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IN VITRO ANTIBACTERIAL EFFICIENCY OF KANAMYCIN SULFATE LOADED CHITOSAN NANO PARTICLES AGAINST SELECTED BACTERIAL STRAINS

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ABSTRACT

Bacterial resistance to antibiotics has been attempted to be resolved by various researchers by discovering new antibiotics and chemically modifying existing antimicrobial drugs. Unfortunately, there is no assurance that newly developed antibiotics will not develop resistance. In connection to this, nanotechnology is the most unique technology that can provide suitable tools for the effective targeted delivery of drugs into specific cells. The aim of my study was to formulate and optimize chitosan nanoparticles for Kanamycin sulfate delivery to improve bioavailability and prevent drug resistance. In this work Kanamycin is the drug of choice for demonstrating the efficacy of chitosan nanoparticles against bacterial resistance. The ionic gelation technique was used to formulate kanamycin sulfate to be encapsulated in chitosan nanoparticles. The anti-bacterial efficacy of kanamycin loaded chitosan nanoparticles was evaluated against selected bacterial strains, namely *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Escherichia coli* and *Klebsiella planticola of* clinical origin. In the present study, the maximum activity was found in *Escherichia coli* followed by *Klebsiella planticola, Bacillus subtilis, Streptococcus pyogenes* and *Staphylococcus aureus*. The study suggested that the Kanamycin sulfate showed better activity than the conventional Kanamycin sulfate.

KEYWORDS: Kanamycin sulfate; Chitosan, Nanoparticles; Bacterial resistance.

INTRODUCTION

Antibiotic resistance is a major health problem worldwide. It has been estimated that each year in US approximately 2 million people are infected with antibiotic resistant bacteria and

23,000 die as a result of these infections.[1] Kanamycin is an aminoglycoside antibiotic, produced by Streptomyces kanamyceticus and is active against Gram positive, Gram negative and acid-fast bacteria. Conventionally the bacterial infections have been treated by penicillin and kanamycin.[2] However, kanamycin has been replaced by gentamycin due to the emergence of resistant organisms. During the last decade various resistance mechanisms such as decreased uptake, increased efflux of drug from bacterial cell, formation of biofilms to avoid contact with antibiotics have been identified, which lead to the failure of the treatment. Instead of developing novel antibiotics to combat infections, which is very expensive, it is advisable to design novel delivery strategies. One such approach is the use of nanotechnology, which will facilitate effective delivery to the infection site, and regulate the amount and frequency of the dosage, thereby preventing toxicity. Previously, scientists have used various approaches to overcome penicillin resistance viz silver nano conjugates, polyacrylate nanoparticles, squalene nanoparticles and PEG modified dendimers. The present study aimed to formulate kanamycin loaded chitosan nanoparticles using the ionic gelation technique[3] in order to increase the efficacy of the drug and its stability.

MATERIALS AND METHODS

Materials

Kanamycin sulfate salt (1653 unit/mg) and Chitosan (DD: 75%, low molecular weight) were purchased from Sigma Aldrich, USA. All other chemicals used were of analytical grade.

Methods

Formulation of Kanamycin sulfate – chitosan nanoparticles

Kanamycin sulfate loaded chitosan nanoparticles was prepared by the ionic gelation method which used formaldehyde as a cross linking agent.[3] Extensive pre-formulation studies were performed with varying polymer and cross linker concentration. Briefly, 1% w/v chitosan solution was prepared in 1% v/v glacial acetic acid that had a pH of 4. The pH was increased to 6 by adding 1ml of 1N NaOH. The chitosan gel was homogenized at 3600 rpm for 30 minutes and during homogenization 1 ml of 1% w/v of kanamycin sulfate was added. During the formulation of nanoparticles, sonication was done for 30 seconds at a pre-determined time interval. The kanamycin sulfate loaded nanoparticles were prepared upon adding 500 µl of formaldehyde in a drop wise manner and homogenization was continued for one hour.

Physicochemical characterization

The Morphology[4] of nanoparticles was visualized under TEM with an accelerating voltage of 100 kv. A drop of the sample was placed on to a carbon coated copper grid and dried to leave a thin film. The film was then stained with 1% phosphotungstic acid. The grid with the film was then dried at an ambient temperature and viewed under TEM (Hitachi, Japan) and photographs were taken at a suitable magnification. The zeta potential, size and PDI [4] of kanamycin sulfate loaded chitosan nanoparticles were measured using the Nano – ZS zeta sizer, Malvern Instruments, UK.

