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Development of Sustained Release Nateglinide Loaded PLGA Nanoparticle: *In vitro-In vivo* Study

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ABSTRACT

In present study long-acting biodegradable Nateglinide loaded Poly (lactic-co-glycolic) acid nanoparticleformulation is reported for treatment of Type 2 Diabetes Mellitus (T2DM).Different formulations were prepared by varying drug: polymer ratio (1:1, 1:2, and 1:3) and polyvinyl alcohol as stabilizer in 0.5-1.5% concentration range by emulsificationsolventevaporation technique. Optimization was carried out by evaluating entrapment efficiency and particle size. Optimized formulation was characterized for *in vitro* drug release, surface charge property, SEM, chemical incompatibility and *invivo* evaluated for blood glucose lowering property in rat model. The obtained resultsindicatethat formulation composition containing drug:polymer ratio 1:3 and stabilizer concentration 1.5% gives high encapsulation efficiency (82.15%) with mean particle diameter of 216 nm and drug release for 72h. Drug release data was best fitted to Higuchi model indicating drug release was mainly took place by diffusion mechanism. Oraladministrationof optimized nanoparticle formulation showed two fold better activity as compared to standardNateglinide formulation($P<0.05$). Study suggested that Nateglinide loaded controlled PLGA nanoparticle can be used for better control of blood glucose level by sustaining its release.

Keywords: Nateglinide, PLGA, Poly vinyl alcohol, nanoparticle, Diabetes mellitus

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INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder in which human body does not produce or properly uses insulin. T2DM is characterized by constant high levels of blood glucose. Nateglinide (NTG) is a drug used to lower blood sugar (glucose) levels in patient suffering from T2DM given by oral route[1] and belongs to a class of drugs called meglitinides. Approximately 90% of patients with diabetes have T2DM which usually occurs in adults and is associated with obesity and a strong family history of diabetes. The major cause of T2DM is reduced secretion of insulin from the pancreas after meals and also due to resistance of the body's cells to the effect of insulin which is to stimulate the cells for removing glucose from the blood[2, 3.]

NTG stimulates cells in the pancreas to produce insulin in a manner similar to the class of drugs called sulfonylureas e.g. glyburide which is also recommended in T2DM. However, NTG appears to have a faster onset and a shorter duration of action than sulfonylureas. Although NTG is a drug of choice for management of T2DM but it possesses a shorter half-life (1.5h). So a new dose is required before every meal which makes this therapy inconvenient. This problem can be overcome by formulating a sustained release dosage form. Application of nanotechnology in drug delivery system has opened up new area for research in sustained release of drugs[4]. The Sustained release of the drug from the nanoparticles could maintain the therapeutic concentration for longer duration. Nanoparticles are one of the multipariculate delivery systems used to prolonged or controlled drug delivery system so as to improve bioavailability as well as stability [5]. Nanoparticle can also offer advantage like limiting fluctuations within therapeutic range. Although a number of different polymers have been investigated for formulating biodegradable nanoparticles, poly (lactic-acid) (PLA) and its copolymers with glycolic acid (PLGA) have been extensively used for controlled drug delivery systems. The active substance Nateglinide is hydrophobic molecule and ideal model drug for incorporation in systems prepared by emulsion–solvent evaporation technique. Thus the aim of this study was to develop NTG loaded sustained release nanoparticulate system based on the biodegradable polymer PLGA.

MATERIALS AND METHOD

Materials

Nateglinide was obtained as a gift sample from Cipla Pharmaceutical Ltd. (India). Poly(lactic-co-glycolic) acid (PLGA 50:50)obtained as gift sample from SPARC Baroda, (India), PVA obtained from SD Fine Chemicals (Mumbai, India). Other chemicals used were of analytical grade.

Preparationof NTG-PLGA nanoparticles

Anattempthasbeenmadetooptimizethenanoparticlesformulationusingvarious formulationparameterslikedrug:polymerratio (1:1,1:2,1:3) and different stabilizer/surfactantconcentrationbymodifiedemulsificationsolvent evaporationtechnique, table 1[6].In brief,PLGAandNTGwere dissolvedin5mldichloromethaneusinga vortexshakertoformahomogeneoussolution.Thishomogeneous solutionwasslowlydropwiseaddedto20mlofaqueous surfactant solution(PVA, 0.5-1.5%w/v)usinghighpressurehomogenizer(IKA®,Japan)toformanemulsion.The formed emulsionwasstirredonlaboratorymagneticstirrer(REMI,India) at 25°C for6h followed by centrifugation (REMI, India) for 30 min at 19000×g. Aftercentrifugationthesupernatantwasdiscardedandthepelletsobtainedwerewashedwithsamevolume ofdistilledwaterasofthesupernatantandagaincentrifugedat 19,000×gfor15min.Thewashingprocesswasrepeatedthreetimesandthewashed nanoparticles weresubjectedtofreezedrying(Lark, India).Freeze dried product was evaluated for physicochemical property.

