Design, Synthesis and Biological Evaluation of Novel Prodrug of Alendronate

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ABSTRACT

The objective of this research work was to develop and evaluate novel alendronate-chitosan (AL-CH) complex for the enhancement of bioavailability of alendronate. AL-CH complex was prepared by phosphoramide coupling reaction between thionyl chloride activated hydroxyl groups of alendronate and amino group of chitosan. The complex formation was characterized by FTIR, XRD and zeta potential measurements. Drug release and in vivo pharmacokinetics of the developed complex was evaluated. Phosphoramide bond formation between alendronate and chitosan was confirmed by FTIR. AL-CH complex was found to be amorphous with zeta potential of + 16.4 mV, in contrast to alendronate, which was crystalline with zeta potential of -2.3 MV. Drug release studies showed that alendronate was released in a sustained pattern from complex over a period of 8 hrs. Compared to AL solution, AL-CH complex demonstrated significantly higher ($p < 0.001$) intestinal permeability as determined by non-everted rat gut technique. The in vivo study in rats demonstrated ~ 8-fold higher bone deposition of AL following oral administration of AL-CH complex compared to AL solution. In conclusion, AL-CH complex is a promising formulation for the delivery of alendronate sodium enhancing its bone deposition.

Key Words: Alendronate, Chitosan, Phosphoramide bonding, Osteoporosis, Prodrug

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INTRODUCTION

Osteoporosis has emerged as a global public health issue mostly affecting 200 million elderly people. About 80% of females above 65 years of age; 40% younger postmenopausal women and 39% of males on glucocorticoids therapy are at a higher risk of developing potentially fatal osteoporotic fractures [1-3]. It is a metabolic skeletal disorder categorized by low bone density and micro architectural declining of bone tissue that results in a higher probability of bone fragility and an increased fracture rate. Despite numerous decades of development, bone-specific delivery is still constrained by the distinguishing anatomical features of bone [3]. Therefore, bisphosphonates that belong to a class of synthetic moieties structurally related to pyrophosphate were recognized as first-line drugs for treating bone diseases [4, 5]. Alendronate sodium (4-amino-1-hydroxybutylidene-1,1-bisphosphonate) AL, was the first orally active nitrogen-substituted bisphosphonate approved by the US-FDA for the prevention and treatment of broad range skeletal disorders viz. osteoporosis, Paget's disease, hypercalcemia of malignancy and metastatic bone disease. AL has been demonstrated as a bone constructing anti-resorptive agent, which is used as a potent inhibitor of osteoclastic hyperactivity and has a more binding affinity to hydroxyapatite [6, 7].

AL belongs to BCS class III drugs that depict low permeability because it suggests five pKa values that impart a high degree of ionization at almost every pH except 7.4. Oral administration of this moiety is quite challenging because of its poor oral bioavailability of 0.6-0.7%. The drug gets absorbed from the upper part of the small intestine through the paracellular pathway and

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ultimately reaches to the bone where it gets accumulated at active remodeling site and remains there for many years [8, 9]. Low systemic availability and unenviable gastric effects associated with AL pose a significant constraint to oral delivery of this drug. To overcome these issues, alternative approaches have been explored that include dry powder inhalers; mucoahesive liposomes; enteric coated nano-liposomes; and solid lipid nano-particles [10-13]. In the present studies, we have explored the possibility of developing polymeric complexes of AL called as a prodrug approach to improve its oral bioavailability. The prodrug approach has attracted considerable attention due to its particular therapeutic properties, such as prolonged half-life and enhanced bioavailability. Hence, it could serve as an effective tool to overcome the drawbacks associated with oral delivery of AL. Chitosan (CH), a cationic polysaccharide, was selected for the conjugation purpose in the current project because of its biocompatibility, biodegradability, and non-irritability upon oral administration [14, 15]. Thus, the aim of current research is to develop chitosan based polymeric complexes of AL for its bioavailability enhancement.

MATERIALS AND METHODS

Materials

Alendronate Sodium (AL) was kindly supplied by (Saja Pharmaceuticals Co. Ltd., Jeddah, Saudi Arabia). Chitosan (ChitoClear^{TM}, degree of deacetylation 96%, viscosity 15cps) was purchased from Primex ehf (Siglufjordur, Ice-land). Thionyl chloride was purchased from Sigma (India). All of the other utilized chemicals were of analytical reagent grade.