***In vitro* release profile**

Briefly, 2 ml of kanamycin sulfate loaded chitosan nanoparticles were taken in 50 ml of phosphate buffer of pH 6.0 in a beaker. The nanoparticles were stirred in a magnetic stirrer at 50 rpm for 48 hrs at a temperature of 25^0C. The samples were withdrawn at pre-determined time intervals of 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 hrs and centrifuged at 15000 rpm to separate the entrapped drug. The samples were then assayed for drug content using a UV spectrophotometer (Schimadzu, Japan) at 279 nm.[5] The values were determined by extrapolation on a standard graph.

Assessment of antibacterial activity

Five bacterial strains *Bacillus subtilis, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli* and *Klebsiella planticola* were collected from Jazan hospital, Jazan, KSA and used in this study. The cultures were subcultured and incubated at 37^0C for 24 hours. To examine the antibacterial action of kanamycin sulfate loaded nanoparticles, the selected bacteria were grown in nutrient broth and incubated at 37^0C for 24 hours. The MIC was determined in Nutrient Broth (NB), (Hi-Media, India) using two-fold serial dilutions of chitosan nanoparticles with initial bacterial inoculums of 10^{-6} CFU and the time and temperature of incubation being 24 hours and 37^0C, respectively. The MIC is the lowest concentration of antimicrobial agent which when observed visually completely inhibits the growth of the microorganisms. The test was performed in triplicate to confirm the value of MIC for each tested strain. A specified quantity of Muller Hinton agar plates was prepared and kept in an aseptic condition. The agar well diffusion and agar disc diffusion techniques were performed for the kanamycin nanoparticle and standard kanamycin disc (5 mcg disc) respectively. After 24 hours of incubation at 37^0 C, the zone of inhibition was measured and tabulated.

Statistical analysis

All of the experiments were performed in triplicate (n= 3) and the data were subjected to the student T test; the level of significance is $P < 0.001$ using the Graph pad Instat software system, USA.

RESULTS AND DISCUSSION

Formulation and Physicochemical characterization

Preformulation studies were performed to standardize the formulation. In this study the formulations was standardized based on the size, PDI and zeta potential by varying the polymer and cross linker concentration. In this study the kanamycin sulfate loaded chitosan nanoparticle was prepared by using ionic gelation technique. Table 1 and 2 summarizes the effect of polymer and cross linker concentration on size and PDI. The nanoparticle showed the Z average diameter of 585.9 nm, the poly dispersity index (PDI) was 0.63 and Zetapotential was 42.4 mv when 0.1% w/v of chitosan polymer and 500 µl formaldehyde was used as a cross linker. However, this parameter was highly influenced by sonication process (Table 3). After sonication the size of nanoparticle was reduced to 390 nm with a PDI of 0.3. It was quite interesting to note that the zeta potential was increased from 42.4 to 62.8 mV. A similar type of study was reported by pertha et al., 2010.[6] From the TEM analysis it was understood that the particles were discrete and homogenous but not spherical in shape. The formulation was more successful since the TEM analysis showed homogenous and discrete particles. Similar findings have been reported earlier when penicillin was encapsulated in chitosan nanoparticles.[3]

In vitro release pattern

The release of kanamycin from chitosan nanoparticles was characterized by a biphasic pattern with an initial release of up to 2 hours considered as a zero order (Fig.1.). After 2 hours the release was much more sustained and considered as a first order release. The *in vitro* release proved that approximately 92% of the drug was released within 24 hours. This shows that the kanamycin is successfully released from the chitosan nanoparticles without any interruption. The linear regression analysis was performed and based on Run's there are 7 points above, 1 point below the line and 2 points were run (Fig. 2.). There were fewer runs than expected. The p value was found to be 0.2500, showing no significant departure from the linearity. Therefore, study suggested the release pattern is in linear fashion. Similarly, an earlier report

showed that the insulin loaded chitosan nanoparticles release insulin in a biphasic pattern with an initial release within 15 minutes.[7]

