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ORIGINAL RESEARCH PAPER

Evaluation of loading efficiency of azelaic acid-chitosan particles using artificial neural networks

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ABSTRACT

Objective(s): Chitosan, a biodegradable and cationic polysaccharide with increasing applications in biomedicine, possesses many advantages including mucoadhesivity, biocompatibility, and low-immunogenicity. The aim of this study, was investigating the influence of pH, ratio of azelaic acid/chitosan and molecular weight of chitosan on loading efficiency of azelaic acid in chitosan particles.

Materials and Methods: A model was generated using artificial neural networks (ANNs) to study interactions between the inputs and their effects on loading of azelaic acid.

Results: From the details of the model, pH showed a reverse effect on the loading efficiency. Also, a certain ratio of drug/chitosan (~ 0.7) provided minimum loading efficiency, while molecular weight of chitosan showed no important effect on loading efficiency.

Conclusion: In general, pH and drug/chitosan ratio indicated an effect on loading of the drug. pH was the major factor affecting in determining loading efficiency.

Keywords: *Azelaic acid, Artificial neural networks (ANNs),Chitosan, Loading efficiency*

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INTRODUCTION

Chitosan, as a natural linear polysaccharide, is composed of D-glucosamine and N-acetyl-D-glucosamine units which may be prepared by the alkaline N-deacetylation of natural chitin [1-4]. Chitosan and its derivatives are nowadays being used in hydrogels, nano/micro-ûbres and particles as well as scaffolds for biomedical applications, such as tissue engineering. Furthermore, they are being applied in areas such as water purification, textiles, cosmetics, food and agriculture [5-9]. Azelaic acid is a natural organic dicarboxylic acid, which is obtained by oxidation of oleic acid [10]. It has been shown to be a competitive inhibitor of oxidoreductive enzymes such as thioredoxin reductase, mitochondrial oxidoreductases, tyrosinase and DNA polymerase [11-14]. Moreover, the drug is used as an oxygen scavenger and inhibits oxyradical activity by neutrophils in cell cultures [15, 16]. It has been used clinically for many years in treatment of acne vulgaris as well as in epidermal hyperpigmentary disorders [17].

Drug loading efficiency is an important property in drug-loaded systems. Particularly, in case of expensive drugs, the drug loading efficiency becomes more important [18]. Loading of drug to carrier particles can protect the drug from loss and inactivation. Also, it helps to save drug activity for

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prolonged durations and reduce its toxicity by decreasing frequency of usage [19].

Preferably, a high drug loading is aimed in design of drug delivery systems as this improves the efficiency of such system. Commonly, drug loading occurs by either adsorption onto carrier particles or entrapping during the formulation [20]. The efficiency of drug encapsulation/incorporation onto a carrier is a very important factor that influences the selection process of drug and carrier [21].

Studies show that when employing complexation between a drug molecule with a nano/micro-particle with opposite charge, loading efficiency depends on many different parameters. For instance, loading efficiency of bovine serum albumin has been shown to be related to deacetylation degree and molecular weight of chitosan. Also, increase in chitosan concentration caused a reduction in loading efficiency [22]. Several parameters influence the loading of drug into chitosan. For instance, effect of chitosan molecular weight (MW) on drug loading in chitosan particles was studied [23]. The method of preparation has an impact on drug loading. For instance, when using combined method of mixing and absorption for loading Hb into microcapsules, loading efficiency was found to be maximum (19.9%) [24]. In our work, effect of three independent parameters, namely, pH, ratio of azelaic acid/ chitosan and molecular weight of chitosan, on loading efficiency was studied. The influence of pH on drug loading in chitosan particles has already been reported. By increasing the pH value, drug loading in chitosan has been reported to reduce [25]. Similarly, molecular weight of chitosan has also been shown to be effective on loading efficiency. Often, by increasing in chitosan MW, the loading efficiency of chitosan for a drug increases [26].