Preparation of AL-CHcomplex

AL-CH complex was efficiently synthesized through amide coupling reaction [16]. Briefly, 5% w/v solution of alendronate in benzene was refluxed for one hour with thionyl chloride (1 ml) and the reaction mixture was evaporated to dryness. To the activated alendronate, 2% solution of chitosan in acidified aqueous solution (10 ml) was added and refluxed for two hours. Further, to this reaction mixture 10 ml of 5% NaOH aqueous solution was added and precipitates thus obtained was filtered off and washed. The formation of complex was confirmed by FT-IR analysis, XRD and zeta potential measurements.

Characterizationof AL-CH complex

FT-IR analysis

The chemical structure of the AL-CH complex was characterized using FT-IR spectroscopy in order to identify the linkage generated between AL and CH. Briefly, AL, CH and AL-CH complexwere separately mixed with KBr (1:1) and converted into a pellet. Each pellet was scanned between 4000 to 500 wavelength ranges (cm^{-1}) and compared with standard peaks of drug and polymer.

X-ray diffraction (XRD)

The formation of the complex between AL and CH was characterized by XRD. Physical state of AL, CH and AL-CH complex were resolved by X-ray diffractometer with a scanning speed of 3 min^{-1}, in a parallel setup including Ni-filtered Cu-Kα radiation over a range of 2θ from 5º to 60º with the scattering angle of $q= 4\pi\sin\theta/\lambda$, where λ is X- ray wavelength.

Zeta potential

The formation of a complex between AL and CH was further characterized by zeta potential changes. Zeta potential measurements were done with a disposable capillary cell with a volume of 1 mL using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Each analysis was performed in triplicate.

Drug release

Invitro release studies were performed on AL (70 mg), marketed formulation of AL (FOSAVANCE®, 70 mg) and AL-CH complex equivalent to 70 mg of AL using500 mL of 0.1 N HCl (pH 1.2). Release medium was stirred with the help of magnetic stirrer at 150 rpm. Samples were withdrawn at predetermined time intervals (0.25, 0.5, 1, 2, 4, 8, 10, 12 h) with replacement with fresh medium. Concentration of AL was determined by UV-visible spectrophotometry method.

Intestinal permeation by gut sac method

Male Wistar rats, weighing 200 ± 20 g, were used for this study. The animals were kept under standard laboratory conditions (temperature 25 ± 2°C, relative humidity 55 ± 5%) and were housed in polypropylene cages with free access to standard laboratory diet. Permeation study was carried out as reported with slight modifications. Animals were kept without food for 12 h, but water was allowed ad libitum before experimentation. Animals were sacrificed by cervical dislocation under excessive ether anesthesia. Ileum was taken out, washed with saline and one end of the segment was ligated with thread, while the other end was mounted on a port of an in house developed assembly to conduct the study. Sac of each segment was either filled with 1.0 ml of AL solution (1 mg/mL) or AL-CH complex suspension (equivalent to 1 mg of AL), incubated pre-oxygenated and pre-warmed (37 ± 0.5°C) Tyrode's buffer (10 mL). Sample (0.5 mL) was collected at different time intervals 15, 30, 60, 90, 120 and 240 min and replenished with the same volume of fresh Tyrode's buffer solution. All the samples were analyzed by HPLC.

In vivo Bone deposition

After an overnight fast, rats (N = 6) received per oral (PO) dose of AL solution (4 mg/kg) and AL-CH complex suspension (equivalent to 10 mg/kg of AL). The PO solutions and suspensions were prepared with saline and

the administered volume was 1.0 ml. Twenty-four hours later, the animals were euthanatized by overexposure to ether, and the femur and tibia boneswere removed. Bones werethen carefully dipped in saline solution to extract the deposited AL and the concentration of AL was analyzed by HPLC method to determine extent of bone deposition. Data was expressed as mean ± S.D and compared by applying paired t test using software GraphPad Instat 3 (USA). $p< 0.05$ was considered as the level of significance.

HPLC Analysis

HPLC method previously developed was used in the present study [17]. Chromatographic separations and subsequent quantifications were carried out at room temperature using reversed phase HPLC column. To each of the sample, sixty microliter of the o-phthalaldehyde reagent in the presence of sodium sulfite was added and the volume was made up to 1.0 ml using 0.05 M NaOH. Fifty microliter of each solution was injected into the HPLC system and chromatograms were monitored by a PDA detector at a wavelength of 333 nm. An isocratic elution system was employed with a mobile phase consisting of a mixture of phosphate buffer pH 9.6: acetonitrile (80:20) containing 3.0 % tetrabutylammoniumperchlorate at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Preparation of AL-CH complex

