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Zn–Al–NO_3-layered double hydroxides with intercalated diclofenac for ocular delivery

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ABSTRACT

This study was aimed to evaluate the potential use of a drug delivery system, drug-layered double hydroxide (LDH) nanocomposites for ocular delivery. Diclofenac was successfully intercalated into Zn–Al–NO_3–LDH by coprecipitation method. The nanocomposites were characterized by particle size, elemental chemical analysis, thermogravimetric analysis, etc. A tilt bilayer of diclofenac molecules formed in the interlayer with the gallery height of 1.868 nm. In vivo precorneal retention studies were conducted with diclofenac sodium (DS) saline, diclofenac–LDH nanocomposite dispersion, 2% polyvinylpyrrolidone (PVP) K30–diclofenac–LDH nanohybrid dispersion and 10% PVP K30–diclofenac–LDH nanohybrid dispersion, separately. Compared with DS saline, all the dispersions have extended the detectable time of DS from 3 h to 6 h; C_{max} and AUC_{0-t} of diclofenac–LDH nanocomposite dispersion showed 3.1-fold and 4.0-fold increase, respectively; C_{max} and AUC_{0-t} of 2% PVP K30–LDH nanohybrid dispersion were about 5.3-fold and 6.0-fold enhancement, respectively. Results of the Draize test showed that no eye irritation was demonstrated in rabbits after single and repeated administration. These results suggest that this novel ocular drug delivery system appears to offer promise as a means to improving the bioavailability of drugs after ophthalmic applications.

1. Introduction

Recently there has been a rapid expansion of the development of bioinorganic hybrid systems for drug delivery (Li et al., 2010). Layered double hydroxides (LDHs) or anionic clays are a class of synthetic two-dimensional lamellar compounds with positively charged layers and charge-balancing anions located in the interlayer region. They have the general formula $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}(A_{x/n}^{n-})\cdot mH_2O$, where M^{2+} and M^{3+} are divalent and trivalent metal ions, A^{n-} is the anion in the interlamellar region, and m is the amount of water present in the same region. LDHs are also widely known as hydrotalcite-like compounds due to their structural similarities to hydrotalcite, a mineral with the formula $Mg_6Al_2(OH)_{16}CO_3\cdot 4H_2O$. LDHs have been synthesized by direct methods, of which the most frequently used is coprecipitation method, and indirect methods, such as anionic exchange using LDHs as precursors. Advanced applications have sought to synthesize a large range of LDHs varying either the divalent and trivalent cations or the interlamellar anions, which can be of an inorganic or organic nature. Actually, there is an increasing interest in LDHs because they can be used for numerous applications, for example, catalysts and catalyst supports, trapping agents for anionic contaminants, ion exchangers, sorbents, and medical applications. In particular, LDHs can be efficient delivery vehicles of drug, nucleotides/genes (Ladewig et al., 2010), and biomedical and other functional molecules because these compounds, intercalated in LDHs, are slowly released, thus reducing the frequency of dose. Oral and injection delivery are commonly used for administration of drug-LDH nanocomposites (Li et al., 2004; Qin et al., 2010) and ocular drug delivery of LDHs has not been reported so far.

Ocular drug delivery has remained as one of the most challenging task for pharmaceutical scientists. The unique structure of the eye restricts the entry of drug molecules at the required site of action. Generally less than 5% of administered drug enters the aqueous humor. So, in order to maintain the therapy concentration, the agents need to be frequently dosed resulting in poor patient compliance. In the last decade, in addition to the commonly used dosage forms such as solutions, gels, ointments, and aqueous suspensions, main focus was given to colloidal systems consisting of micro/nanoparticles, micro/nanoemulsions, nanosuspensions, liposomes, dendrimers and niosomes (Gaudana et al., 2009). Diclofenac, a phenyl acetic acid nonsteroidal anti-inflammatory drug, is available as sodium salt. Aqueous diclofenac sodium (DS, 0.1%, w/v) solution is applied topically in the eye to manage pain in corneal epithelial defects following surgery or accidental trauma, treatment of postoperative ocular inflammations,

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chronic noninfections inflammations, and prevention of intraoperative miosis during cataract surgery and for symptomatic relief of seasonal allergic conjunctivitis. About 90% of the dose applied topically in the eye from such solutions is lost due to precorneal losses (Ahuja et al., 2006).

Several papers in the literature report studies on DS of different topical administrations, such as liposome (Li et al., 2009), nanosuspension (Agnihotri and Vavia, 2009), polymeric nanoparticles (Valls et al., 2008), and solid lipid nanoparticle (Shen et al., 2009). However, the short residence time of these colloidal systems in the ocular mucosa represents a limitation in the therapy of eye diseases. Ideal ocular delivery systems are easy to administer, require decreased administration frequency, and provide controlled and possibly sustained drug release in order to increase therapeutic efficacy and patient compliance.