However, majority of works in this area are employing one-factor-at-a-time approach, with its inherent drawbacks such as lack of accurate estimates in the predicted effects and imperfect coverage for the factor space [27]. Furthermore, effect of ratio of polyanion/chitosan on loading efficiency has been rarely reported- with increasing the ratio of tripolyphosphate /chitosan, the loading efficiency increases reduced [28].

Artificial neural networks (ANNs) as approaches to generate models by mimicking biological neurons, are usually used to estimate complex relationships between a large number of inputs and outputs [29]. ANNs are computational networks which attempt to find patterns in data, by using algorithms to organize and control its embedded functions [30]. ANNs work based on a brain with the arrangement and interconnection of neurons in various layers to create neural networks [31].

Compared with other classic modeling techniques such as response surface methodology (RSM), ANNs have demonstrated satisfying in terms of their prediction and estimation capabilities [32]. In present study, An ANNs model was used to investigate the interactions between the three possibly important independent variables and their effects on loading of azelaic acid in the chitosan particles.

MATERIALS AND METHODS

Materials

Chitosan used in the study was supplied by Zhengzhou Sigma Chemical Co. (China) (MW= 7, 30, 100 and 500 kDa, deacetylation degree> 80%). Azelaic acid was gifted by Sepidaj Pharmaceutical Co (Iran). Amicon® Ultra 15 mL Filters were supplied by Merck chemicals (Germany).

Loading of azelaic acid in chitosan

Chitosan was added to solution of azelaic acid (10mg/ml) and vigorously stirred until dissolved (~45-60 min). 30 solutions of azelaic acid in chitosan were prepared (pH: 5-7, Ratio of azelaic acid/ chitosan: 0.5-0.9, Chitosan MW: 7, 30, 100 and 500kDa). Solutions were filtered by Amicon® Ultra 15 mL (Membrane NMWL, 3 and 10 kDa) at 5000rpm.

Free azelaic acid was determined by HPLC method (KNAUER smartline Manager 5000, pump 1000, UV-visible detector 2500, Teknokroma C-18 column, 150mm×4.6mm i.d., 5ìm particle size).

The mobile phase was prepared by adding 0.77g of ammonium acetate in 1000 mL of methanol-water (2:3) and *adjusted* to *pH* 5.0 ± 0.1 with adding acetic Acid (96%), volume of injection was 20ìl. The column temperature was retained at 25ºC [33].

ANNs modeling

The ANNs Model was made by INForm V4.02 (Intelligensys, UK).

Then, the model was used for showing the relations between the inputs and the output were. The 3D response surfaces illustrated the impact of two inputs on the output when the third input was fixed.

Table 1. The training, test and unseen data sets used in ANNs modeling

pH	Ratio of azelaic acid/chitosan	chitosan MW (kDa)	Measured free azelaic acid (mg/ml)	Predicted free azelaic acid (mg/ml)	Error (mg/ml)
			Unseen data		
5.3	0.85	100	2.199	2.585	0.386
5.6	0.65	7	3.704	3.310	-0.394
5.9	0.6	500	2.972	3.371	0.399
5.9	0.75	30	3.324	3.756	0.432
6.2	0.5	7	3.608	3.407	-0.201
7	0.75	100	4.305	4.178	-0.127
			Training data		
5	0.7	7	1.853	2.058	0.205
5	0.85	30	1.191	1.366	0.175
5.3	0.7	7	4.492	2.937	-1.556
5.3	0.65	30	2.101	2.731	0.630
5.3	0.9	500	2.874	2.657	-0.217
5.6	0.9	30	3.321	2.930	-0.392
5.6	0.8	100	3.205	3.436	0.231
5.6	0.7	500	3.550	3.423	-0.127
5.9	0.9	100	3.474	3.508	0.034
5.9	0.55	500	3.038	3.018	-0.020
5.9	0.7	7	4.067	3.739	-0.328
5.9	0.8	100	3.353	3.736	0.383
6.2	0.85	30	4.108	3.843	-0.265
6.2	0.6	100	3.415	3.796	0.381
6.2	0.55	500	3.939	3.535	-0.404
6.5	0.8	7	3.853	4.003	0.150
6.5	0.85	30	4.363	3.975	-0.388
6.5	0.75	500	3.943	4.096	0.153
7	0.9	7	4.043	4.097	0.054
7	0.6	30	4.246	4.196	-0.050
7	0.7	500	4.266	4.252	-0.014
			Test data		
5	0.5	500	0.912	0.906	-0.006
5.6	0.85	7	4.886	3.209	-1.677