AL is an anionic drug consisting of phosphonate groups having free hydroxyl (OH) groups. CH is a cationic water soluble macromolecule with active amine-functional groups. The complex between AL and CH was attempted in order to suppress charge on AL and it is well known that chitosan also acts as a penetration enhancer thus complex between alendronate and chitosan might increase the bioavailability of alendronate [14, 15]. The complex between AL and CH was formed by first converting free hydroxyl groups of AL to corresponding halogen on treatment with thionyl chloride through a substitution reaction - specifically, an SN2 mechanism. The corresponding halogen groups are readily available for reaction with amine groups of chitosan. The CH was thus covalently attached to AL through phosphoramide linkage that is known to be cleaved under physiological conditions. The byproducts of the reaction, i.e. HCl and sulfur dioxide are easily separated as they exist as gases and are bubbled away. The schematic representation of complex formation is depicted in Fig. 1.

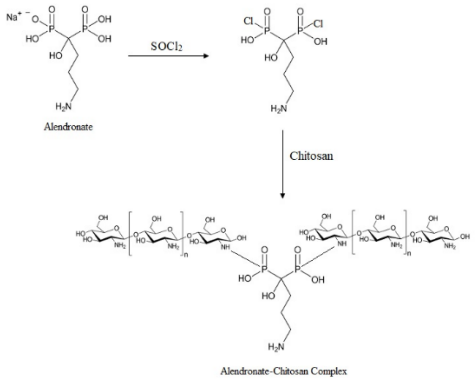

Fig. 1. The schematic representation of complex formation

FT-IR analysis

To confirm the complexation between AL and CH, FT-IR analysis has been conducted (Fig. 2).

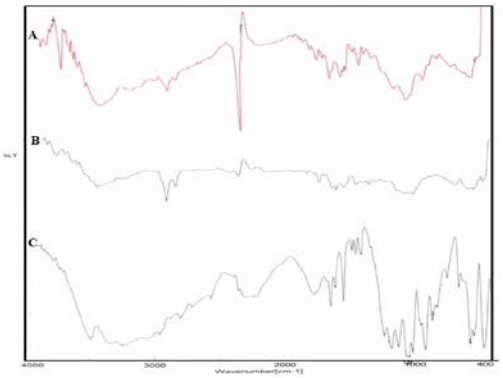

Fig. 2. FT-IR analysis of AL-CH complex (A), Chitosan (B), and Alendronate (C)

FTIR spectra of AL-CH complex shows the following changes which confirms the formation of phosphoramide linkage between AL and CH.

(a) P-O stretching peaks at 823.46 cm^{-1} and 746.32 cm^{-1} which were present in AL spectra (Fig. 2C) were absent in the spectra of AL-CH complex (Fig. 2A).
(b) O-H stretching peak at 3542.72 cm^{-1}(Fig. 2C) was absent in the spectra of AL-CH complex (Fig. 2A).
(c) Slight decrease in intensity of P=O stretching peak from 1178.29 cm^{-1} to 1064.51 cm^{-1} was observed (Fig. 2A).
(d) Appearance of sharp peak at 2362.37 cm^{-1}, which corresponds to phosphine group (P-H stretching) (Fig. 2A).
(e) The appearance of C-H stretching at 2923.56 cm^{-1} in spectrum indicates complexation of chitosan with AL (Fig. 2a).

XRD

XRD studies were conducted in order to predict the atomic orientation and phase determination of pure AL and AL-CH complex.

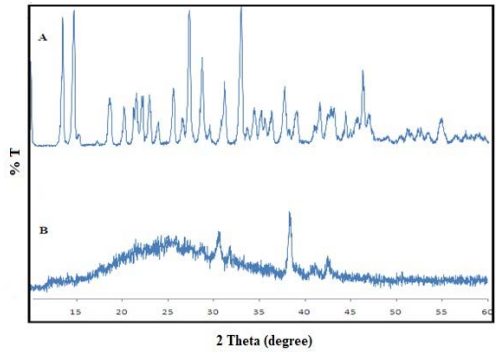

Fig. 3. The XRD of Alendronate (A) and the AL-CH complex (B)

Numerous high intensity visible peaks were recorded from 10º to 60º at a diffraction angle of 2θ, which correspond to the crystalline behavior of AL (Fig. 3A), whereas the diffraction pattern of prepared AL-CH complex exhibited a reduction in both the number and intensity of peaks (Fig. 4B). New low intensity peaks were observed at 30.5º and 38.1º while the rest of all other intense peaks were disappeared (Fig. 3B). The disappearance of Bragg's peaks in the diffraction pattern of AL-CHcomplex is suggestive of decrease in crystallinity due to lattice distortion upon attachment of CH to AL.