Assessment of antibacterial activity

In this study the MIC of conventional kanamycin was found to be 10^{-2} and that of kanamycin loaded chitosan nanoparticles was found to be 10^{-3}. The zone of inhibition of kanamycin nanoparticles was higher when compared to a standard kanamycin disc (Table 4). In the present study the maximum activity was found in *Streptococcus pyogenes* followed by *Bacillus subtilis* and *Staphylococcus aureus* (Fig. 3.). Earlier reports showed that the electrostatic attraction between negatively charged cell membrane of microorganisms and positively charged nanoparticles are linked and exhibiting the antibacterial effect by releasing the drug in sustained manner.[8] It is very obvious that chitosan is a polycationic polymer that can easily attach to negatively charged microbial cells and kanamycin released in sustained manner and exhibited the antibacterial effect.

Table 1: Effect of cross linker concentration on nano particle size and PDI

Polymer	Formaldehyde	Various parameters		
		Size (nm)	PDI	Zeta potential (mV)
0.1%	250 µl	644.2	1.00	35.4
0.1%	500 µl	585.9	0.63	42.4
0.1%	1000 µl	1057	0.7	37.3
0.1%	1500 µl	1167	0.8	33.7

Table 2: Effect of polymer concentration on nano particle size and PDI

Polymer	Formaldehyde	Various parameters		
		Size (nm)	PDI	Zeta potential (mV)
1%	500 µl	700	1.00	65.4
0.5%	500 µl	638.5	0.83	52.4
0.25%	500 µl	616.12	0.7	49.6
0.1%	500 µl	585.9	0.63	42.4

Table 3: Effect of Sonication process on nano particle size and PDI

Polymer	Formaldehyde	Before sonication			After sonication			
		Size (nm)	PDI	Zeta potential (mV)	Sonication time (Sec)	Size (nm)	PDI	Zeta potential (mV)
0.1%	250 µl	644.2	1.00	35.4	10	497.2	0.59	55.4
0.1%	500 µl	585.9	0.63	42.4	15	401.1	0.48	62.8
0.1%	1000 µl	1057	0.7	37.3	20	390	0.3	52.3
0.1%	1500 µl	1167	0.8	33.7	25	661	0.51	46.8

Table 4: Spectrum of anti bacterial effect of kanamycin nanoparticles and standard kanamycin disc against specified organisms

Organism	Zone o Inhibition (mm)	
	Kanamycin chitosan Nanoparticles	**Kanamycin disc (5 mcg/disc)**
B.subtilis	12.3 ± 0.57	13.8 ± 1.57
S.aureus	10.1 ± 1.73	12 ± 1.15
S.Pyogens	15.6 ± 0.57	12.1 ± 1
E.Coli	16.3 ± 1.52	27.1 ± 0.2
Klebsiella	17.6 ± 1.7	21.6 ± 1. 2