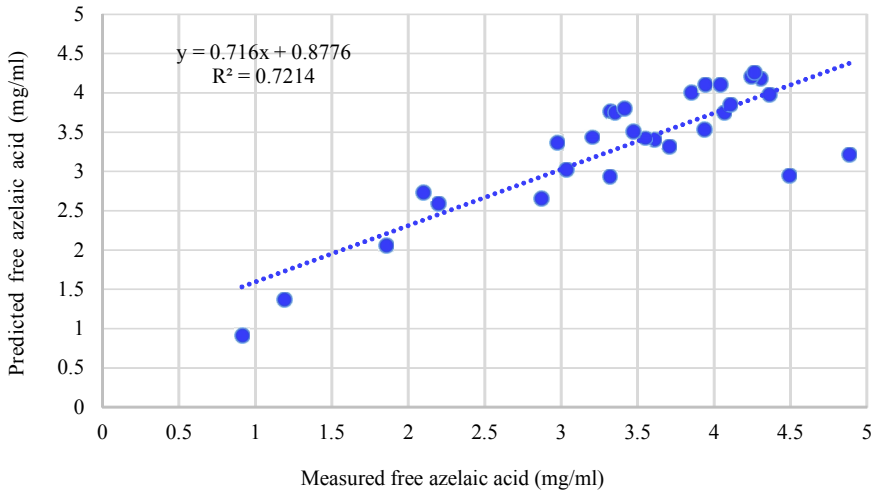

Fig. 1. Measured vs predicted values for training data

Data collection, Network Training, and Model Generation

The examination data were divided randomly into three data sets: 21 for training set, 2 for testing set, and 6 for unseen (validation set) that were used to train the network, stop overtraining, and assess the predictive ability of the trained network, respectively [34]. Coefficient of determination (R^2) was applied to validate the predictability and quality of the model [31]. The ANNs model with a determination coefficient value near to "1" suggests an appreciable predictability. The experimentally measured and predicted values for the unseen data are shown in the Table 1 and Fig 1.Training parameters used for ANNs modeling are listed in Table 2.

Scanning electron microscopy and Fourier Transform Infrared Spectroscopy studies

Scanning electronic microscopy (SEM) of optimal azelaic acid-loaded chitosan particles was performed using Zeiss DSM-960A Scanning Electron Microscope instrument under voltage at 5 kV. Also, Fourier Transform Infrared Spectroscopy (FT-IR) for azelaic acid, chitosan and drug-loaded particles were made by bruker equinox 55 FT-IR instruments. All spectra were recorded with the resolution of 4 cm^{-1} in the range of 600 - 4000 cm^{-1}.

RESULTS

The trained ANNs model for predicting free azelaic acid (mg/ml), showed R^2 values of 0.73 and 0.74 for the unseen and train data, respectively, which indicates a quality predictive ANNs model. To achieve insight into the interactions between the data the inputs and the output, as previously described [35], one input was fixed and the effect of the other inputs was checked using the response surfaces generated by the software (see Figs 2 to 4). In order to investigate