Zeta Potential

Alendronate has five pKa values (0.8, 2.2, 6.3, 10.9 and 12.2 of the protonized amino group) with a negative zeta potential of -2.3 mV at physiological pH (Fig. 4A). The zeta potential of CH was highly positive with a value of +26.2 mV owing to the presence of cationic amino groups (Fig. 4B). The zeta potential values of prepared AL-CH complex was found to be +16.4 mV indication charge suppression owing to complex formation (Fig. 4C).

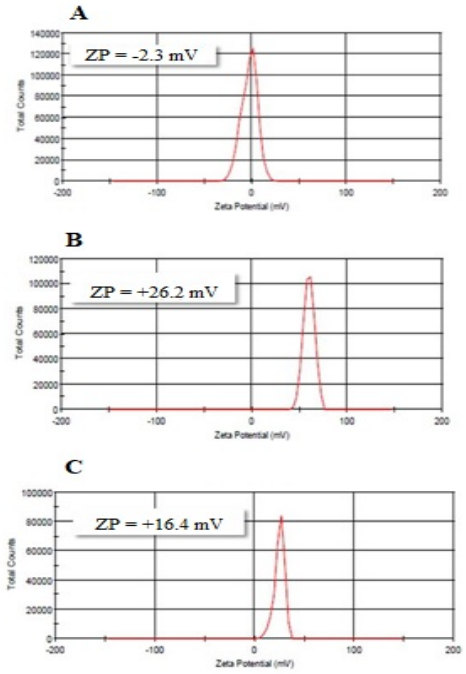

Fig. 4. The zeta potential of Alendronate (A), Chitosan (B), and AL-CH complex (C)

Drug content and Invitro release

The content of AL in the complex was evaluated by HPLC method previously reported for alendronate. The content of AL in complex was found to be 27.6 ± 1.5%. To check the release of the AL from complex and to determine release behavior, drug release studies were conducted in 0.1 N HCl (pH 1.2) for 2 h and phosphate buffer pH 7.4 for 8 h. Comparisons were done using pure AL and marketed formulation Fosamax (Fig. 5).

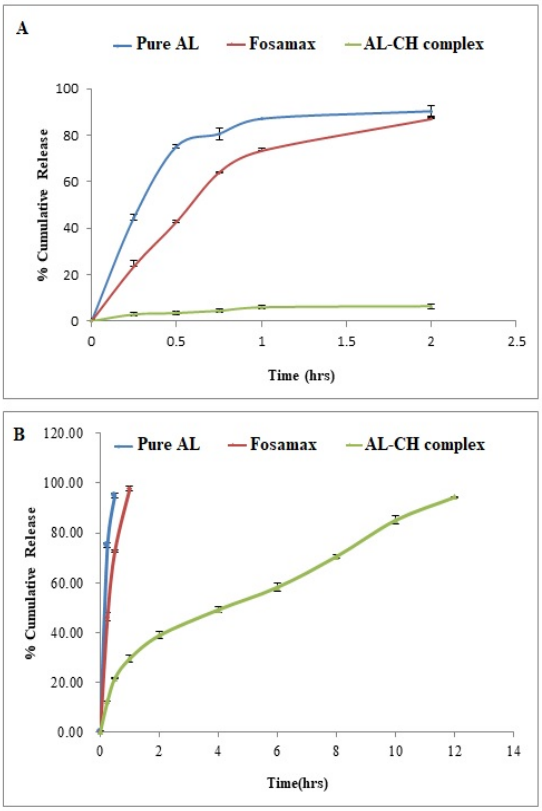

Fig. 5. HPLC determination of the content of AL in the complex

After 2 h in 0.1 N HCl around 6% of AL was released from AL-CH complex compared to more than 75% for both pure AL and Fosamax (Fig. 5A). Comparison of dissolution profile in pH 7.4 phosphate buffer showed that 100% AL was release from pure AL and Fosamax within 45 minutes (Fig. 5B). In comparison to this 94% of AL was released from complex at 8 h time point (Fig. 5B). The results indicated that AL is released from the complex under physiological conditions. Further, AL-CH complex resulted in the sustained drug release. In order to determine the apparent rate constant of AL release, the dissolution profiles were fitted to various release models, (Fig. 6) and from the correlation coefficient it was found that AL-CH complex exhibited a Higuchi model (r^2 = 0.995) and Korsemeyer-Peppas release patterns (r^2 = 0.957, n = 0.647), respectively.