Table 1.Formulation composition of NTG loaded nanoparticles

Compositions	1	2	3	4	5	6	7	8	9
Nateglinide(mg)	60	60	60	120	120	120	180	180	180
PLGA (mg)	60	60	60	120	120	120	180	180	180
PVA (%)	0.5	0.5	0.5	1.0	1.0	1.0	1.5	1.5	1.5

DRUG POLYMER INTERACTION

Differential scanning calorimetry (DSC)

Drug polymer incompatibility was evaluated by differential scanning calorimetry method. DSC analysis was performed using PERKINELMER DSC Pyris -6 (USA) on 2 to 8 mg sample. Sample was heated in an aluminum pan at a rate of 10°C/min within a 30 to 300°C temperature range under a nitrogen flow of 20 ml/min. An empty sealed pan was used as a reference.

CHARACTERIZATIONOFNANOPARTICLES

Particle sizedetermination

Themeanparticlesizeoftheformulationswasdeterminedbyparticlesizeanalyzer (MalvernS4700PCSSystem,Malvern,UK).Theanalysis wasperformed on suitably diluted sample with filtered water (0.22µmfilter)at25°C.Foreachsample, measurement was carried out in triplicate. Values reportedarethe mean diameter.

Surfacecharge property

Nanoparticleswerecharacterizedwithrespecttozetapotential (z)byusingLaser Doppler Anemometry(MalvernZetasizer,Malvern,UK). All theanalysis was performed on appropriately

diluted samples with triple filtered distilled water.

Entrapmentefficiency and drug content

Freezedriednanoparticlesequivalentto10mgofdrugwereweighedanddissolvedin 10mlofmethanoland drug content was analyzedbytheUV-Visible Spectrophotometer (Shimadzu UV-1650, Tokyo, Japan). Drug wassoluble in methanolwhereasPLGAwasinsolublein it. NTGentrapment efficiency(%w/w)was calculated by following equation[7]:

Entrapment efficiency= amountof total NTGin nanoparticle – amount of free drug /amount of total NTG in nanoparticle × 100

***Invitro*drug releasestudy**

*Invitro*drugreleasestudywasperformed using equilibrium dynamic dialysis technique[8, 9]. Inbrief, 60 mg equivalent drugloaded PLGA nanoparticles were redisperse in 2.5 ml buffer, suspended in dialysis bag (Spectra Por, cut off size 10kD) in 350 ml of phosphatebufferpH7.4 and kept for string on magnetic stirrer at 50rpm and 37 °C temperature.Afterspecified timeintervals,5mlof thealiquot was withdrawn and centrifuged at 20,000 rpm for 30 min. Aliquot was analyzed byUV-spectrophotometry at258nm λmax. Dissolution medium was replenished with5 mlof fresh phosphatebuffer to maintain the sink condition. The release experiment was monitored for over a period of 72h.

Scanning electronmicroscopy

Morphologicalevaluationofthenanoparticleswasperformedusingscanningelectron microscope. Scanning electron micrographs were taken using a Philips XL 20 (Philips, Eindhoven, Netherlands). Samples were fixed on an aluminum stub with conductive double sided adhesive tape (Leit-Tabs, Plano GmbH, Wetzlar, Germany) and coated with gold in an argon atmosphere (50 Pa) at 50mA for 50 s (Sputter Coater, Bal-Tec AG, Liechtenstein)

***In vivo*study**

*In vivo*studywasconductedattheAnimalHouseofFacultyofPharmacy,VNS Group of Institutes (Reg.No.778/03/C/CPCSEA-03.09.03). All ethical manners regardingtheuseof animals in scientificresearchwerecarefullyconsidered. Diabetes induced by streptozotocin in rat model was selected forin vivo experimental studies. Wistar strain malealbinoratsweighing200-250gwereused.Ratswere acclimatizedtotheirenvironment25±2Cand70±5%RHundernaturallight–dark conditionsfor15 days beforedosing.