Table 2. The training parameters set with INForm v4.02

parameters	sets
No. of hidden layers	1
No. of nodes in hidden layer	4
Back propagation type	Incremental
Back propagation parameters (Momentum factor)	0.8
Back propagation parameters (Learning rate)	0.7
Target Epochs	1000
Target MS error	0.0001
Random seed	10000
Smart stop(Minimum iterations)	20
Smart stop enabled	On
Smart stop (Auto weight)	On
Iteration overshoot	200
Transfer function	Asymmetric Sigmoid
Transfer function Output	Linear

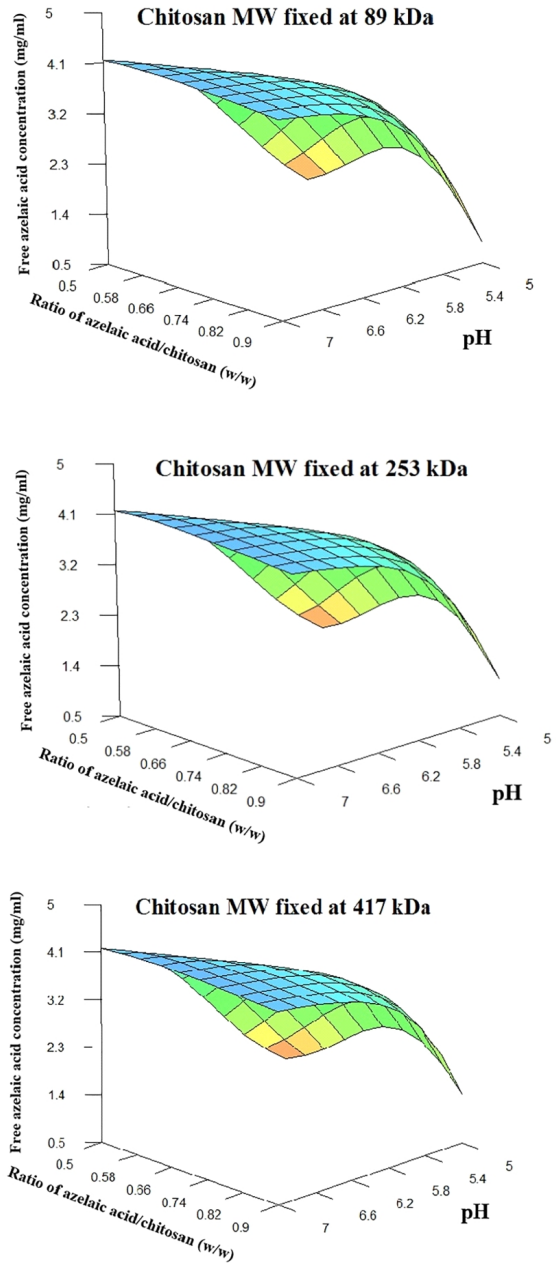

Fig. 2. 3D plot of pH, ratio of azelaic acid/chitosan and free azelaic acid concentration driven by the ANNs model

the effect of pH and ratio of azelaic acid/chitosan on loading of azelaic acid in chitosan (Z-axis: free azelaic acid in the solution, mg/ml), molecular weight of chitosan was fixed at 89, 253 and 417kDa. The 3D response surfaces are shown in Fig 2.

From the details, increasing the pH typically leads to increase in free azelaic acid. Also, when pH is low or medium, the ratio of azelaic acid/chitosan of ~ 0.7 is required to obtain the most free azelaic acid (i.e. minimum loading efficiency). As the details show, the

