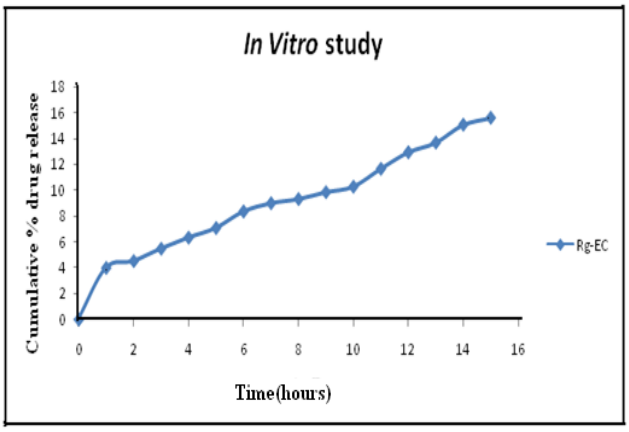

Fig. 7 In-vitro release of Repaglinide from polymeric nanoparticles of 1:4 ratio.

The *in-vitro* release of drugs from nanoparticles may approximate the drug release profile inside the body although the rate is usually faster *in-vivo* due to the presence of enzymes and surfactants in biological fluids. An in vitro dissolution medium mimics the pH and salt concentrations in the body. Particularly for hydrophobic drugs, it is critical during dissolution testing that sink conditions are maintained and pH and salt concentration of biological fluid are approximated. The in vitro release profile (Fig.7) suggested that fifteen hours might be needed for the release of 15% dose, but this might not be consistent completely with the in vivo absorption and 100% release occurred at 5th day. It is not always easy to estimate the bioavailability exactly from the in vitro release because the situations are different between the in vitro and in vivo releases [58]. For example, Tuncel reported that the release was faster *in- vivo* than *in-vitro* [59]. For the present results, the consistency between *in-vitro* release and *in-vivo* absorption appeared not to be complete but fairly good. The *in-vivo* release and absorption features in the small intestine will have to be investigated in more detail to discuss relationship of them exactly. The increase in drug content in the particles influence the absolute release profiles of the drug, in such a way that, it increases the induction period and the cumulative amount of drug released at any given point of time. The drug content which is closer to the surface of the nanoparticle is responsible for an increased initial burst and the drug in the core of nanoparticles is responsible for a prolonged drug release from the polymer [38].

3.10. Evaluation of *In-vitro* release kinetics

In order to determine the release model which best describes the pattern of drug release, the *in-vitro* release data were substituted in zero order, first order and diffusion controlled release. The zero order rate describes, the systems, where the drug release rate is independent of its concentration. Cumulative amount of drug release vs time for zero order kinetics (Fig. 8), the first order rate, which describes the drug release rate, is dependent of its concentration and it's the cumulative percentage of drug remaining in log scale vs time (Fig 9). Higuchi model [60, 61] describes the release of drugs from an insoluble matrix as a square root of time dependant process, based on Fickian diffusion (Fig 9). The release constant was calculated from the slope of the appropriate plots and the regression co- efficient (r^2) was determined and the results are tabulated in Table 2. In this Rg- EC preparation the in vitro release kinetics was best explained by zero order equation, as the plots showed the linearity (r^2= 0.968), first order (r^2= 0.986), followed by Higuchi equation (r^2= 0.953). First order showed high correlation than other models. The data were also plotted in accordance with the Hixson crowell cube root law which indicates the progressive dissolution of the matrix as a function of time (Fig. 11).

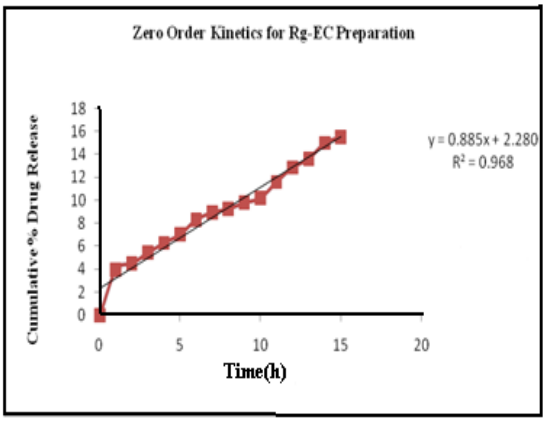

Fig. 8 Zero order kinetics data of Rg – EC nanoparticle preparation.