Induction ofdiabetes

Diabetes was induced by following reported method with some modifications[10].Streptozotocin(SiscoResearchLab)wasdissolvedinicecoldnormalsalinesolution

and60mg⁄kgfreshsolutionswereinjectedintraperitoneallytoovernight-fastedrats. Bloodsampleswerechecked 48 hlaterusingSugarScan(Thyrocare)andratswith bloodglucosevaluesbetween300and400mg⁄dlwereconsidered diabetic and wereincluded in the experiments.

Experimentalprocedure

Rats wererandomlydivided into fourgroups ofsixanimals each group. Group 1(NormalControl) containratswithbloodglucoselevelrangingbetween80-90 mg/dl, treated with vehicle only. Group2(DiabeticControl)consist of ratswithbloodglucoselevelrangingbetween300-400 mg⁄dl, treated with vehicle only. Group3(Standardtreated), diabeticratstreatedwithstandardNTG(800µg⁄kg, three times in a day) oral up to 7 days. Group4**,** diabeticratstreatedwithNTGNP 08(Equivalentto 800µg⁄kgthree times in a day,oral for 7 days.Blood glucoselevelsweremeasuredinallratsusingSugar Scanglucometserat24hintervals over the7daytreatmentperiod.

Bloodsampling

Approximately0.5mlbloodwas withdrawnfromallanimalsbelonging toGroup1to4bytailpuncturemethod.Bloodsamplesweretakenasperttheschedule mentionedinprotocol(0,1,6,12h,1,2,3,4,5,6and7day). One-wayanalysisofvariance(ANOVA)wasusedtocomparedatabetweenallgroups inthestudy.Differences were considered significant when $P<0.05$.

Stability study

Theselectedformulationsweresubjectedtoacceleratedstabilitystudiestoevaluate effectofstress conditionsaccordingtoICHguidelines.Formulationswerepackedin 0.044mm Bi laminated aluminum foil and subjected to elevated temperature and humidityconditionsof40C±2C/75±5% RH. Sampleswerewithdrawnattheendof1,2and3 months and evaluated forparticle diameter, drug content and drug release.

RESULTS AND DISCUSSION

DSC study

TheDSCthermogrampresented in figure 1 showed a sharp enothermic at 137°C corresponding to melting point of B form of drug. Retention of this endothermic in drug polymer mixture indicated absence of chemical interaction between drug and polymer.