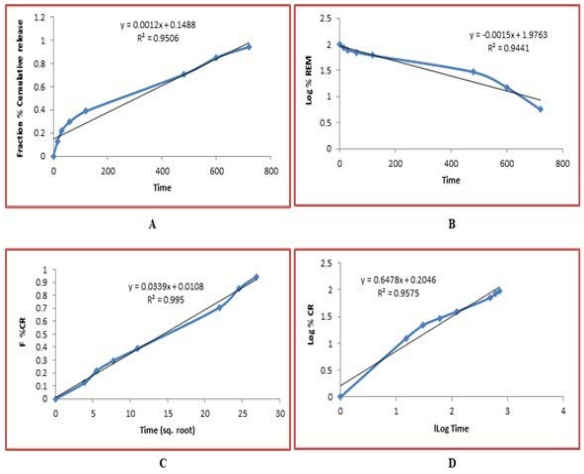

Fig. 6. Release kinetic of Alendronate

Intestinal permeation by gut sac method

CH is well known oral permeation enhancer [14, 15]. To evaluate the effect of CH on AL permeation, we carried out intestinal permeation by ex vivo gut sac method. In the present study, non-everted intestinal sac method was adopted for the assessment of permeability of AL-CH complex. Ruan et al. reported the applicability of non-everted rat intestinal sac method and revealed a good relationship between the permeability of drugs and their corresponding human absorption data for 11 marketed compounds [18]. Permeation profile of AL-CH complex and AL solution across the rat ileum is shown in Table 1.

Table 1. Intestinal Permeability of AL from AL solution and AL-CH complex (n=3)

Time (min)	Cumulative AL Permeability (µg)	
	AL solution (mean±sd)	AL-CH complex (mean±sd)
15	0.0 ± 0.0	18.9 ± 1.2 (1.89%)
30	0.0 ± 0.0	45.0 ± 3.4 (4.50%)
60	0.0 ± 0.0	80.8 ± 6.9 (8.08%)
90	0.0 ± 0.0	91.7 ± 5.5 (9.17%)
120	0.0 ± 0.0	98.3 ± 8.7 (9.83%)
240	0.0 ± 0.0	120.5 ± 8.4 (12.5%)

The result showed that AL was not permeated through rat ileum from solution formulation. In contrast, AL significantly higher ($p < 0.001$) AL permeation was observed when it was complexed with CH. The molecular weight of alendronate is 249 grams/mole and its octanol/buffer partition coefficient is 0.0017 independent of pH, all properties indicating a large, hydrophilic drug preventing both transcellular and paracellular absorption [7-9]. Additionally, the brush-border membrane is negatively charged and will often repel the negatively charged phosphate groups on the alendronate from the epithelium and tight junctions. However, enhanced permeation as observed for AL-CH complex could possibly be due to (1) suppression of charge of AL due to complexation with CH, and (2) ability of CH to improve permeation through the opening of tight junctions [15]. The results showed that AL-CH complex improves AL permeation and thus would result in bioavailability enhancement.

In vivo Bone Deposition

It is well known that following oral administration the concentration of alendronate in plasma is too low for accurate measurement, impeding the use of plasma concentrations in assessing oral absorption. Further, alendronate redistributed extensively from non-calcified tissues to the bone, thus absorption can be estimated by measuring the amount of AL deposited in bone. Assuming linear kinetics of bone uptake, any change in the concentration of alendronate in the bones should reflect a change in absorption. The concentrations of alendronate in bones after POadministration of AL solution and AL-CH complex are shown in Table II.

Table 2. Concentration of Alendronate in bone of rat after oral administration of AL solution and AL-CH complex

Rats	In vivo bone deposition of AL (µg/g)	
	AL solution	AL-CH complex
1	0.16	1.55
2	0.19	0.87
3	0.20	1.38
4	0.11	1.45
5	0.09	1.26
6	0.23	1.18
Mean ± s.d.	0.16 ± 0.054	1.28 ± 0.241

The concentrations of AL in the bones were significantly higher for the AL-CH complex group(1.28 ± 0.241 µg/g) compared to AL solution group (0.16 ± 0.054 µg/g) indicating nearly 8-fold higher bone deposition.

CONCLUSIONS

In the present work, we synthesized AL-CH complex, and reported the physicochemical characteristics and pharmacokinetics of the new prodrug complex. AL-CH complex showed prolonged release of AL and 9-fold higher oral bioavailability as compared to AL solution in a rat model. Taken together, CH-based complexes may be used as a promising oral delivery platform for highly ionizable and poorly permeable drugs. We are currently

synthesizing other CH-based conjugates with different types of drugs in the same direction.

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