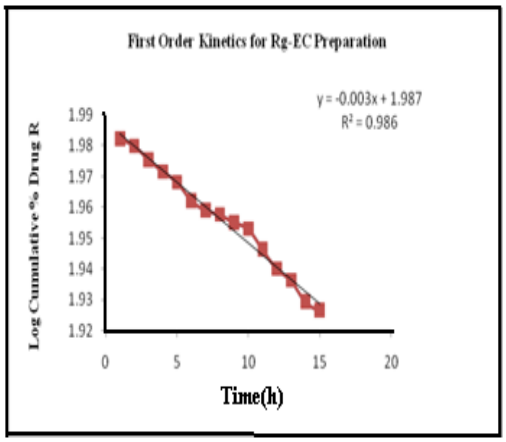

Fig. 9 First order kinetics data of Rg – EC nanoparticle preparation.

Table 2 Release kinetics data of Rg-EC polymeric nanoparticle

Equation	Zero order	First order	Higuchi	Korse meyer	Hixson –crowell
r^2	0.968	0.986	0.953	0.969	0.985

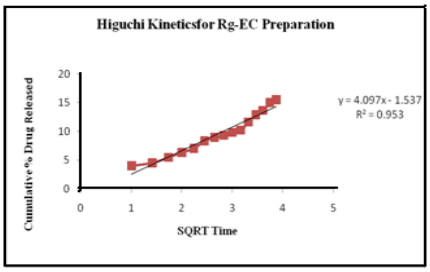

Fig. 10 Higuchi eqation data of Rg – EC nanoparticle preparation.

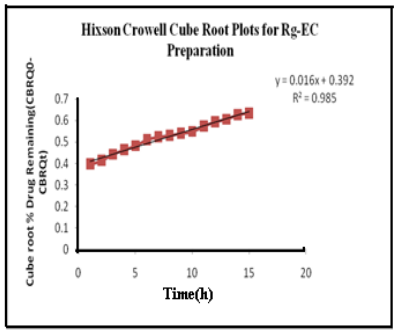

Fig. 11 Hixson- Crowell equation data of Rg – EC nanoparticle preparation.

To explain the mechanism of drug release, Korsemeyer– Peppas equation has been applied (cumulative percentage drug release in log scale Vs time) and good linearity (r^2= 0.969) has been observed. Generally, the release rate observed is a cumulative effect of drugs solubility (influenced by its structure, molecular weight and pKa) polymer property (hydrophilicity/ lipophilicity molecular weight) and the relative ratio of drug and polymer. For mechanism of drug release k is the rate constant incorporating the properties of the macromolecular polymeric systems and the drug and n is a kinetic constant which depends on and is used to characterize the transport mechanism. The value of n for a formulation = 0.45 for Fickian (case I) release,> 0.45but < 0.89 for non fickian (anomalous) release and 0.89 for case II (zero order) release and > 0.89 for super case II type of release (Peppas). Case II transport generally refers to the dissolution of the polymeric matrix due to the relaxation of the polymer chain and anomalous transport (Non Fickian) refers to the summation of both diffusion and dissolution controlled drug release. The results are plotted in graphical form (Fig. 12). The release exponent "n" who appears to be coupling of diffusion and erosion mechanism called anomalous diffusion, which indicates that the drug release is controlled by more than one process. Group of researchers also considered the corresponding "n" values as the indication of anamalous release mechanism [61, 65]. The 'n' value of Rg-EC is 0.485 indicates the mass transfer and was found to be less than 0.5 indicating the mechanism of drug release is diffusion controlled (Fickian diffusion) and Korsmeyer- Peppas model showed high correlation between each other (Table 2). From these results we concluded that the release of Repaglinide from the EC matrix was predominently controlled by first order and diffusion mechanism.

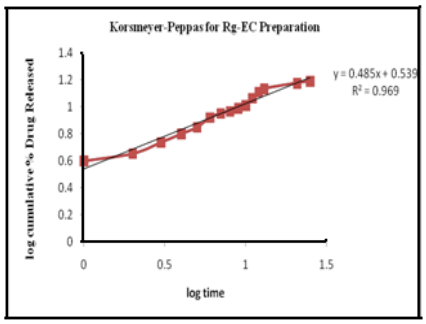

Fig. 12 Korsmeyer –Peppas eqation data of Rg – EC nanoparticle preparation.