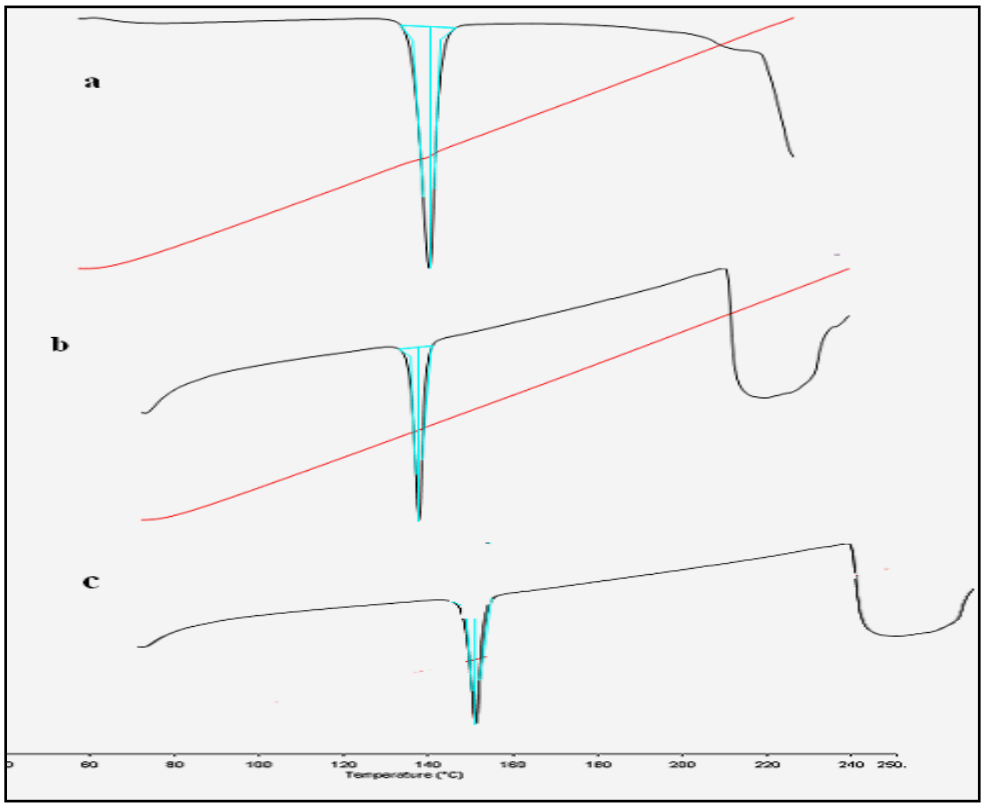

Figure1. Drug polymer stability indicating DSC thermogram (a) Nateglinide and (b) Nateglinide: PLGA mixture and (c) Nateglinide: PLGA nanaparticle

Preparation of nanoparticles

Inordertoobtainemulsified systems,theadditionof energy isafundamentalstep.Emulsificationcanbeconsideredoneofthemostimportantstepsoftheprocess, becauseaninsufficientdispersionof phasesresultsinlargeparticleswithwidesize distribution.Thefinalsizeofthenanoparticlesdependsontheglobulesizethroughout the emulsification process. A reduction of the emulsion globule sizeallows the formation of smaller nanoparticles. All the batches were prepared at 17000 rpm homogenization speed for 10 min and evaluated for particle diameter and drug entrapment efficiency.

Effect of polymer concentration

PLGAcontentwasvariedbetween60-180mg,andtheinfluenceoftheinitial concentration ofpolymeron theparticlesizedistributionwasstudied without inclusion of surfactant. It was observed that increase in PLGA amount gives particle size with increase in diameter, figure 2. Thiswasprobably causedbyincrease inviscosityofdispersedphase(polymersolution),resultinginpoordispersabilityi.e.ahighviscousresistan ceagainsttheshearforcesduringthe emulsification.Coarseemulsionsareobtainedathigherpolymerconcentrations,which

leadtothebuildofbiggerparticlesduringthediffusionprocess.Thisfactisexplained bythegreaterprobabilitythatthedesolvatedmacromolecules(orsmallaggregates formed fromthese molecules)coalesce in a moreconcentratedsolution, thereby forminglargercoacervates orparticles.