Diclofenac has been intercalated into Mg–Al–Cl–LDHs (Ambrogi et al., 2002) and Mg–Al–CO_3–LDHs for oral drug delivery (Dupin et al., 2004; Zhang et al., 2010), and these nanocomposites showed controlled release characteristics. As a matter of fact, the therapeutic efficacy of an ophthalmic drug can be greatly improved by prolonging its contact with the corneal surface. Consequently, the application of new carrier system with sustaining the duration of intimate drug–eye contact would be a promising step towards the management of ocular diseases. It has been reported that positively charged liposomes had a higher binding affinity to the corneal surface than the neutral and negatively charged vesicles as a result of interaction of positively charged liposomes with the polyanionic corneal and conjunctival surfaces, and therefore increase the drug retention and absorption (Fresta et al., 1999). It has been suggested that the major force responsible for the prolonged residence of cyclosporin A in these epithelia might not be the mucoadhesive character of chitosan molecules but the electrostatic interaction between the positively charged chitosan nanoparticles and the negatively charged corneal and conjunctival cells (De Campos et al., 2001).

Taking into account positively charged LDHs and the fact that the cornea and conjunctiva have a negative charge, we supposed the use of LDHs which might interact electrostatically with the cornea and conjunctiva would increase the residence time and concentration of the associated drug, resulting in an enhancement of bioavailability. In our previous work (Wang et al., 2009), diclofenac–Zn–Al–NO_3–LDHs were successfully synthesized and characterized by powder X-ray diffraction, fourier transform infrared and transmission electron micrographs. The sustained release behavior of the vesicles was studied in different media. For the further development of the potential use in ocular drug delivery of diclofenac–LDHs, the nanocomposite structure, formula and properties were characterized in this paper by various methods, such as particle size, elemental chemical analysis, and thermogravimetric analysis. Furthermore, polymer–LDH nanohybrid dispersions were prepared and the precorneal retention and irritation experiments in vivo were evaluated. To the best of our knowledge, there are no reports in the literature on diclofenac- or other drugs-LDH nanocomposites used for ocular drug delivery.

2. Materials and methods

2.1. Materials

Diclofenac sodium was purchased from the Henan Dongtai Pharma. Co., Ltd., China. Polyethylene glycol 400 (PEG 400) and PEG 200 were purchased from Beijing Fengli Jingqiu Commerce and Trade Co., Ltd., China. $Al(NO_3)_3{\cdot}9H_2O$ was purchased from Guangdong Xilong Chemical Co., Ltd., China. $Al(NO_3)_3{\cdot}9H_2O$ was purchased from Shanghai Sinpeuo Fine Chemical Co., Ltd., China. Polyvinylpyrrolidone (PVP) K30, PEG2000 and PEG4000 were obtained from BASF Corp., Germany. Deionized water from which carbon dioxide was removed by boiling under nitrogen was used in all preparations. All other reagents were of analytical grade and were used without further purification.

2.2. Preparation of LDHs

Diclofenac–Zn–Al–NO_3–LDHs were prepared by the coprecipitation method as reported previously (Wang et al., 2009). Briefly, a solution of NaOH (0.56 g, 14 mmol) dissolved in deionized H_2O (3 ml) and a mixture of $Zn(NO_3)_2{\cdot}6H_2O$ (1.19 g, 4 mmol) and $Al(NO_3)_3{\cdot}9H_2O$(0.75 g, 2 mmol) dissolved in deionized H_2O (3 ml) were added dropwise simultaneously to deionized H_2O (70 ml) under N_2 atmosphere with vigorous stirring at 0 °C. The pH of this suspension was maintained at 7.0–8.0 with 20% (w/v) NaOH. The resulting suspension was crystallized at room temperature for 24 h. Then the precipitate was centrifuged at 18,000 rpm for 5 min at 4 °C with a high-speed refrigerated centrifuge (CR21G, Hitachi, Japan), washed several times with decarbonated water. Decarbonated water (15 ml) was added to the precipitate and the mixture was kept at 70 °C for 5 h. This product was denoted as dispersion of Zn–Al–NO_3–LDH (H_2O), which was freeze dried to get powders of Zn–Al–NO_3–LDH for further analysis. Synthesis of Mg–Al–NO_3–LDH (H_2O) was the same as above except that the pH of the synthesis suspension was maintained above 9.0. Diclofenac–Zn–Al–NO_3–LDH (H_2O) was carried out following the same procedure as above except the presence of 0.01 mol/L DS in the medium. In order to study the influence of reaction solvent on LDHs, 10% EtOH or 2% PEG 400 was added to the medium, so diclofenac–Zn–Al–NO_3–LDH (10% EtOH) and diclofenac–Zn–Al–NO_3–LDH (2% PEG 400) were prepared, separately.