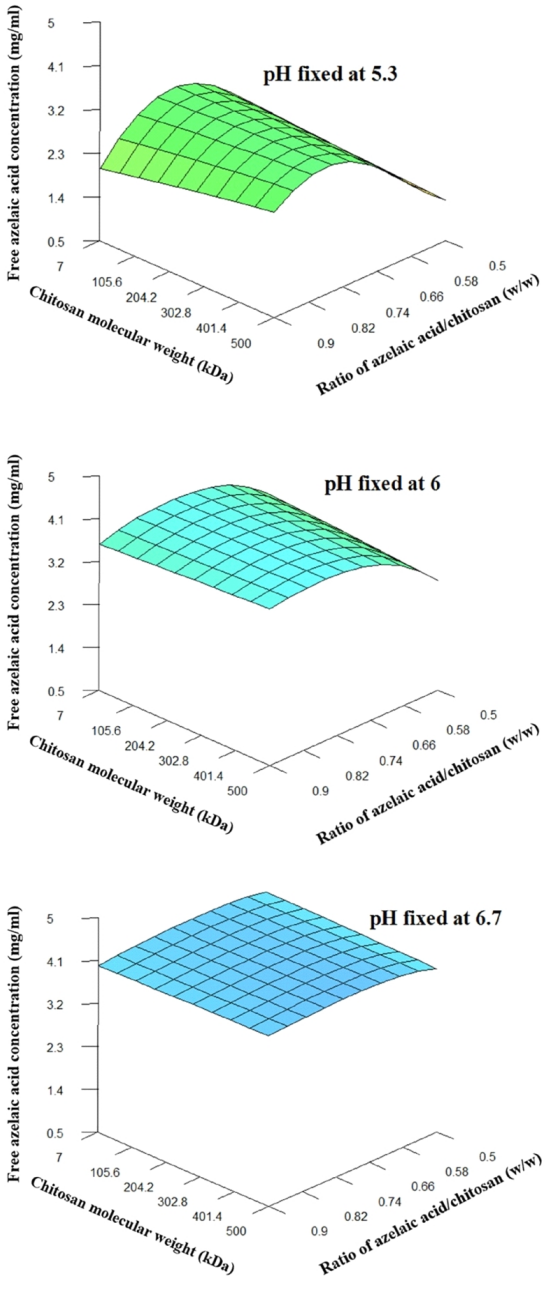

Fig. 3. 3D plot of chitosan MW, ratio of azelaic acid/chitosan and free azelaic acid concentration driven by the ANNs model

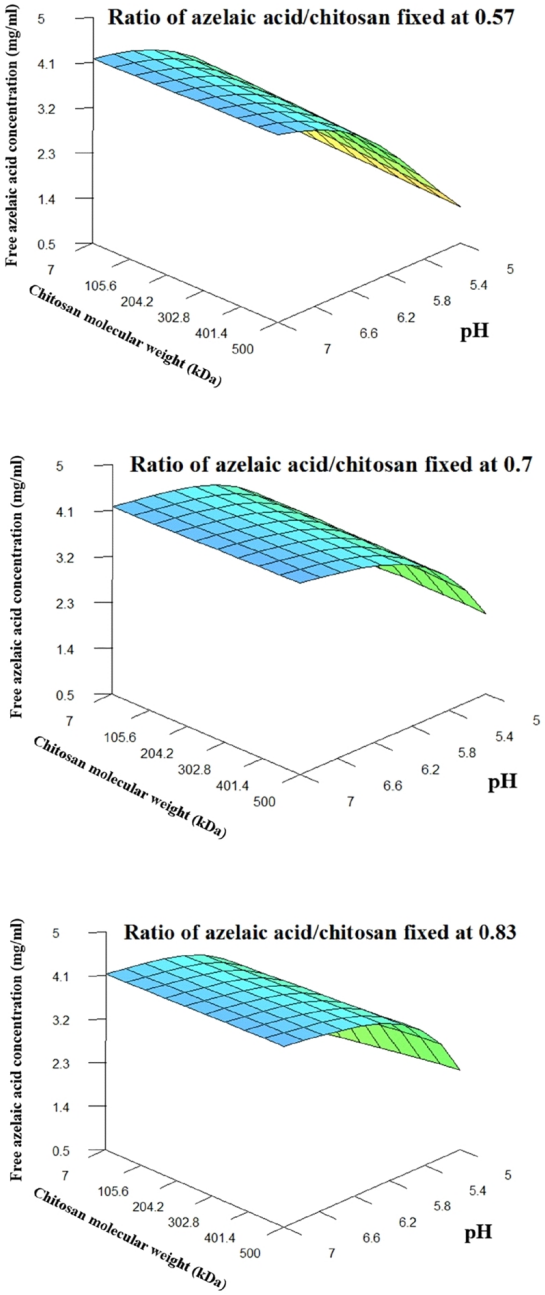

Fig. 4. 3D plot of chitosan MW, pH and free azelaic acid concentration driven by the ANNs model

least free azelaic acid is obtained when pH is minimum and ratio of azelaic acid/chitosan value is the highest possible.