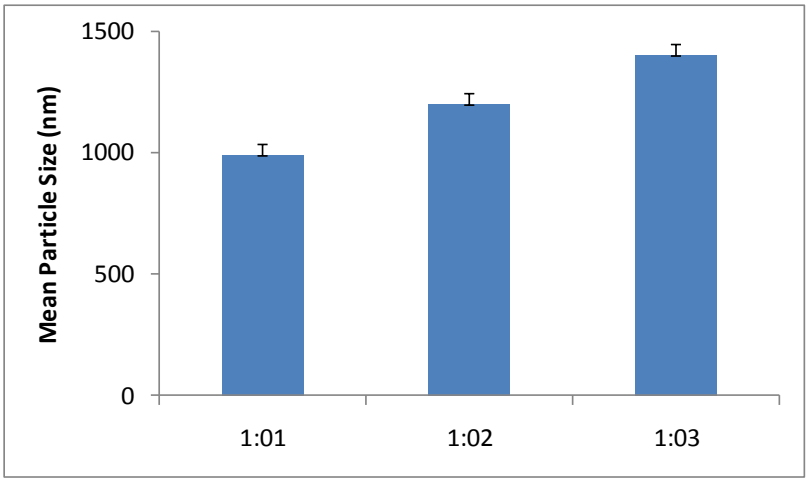

Figure 2. Effect polymer concentrations on formulation particle size

Effect of surfactant content

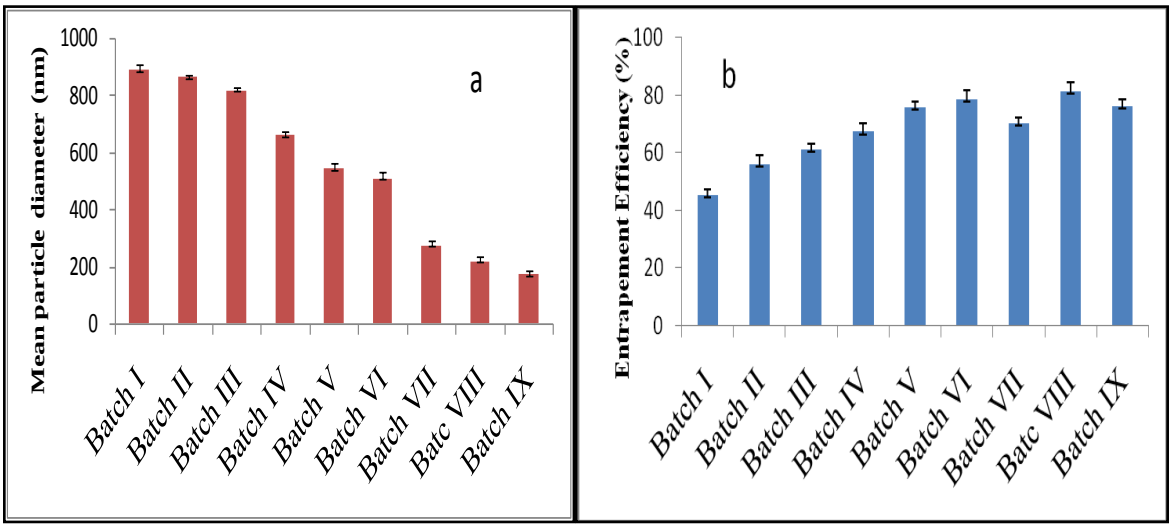

Figure 3. Effect of variable conditions on (a) mean particle size and (b) encapsulation efficiency

Itwasobservedthattherewasdecreaseinparticlesize(285–170nm)whenthePVAconcentrationintheexternalaqueousphasewasincreasedfrom 0.5to1.5%(w/v).Presence of highconcentrationofemulsifier aids in size reduction process and produced nanoparticles of reduced size. This phenomenoncanbeexplained bystabilizingfunction ofanemulsifier.Itiseasy tounderstandthataninsufficientamountofemulsifierwould failinstabilizingallthenanoparticlesandthussomeofthemwouldtendtoaggregate.

Asaresult,nanoparticleswithlargersizewouldbeproduced.Intheemulsion–solvent evaporationmethod,theemulsificationandstabilizationofthe globulesarecrucial factors.Theamountofsurfactantplaysanimportantroleintheemulsificationprocess and in theprotection ofthedroplets, because it can avoid the coalescence of globules. Particle size and drug encapsulation studies showed that nanoparticle prepared from drug polymer ratio 1:3 and stabilizer concentration 1.5 % w/v gives maximum 82% encapsulation efficiency (figure 3and 3b).

Particle size distribution study

Figure 4 shows plot of size distribution by intensity i.e. intensity (%) vssize in nanometer of the optimized formulation NTGNP08. It showed particle size distribution inbetween90to700nm withmaximum intensityof14.5 %ofsizerange200-300nm.Averageparticlesizewasfoundtobe 216nm. Figure 5 shows the histogram of the particle size distribution.