3.11. Haemolytic assay

The data obtained in this assay gives a qualitative indication of the damage caused by polymeric nanoparticles in red blood cell (Fig. 13). The result obviously declared that the nanoparticles are more haemocompatible for drug delivery applications. More over the nanoparticle system showed lysis less than 5% in the whole experimental concentration range. When the nanoparticles are administered into the body for drug delivery or oral detoxification,

detrimental interaction of these particles with blood constituents must be avoided. Based on the report of the present study the nanoparticle dispersion in the experimental range did not show any observational haemolytic toxicity in the red blood cell. It was observed that the haemolytic percentage of the nanoparticle dispersions is independent on its concentrations. According to the ISO/TR 7405-1984(f), the samples were considered as haemolytic if the haemolytic percentage was above 5% [62, 63]. In the present study all the formulations showed haemolytic percentage which was significantly lower than 5%. The results suggested that the nanoparticles were suitable for a wide safety margin in blood – contacting applications and suitable for administration.

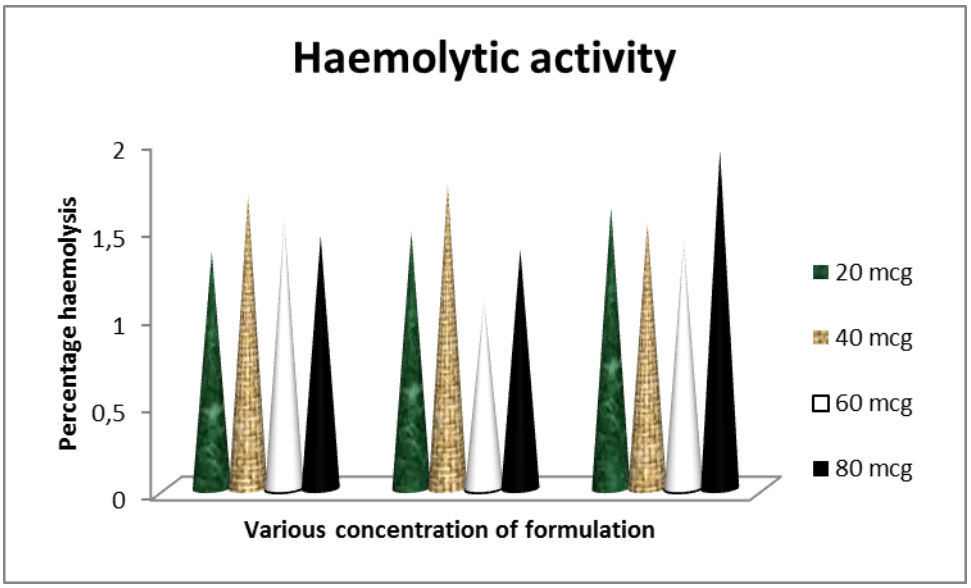

Fig. 13 Heamolytic activity.

4. Conclusion

Repaglinide loaded ethyl cellulose nanoparticles were successfully formulated by solvent evaporation method. The interaction between drug and polymer were clearly illustrated. The results of drug loading and release experiments indicated that this system seems to be a very promising vehicle for encapsulation of water insoluble drugs like repaglinide. The high nanoparticle recovery could reduce the manufacturing costs and its size and morphology could improve the bio distribution and large surface to volume ratio. Therefore, the bioavailability of drug may be improved and may help to reduce the dose of the drug and frequency of administration. These approaches have yielded some good results, and nanoparticle drug delivery systems are demonstrating lot of potential in this area. Increase in release properties by changing various parameters is in hope. Many of these studies are preliminary in nature, and various systems require further toxicity and efficacy data to facilitate their transition to clinical trials. The impact of these techniques in formulation of drug delivery systems and their therapeutic applications will be worth watching during the coming few years. Today's interest in applying nanotechnology to medicine is growing owing to its capacity for producing nanoparticles for use in delivery systems. Finally, because nanotechnology is a novel tool, the concern about the safety of polymeric nanoparticles used in medicine for human health has to be addressed in a sufficient and satisfactory manner. Before polymeric nanoparticles will be used in human applications, it will be necessary to carry out consistent and comprehensive studies to ensure their innocuousness.

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