Fig 3 details the effect of chitosan MW and ratio of azelaic acid/chitosan on loading of azelaic acid in chitosan. The details show that MW is not considerably affecting the concentration of free drug. Also, as described above, maximum or minimum azelaic acid/chitosan ratio is needed to obtain the least amount of free drug, especially when pH is low.

In Fig 4, ratio of azelaic acid/chitosan has been fixed to create 3D graphs of free azelaic acid against chitosan MW and pH. In total, as reported in the previous graphs, chitosan MW shows no remarkable effect on the loading, while pH shows a dominant and direct effect on free drug.

To summarize, the results show that:

- Increase in pH in results in sharp decrease in loading of azelaic acid in chitosan.
- Change in chitosan MW does not make considerable variations in the loading efficiency.
- Azelaic acid/chitosan ratio of ~0.7 leads to decrease in loading of azelaic in the polymer.

SEM results of azelaic acid-loaded chitosan particles (i.e. pH= 5.2, MW= 100KDa, ratio= 0.5) is given in Fig. 5. Microparticles of chitosan are obvious in the graph.

Also, Fig 6 illustrates the FT-IR spectral details of azelaic acid, chitosan and drug-loaded particles.

In azelaic acid-loaded chitosan spectrum, few changes were observed when compared to azelaic acid and chitosan particles (Table 3) [36].

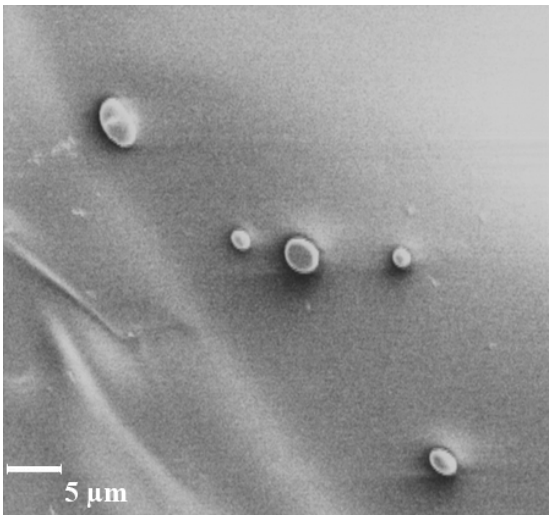

Fig. 5. SEM Micrograph of azelaic acid-loaded chitosan

DISCUSSION

Arguably, the most common carriers for drug delivery purposes are polymeric compounds where drugs are loaded onto non-toxic and biodegradable polymer-based supports. Our work consisted of loading azelaic acid onto chitosan, a biocompatible, biodegradable and economically ideal polymer via electrostatic interactions. An ANNs model was then used to study the effect of three independent variables on loading efficiency of the particles. In this work, to study the interactions between the output and the inputs, we used 3D response surfaces. Response surfaces are created by the ANNs software while other variable(s) are fixed at low, moderate and high values.

mentioned above, it can be concluded that pH has a major impact on loading efficiency. The details also show that chitosan MW has a negligible impact on loading of azelaic acid in chitosan.