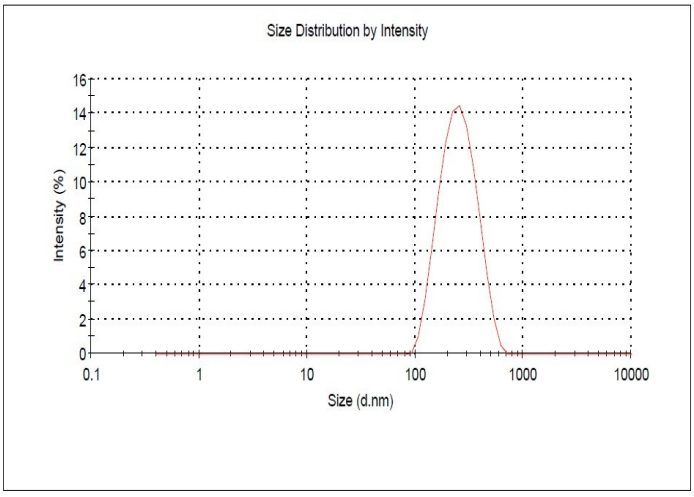

Figure4: Particle sizedistributionofNanoparticlesofNTGNP08

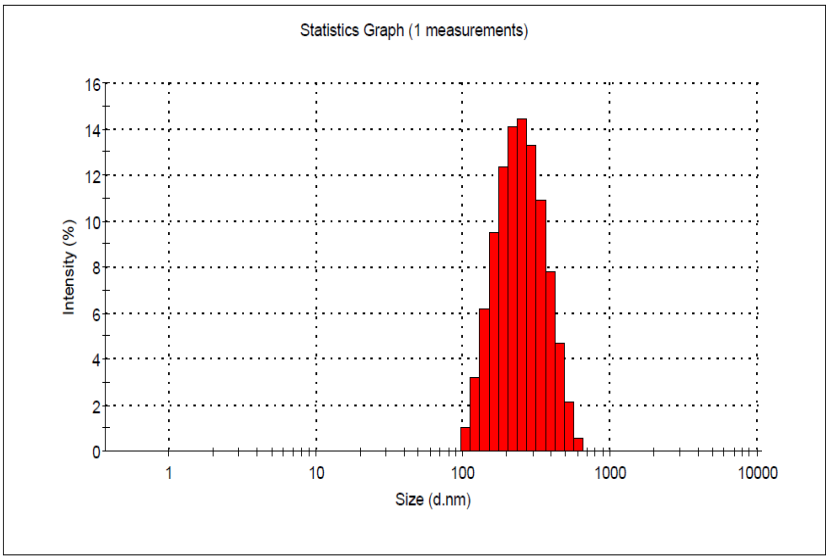

Figure 5.Histogramshowing particle sizedistributionofNTGNP08

Zetapotential

Thezetapotentialisanindexofstabilityofnanoparticles.Thehigherthemagnitude, irrespectiveofthechargetype,higherthestability. Alltheformulations ofnanoparticlesexhibitedhigh zetapotentialsrangingfrom-7.72to-0.473 (figure 6) andthe valuesarenegative.Generallyhighlynegative zeta potentialvaluesareexpectedfor purePLGAnanoparticlesduetopresenceofcarboxylgroupsonthepolymericchain extremities. High zeta potential value indicates good stability of formulation against particle aggregation.

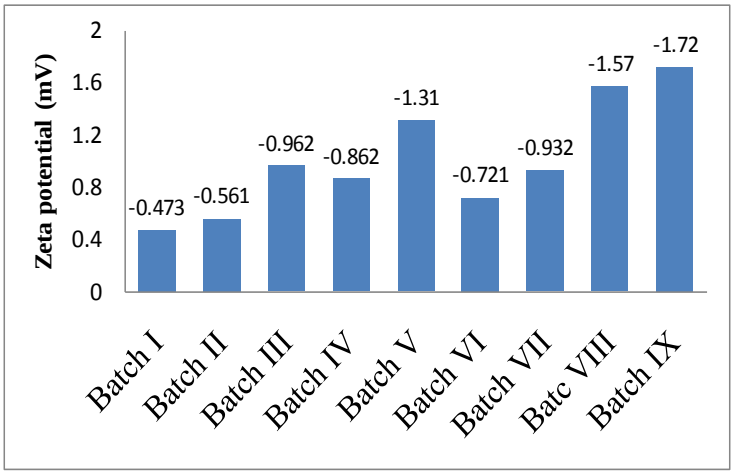

Figure6. Zeta potential ofdifferent NTGNPformulation

Scanning electron microscopy

Morphologicalevaluationofthenanoparticleswasperformedusingscanningelectron microscope. Plain drug was present as rod shape crystal powder whereas optimizedformulationshowedformationofsmallsizenanoparticlesofPLGAwith sphericalshapeanduniformsize figure 7.