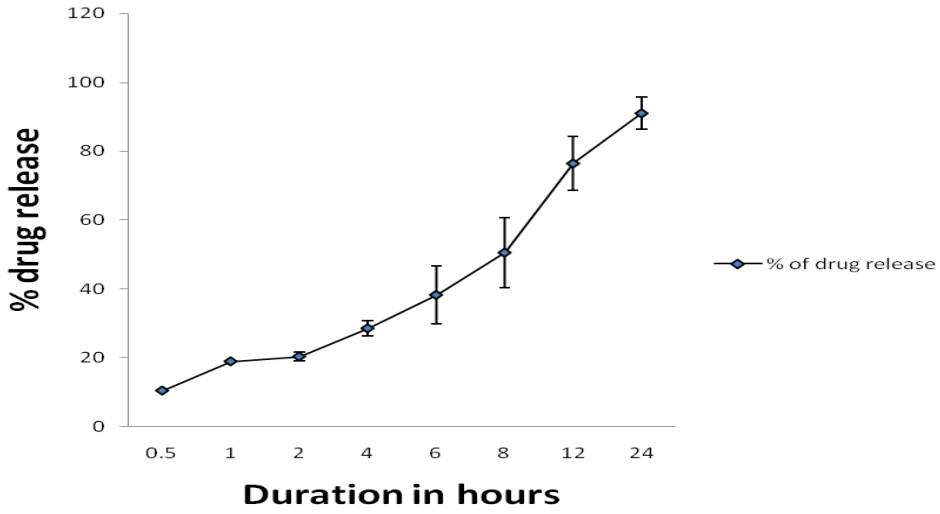

Figure 1: *In vitro* release profile of kanamycin loaded chitosan nanoparticles

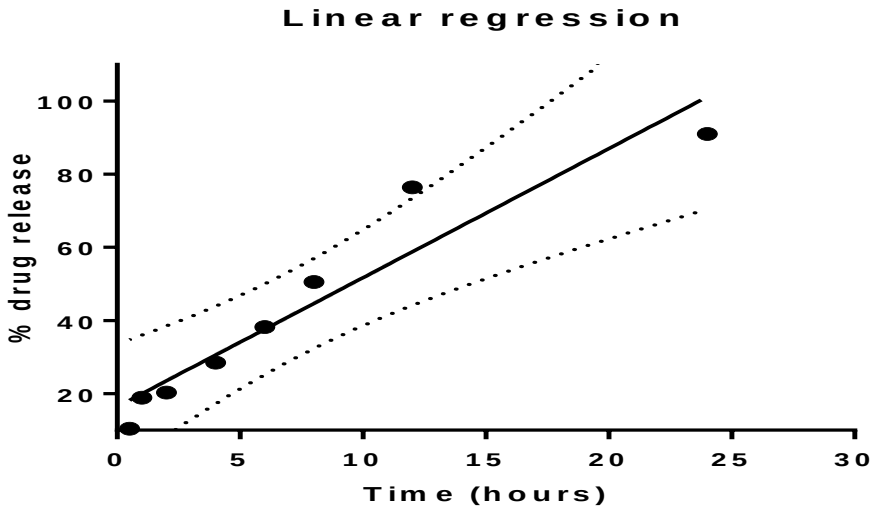

Figure 2: Linear regression plot of *In vitro* release profile of kanamycin loaded chitosan nanoparticle

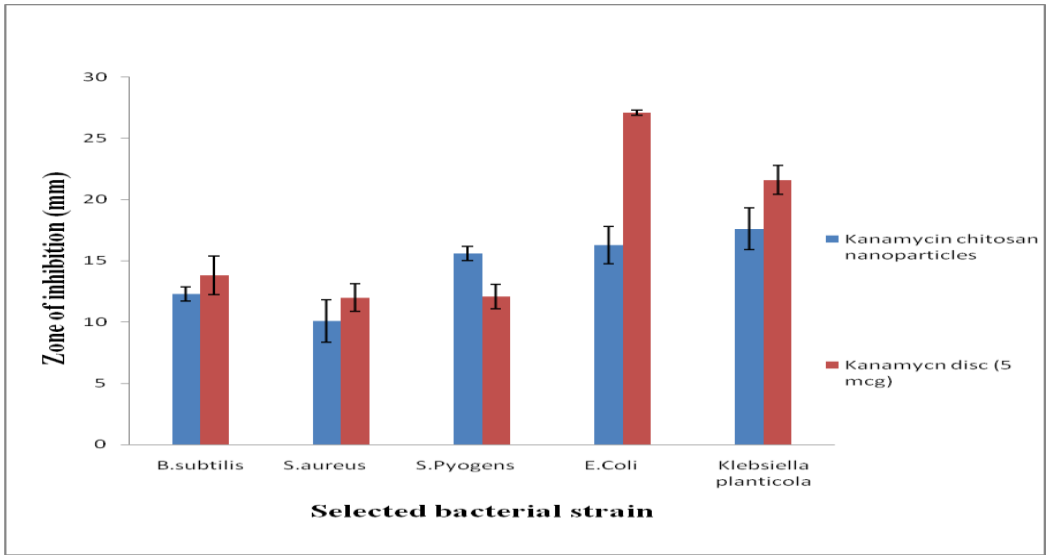

Figure 3: Spectrum of activity against selected bacterial strains

CONCLUSIONS

The preliminary study suggested that a wider spectrum of antimicrobial action is exhibited by chitosan nano kanamycin in comparison to a standard kanamycin disc. However, the toxic profile of nanoparticles has not yet established in order to determine the exact anti microbial action. Therefore, the study is suggesting that kanamyacin can be successfully entrapped in chitosan nanoparticle and their antimicrobial property can be accomplished. Further studies are underway in order to standardize the dosage level and to overcome the lacunae that were experienced in this study.

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