The details indicate that in this work, at minimum pH value (i.e. ~ 5), the most effective interactions may be obtained. At this pH value, the strengths of interactions are highest for obtaining stable particles with highest loading efficiency. In a similar way, when using electrostatic interactions between triphenyl phosphate solution and chitosan, the ionic nature and pH were dominating in specifying the loading efficiency (pH range is between 6 and 8). It was also represented that an optimum pH of the TPP solution (~ 7) was needed to maximize loading efficiency because of a suitable ratio of the anionic and cationic interaction sites [37]. Also, our previous work showed that pH plays an important role in loading efficiency of a protein (i.e. streptokinase), with an optimum value of ~5.1 for obtaining maximum efficiency [25].

When pH is low, the relation between ratio of azelaic acid/chitosan value and loading of azelaic acid follows an interesting pattern. From the details, either increase or decrease in ratio of azelaic acid/chitosan from ~0.7, results in an increase in loading of azelaic acid in chitosan. Gan et al. prepared albumin-loaded chitosan nanoparticles. They reported a decrease in loading efficiency as a result of increasing in mass ratio of chitosan/polyanion [28]. Another work on alginate/chitosan particles showed an optimum value of unity for maximizing the loading efficiency. Decreasing alginate to chitosan mass ratio from the optimum value could