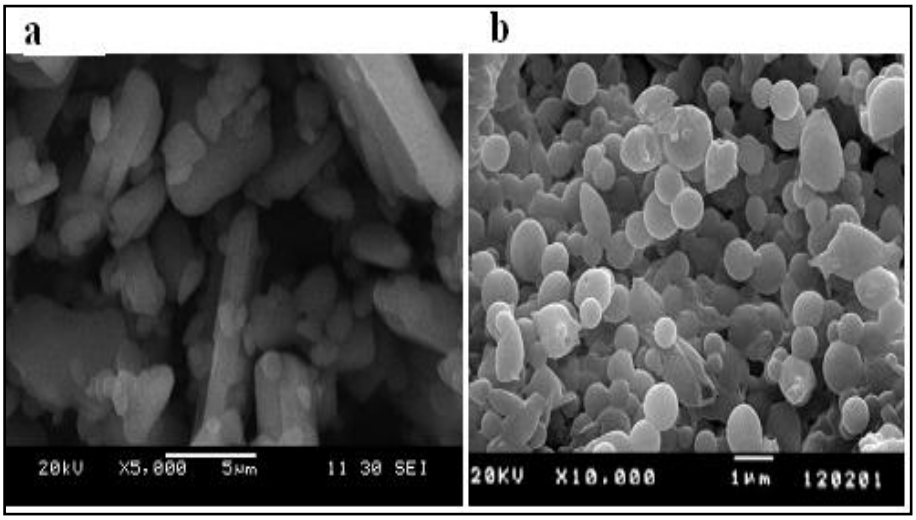

Figure 7. Scanning electron micrograph of (a) drug and (b) optimized nanoparticle

***In-vitro* drug release**

Figure 8showstheinvitroreleaseprofileof optimizedNTGNP 08formulation. NTGreleasefromnanoparticlesappearedto havetwocomponents.Firstaninitialexponential phasereleasing3.873mg(7.918%)in 30minand8.895mg(18.18%)ofthedrugin1hwhichisalmostidenticalwith theconventionaldoseofa60mg tabletfollowedby aslowphasethedrugin 48 h.

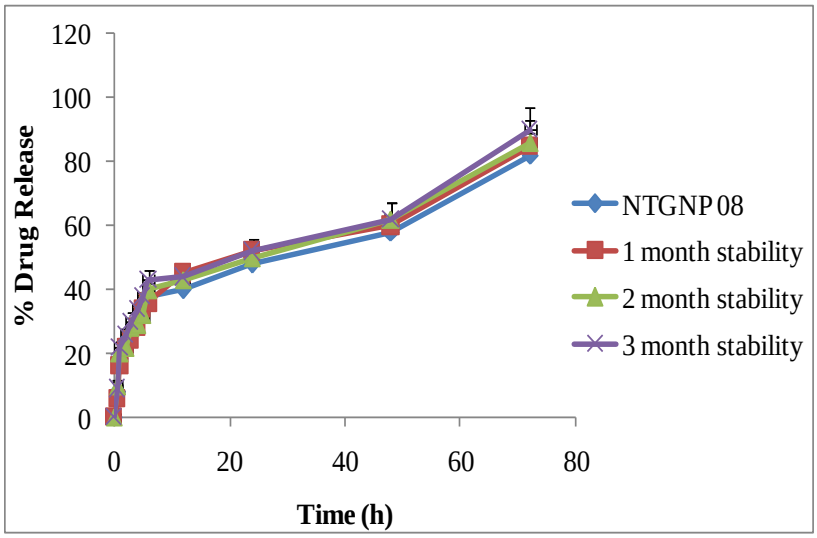

Figure 8. In vitro drug release profile of optimized NTGNP 08 nanoparticle

TheinitialreleasephaseofNTGwasprobablyduetoNTGwhichwasadsorbedor closeto the surfaceofthenanoparticlesandduetodiffusionofthedissolvedNTG withinthePLGAcoreofthe nanoparticleintothereleasemedium.After12h,the releaserateincreasedslightlyuntilthe24hprobablybecauseofalagphasewhen sufficientpolymererosionhastakenplace.Finallythe releaseslowedbutcontinued untilthe72h(82%drugrelease),becausethediffusioncontrolledreleasepatternwas completed and theerosion of polymer was taken place. Basedontheregressioncoefficientsitcanbe concludedthatreleaseofdrugfromPLGAloadednanoparticleisfollowingHiguchi model suggesting that drug release mainly took place by diffusion mechanism, table 2.

Table 2.Release kinetic parameters for optimized batch NTGNP08

	Zero Order	First Order	Higuchi	Hixson Crowell	Korsmeyer Peppas
R^2	0.926	0.937	0.983	0.944	0.953
K	8.485	1.832	15.562	39.475	8.945

Stability study

Formulation kept for stability study found to be showed uniform drug content approximately

99±0.54%. Particle size was get slightly increase due to aggregation of nanoparticles during storage period. Drug release profile was found similar to initial indicating insignificant change and stability.

***In vivo* study**

Oral administrationofstandard NTG (800 µg / kg,t.i.d.) and the formulation (equivalent toapproximately 800µg/kg, t.i.d.) to STZ-diabetic rats significantly reducedbloodglucoselevel 1 h after administration [Figure 9]. Bloodglucose levels in Group1wasnormalthroughoutthestudy.In group 2 ,thediabeticcontrol,blood glucoselevelswereveryhighuntilday4,afterthatdecreased slightly,althoughthe decreasewasnotstatisticallysignificant.Theseobservationscanbeexplainedbythe destructionoftheIsletsofLangerhansbySTZ,whichstartedtorecoverafter4days.

Animalsinthisgroupalsohavesamesurvivalrateasthatofthenormalcontrolgroup butthesignandsymptoms ofdiabetesaremoreseverewithincreaseintheblood glucoselevel andexcessiveurination.

InGroup3initially it wasobserved that therewasdecreasedbloodglucoselevelbutdueto shorter half-lifeofNTGagainthebloodglucoselevelrisesandneeded toadministera newdosewhichwas giventhriceinadayto control the bloodglucoselevel.In Group4,diabeticratsweregiventheformulationNTGNPandafter1hasignificant decreaseinbloodglucoselevelwasthereleaseof surfaceadsorbeddrugimmediateafteradministration.Therewasconstantdecreasein bloodglucoselevelforallsevendays observed whichshowsthatnanoparticlesofPLGA areeffective forcontrolled and sustained drugdeliveryas compared to thegroup3.

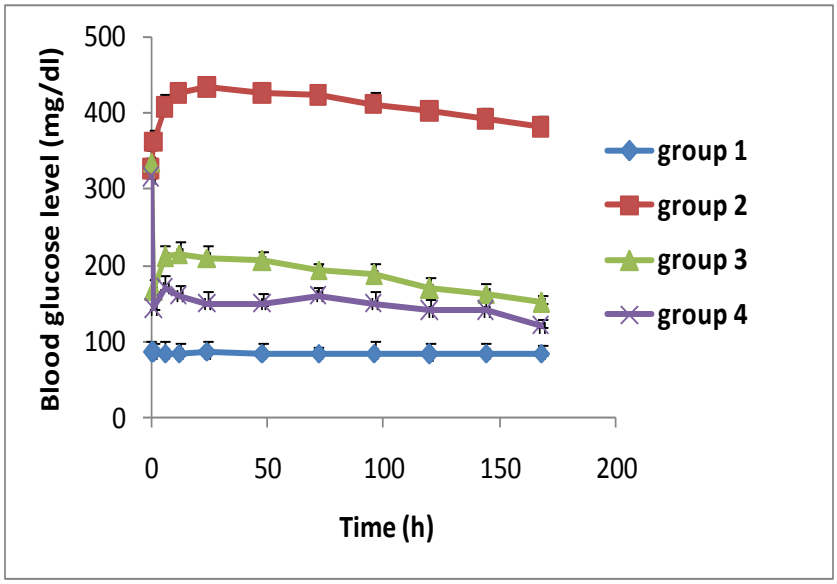

Figure9:Bloodglucoselevels in the four studygroups over 7 days period.

One-way ANNOVA (multiple comparisons) followed byTukey'stestwas used to analyze the in vivo antidiabeticeffectsoftheNPformulationsofNTG.Bloodglucoselevelsingroup3 rats,treatedwithstandardNTG(800µg⁄kg,t.i.d.),weresignificantlylowerthanthose ingroup2($P<0.05$).Ingroup4rats,treatedwithNTGNP,asignificantdecreasein bloodglucoselevelswasseen;therewasaclearlong-lastingeffect untiltheendof experiment($P<0.05$).Comparisonsofresultsbetweengroups3and4indicatedthat theNTGNPformulationwaseffectivetherapeuticallycomparedwithstandardNTG and had aprolonged duration of action.

CONCLUSION

Thepresent study has demonstrated possibility of formulatingalong-acting biodegradable NPformulationofNTGusingtheemulsificationsolventevaporation technique.TheresultsindicatethatNTGcanbeentrappedwithinPLGAnanoparticles in presence of optimum concentration of stabilizerPVA(1.5%). Thesizeof nanoparticlesandtheloadingofdrugincarrierwerehighlydependentonstabilizer and PLGAconcentration.Fromthe studyitcan beconcludedthatthepreparednanoparticlescouldbe used as sustained releasepreparationforeffectivemanagementoftypeIIdiabetesmellituswithreduced dosefrequency,decreasedsideeffectsandimprovedpatientcompliance.

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