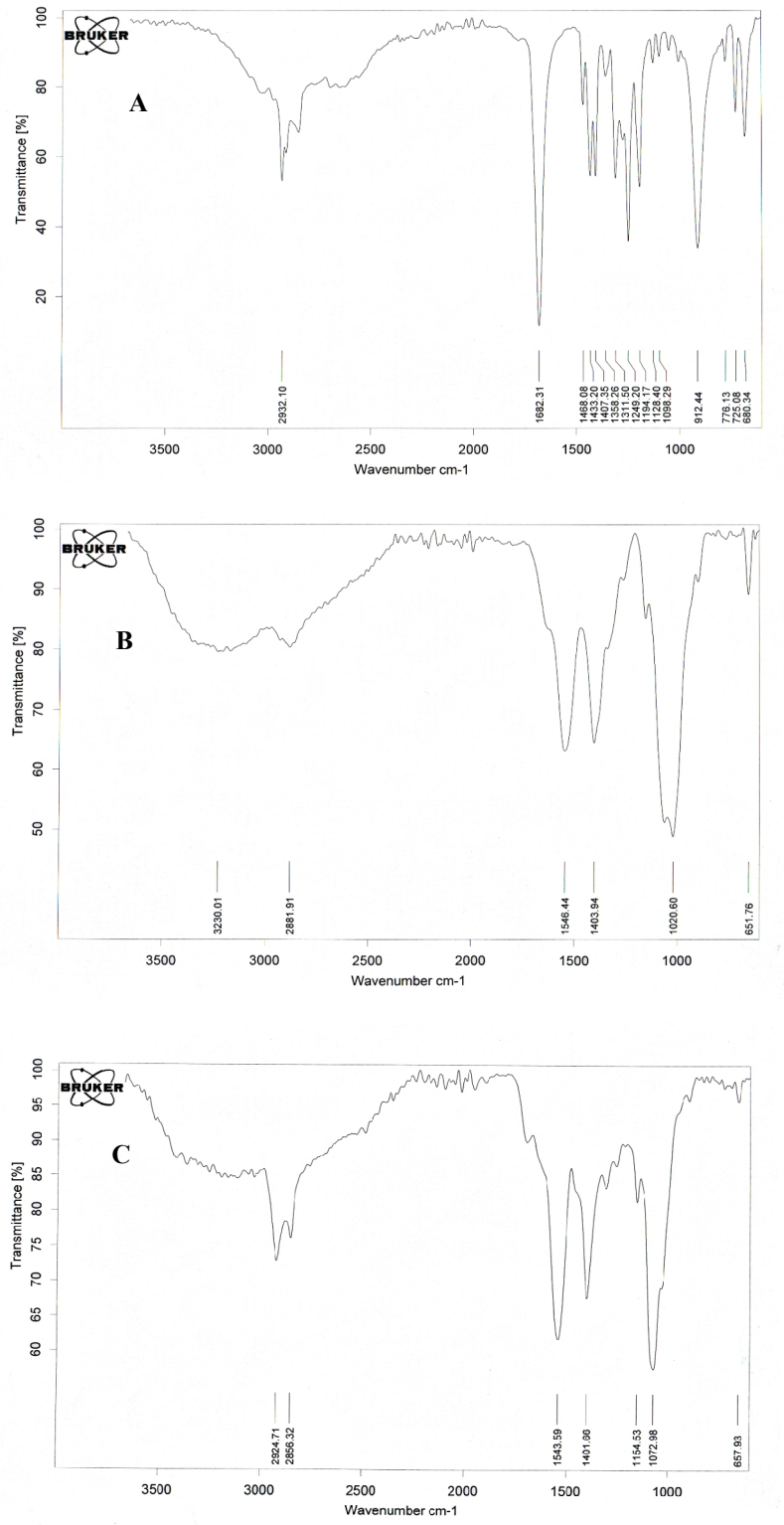

Fig. 6. FT-IR spectrum of azelaic acid (A), chitosan (B) and azelaic acid-loaded chitosan (C).

Table 3. FT-IR analysis of azelaic acid, chitosan

Wave number (cm^{-1})	Assignment
Azelaic acid	
900	Out- of- plane bending of the bonded O–H
1250	C–O stretching vibration
1400	C–O–H in-plane bending
1680	–C=O group
3300-2500	Overlapping stretching vibration of C–H and O–H group
Chitosan	
900	Saccharide structure
1020	C–O stretching in acetamide
1150	–C–O–C in glycosidic linkage
1250	C–O group
1420	Vibrations of OH, CH in the ring
1550	$–NH_2$ bending vibration in amino group
1630	–C=O in acetamide group
2800	Symmetric or asymmetric $–CH_2$ stretching vibration attributed to pyranose ring
3200	$–NH_2$ stretching vibration
Azelaic acid-loaded chitosan	
1250, 1150 and 900	Saccharide structure
1400-1380	Symmetric $–COO^-$ stretching
1540-1500	Symmetric N-H bend
1670-1550	Asymmetric $–COO^-$ stretching
1720-1620	Asymmetric N-H bend
3500-2500	Superimposed -OH and $-NH_3^+$ stretching bend

lead to decrease in electrostatic attractions and increasing the ratio could result in aggregation [38]. In another study, increase in chitosan concentration led to the production of aggregates [39]. In the present work, details show that, either increase or decrease in chitosan mass ratio would result in an increase in the loading efficiency. We believe that by increasing the ratio of drug/chitosan, in fact the chitosan concentration has increased which results in improved loading efficiency. However, above the specified value, the high concentration of chitosan has increased the medium viscosity which reduces the possibility of interactions between azelaic acid and chitosan molecules as previously reported [40, 41].

In this study, chitosan MW did not have a marked effect on loading of azelaic acid in chitosan. Alsarra et al. showed that when using high MW chitosan, a higher loading of lipase may be obtained [26]. While in another study, medium MW chitosan indicated better loading efficiency compared with low and high molecular weights [42]. As in our work chitosan was added slowly to solution of azelaic acid and vigorously stirred, enough time has been provided for the polymer and the drug to interact with each other. Furthermore, concentration of chitosan has been excluded during our modeling procedure. Thus, per unit of mass of the drug, a fixed amount of polymer (i.e. a fixed amount of functional groups) exists in the nanoparticles. It is therefore expected that molecular weight of polymer would not be effective in determining loading efficiency.

CONCLUSION

This investigation showed the interactions of the three input variables on loading of azelaic acid in chitosan. This study indicates that pH and Ratio of azelaic acid/chitosan are potentially the dominant agents that influence the loading of azelaic acid in chitosan. In this case, pH could play an important role in loading azelaic acid to chitosan, showing an optimum value ~5 for obtaining maximum loading efficiency.

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