2.3. Determination of drug loading of diclofenac in LDHs

The loading amounts of diclofenac in LDHs were calculated according to the equation of loading $\% = W_d/W_t \times 100\%$. The W_d was total amount of diclofenac in LDHs and W_t was the total quantity of LDHs. W_d was determined by HPLC. 10 mg powder of diclofenac–LDHs was ultrasounded in the mobile phase (10 ml) until LDH layers were completely dissolved. After dilution with mobile phase the amount of diclofenac was quantified by an HPLC system as the same with that used for diclofenac analysis in the tears in the following section.

2.4. Characterization of LDHs

The particle sizes, zeta potential, powder X-ray diffraction patterns, fourier transform infrared (FTIR) spectra and transmission electron micrographs (TEM) of the LDHs have been studied in our previous paper (Wang et al., 2009). For the further analysis of the diclofenac-LDH structure and formula, C, H, N elemental chemical analysis were carried out in Vario EL III (Elementar, Germany), Zn and Al by using atomic emission spectrometry in a PE5300DV instrument after dissolving the samples in nitric acid. Thermogravimetric analysis (TGA) was carried out by heating the dry powder samples at a rate of 10 °C/min with a flow of air at 200 mL/min over 25–650 °C in TA Instrument TG209C (NETZSCH Instrument Co., Ltd., Germany).

2.5. Preparation of eye drops

The preparation procedures of 0.1% (w/v) DS eye drop (F1) were as follows: Required amount of DS was dissolved in 0.9% NaCl solution and trichlorobutanol with a concentration of 0.25% (w/v)

was added as preservative. 0.1% (w/v) diclofenac–LDH nanocomposite dispersion (F2) was prepared as follows: Required amount of powder of diclofenac–LDH was dispersed in water to get a 0.1% (w/v) diclofenac mixture and 0.25% (w/v) trichlorobutanol was added as preservative. Required quantity of glucose was added to make an isotonic mixture (280–290 mOsm/L) determined by an osmometer (OSMOMAT 030, Gonotec, Germany). 2% PVP K30–diclofenac–LDH nanohybrid dispersion (F3): Required amount of powder of diclofenac–LDH was dispersed in water to get a 0.2% (w/v) diclofenac mixture, and then required amount of PVP K30 was dissolved in water to get a 4% (w/v) PVP K30 solution. Equal volume of the solutions above were mixed to get a 0.1% (w/v) diclofenac mixture with 2% PVP K30. 0.25% (w/v) trichlorobutanol was added as preservative. Required quantity of glucose was added to make an isotonic mixture. 10% PVP K30–diclofenac–LDH nanohybrid dispersion (F4) was prepared similarly. The pH of all formulations was in the range of 7.0–7.4.

2.6. In vivo precorneal retention experiment

Animal experiments were conducted in accordance with the guide for the care and use of laboratory animals of China Pharmaceutical University. New Zealand albino rabbits were used in resident evaluation experiments. Twelve unanesthetized rabbits of either sex, free of gross ocular defects and weighing 2.5–3 kg, were positioned into restraining boxes. Rabbits were then randomly assigned to one of four groups equally: DS in normal saline (F1), diclofenac–LDH nanocomposite dispersion (F2), 2% PVP K30–diclofenac–LDH nanohybrid dispersion (F3) and 10% PVP K30–diclofenac–LDH nanohybrid dispersion (F4). Concentration of diclofenac in all the four formulations was 0.1% (w/v). 50 μl of eye drops was dosed into the lower conjunctival sac of the eyes by a microinjector. According to the following time schedule: 15, 30, 60, 90, 120, 180, 240, 300, and 360 min, 10 μl of tear fluid samples was collected by a glass capillary from the middle of the lower marginal tear strip. Immediately after collection, capillaries were blown under a gentle nitrogen flow into a centrifugal tube. Samples were added with 90 μl methanol to precipitate proteins, vortexed for 2 min, and centrifuged at 10,000 rpm for 10 min at 4 °C with a high-speed refrigerated centrifuge (CR21G, Hitachi, Japan). 20 μl of the supernatants was injected into the HPLC system. The areas under the DS concentration versus time curves in t min ($AUC_{0\rightarrow t\ \mathrm{min}}$) were calculated using the trapezoidal rule. The time t was the one at which the concentration of DS could be quantitatively analysed by HPLC.

The concentration of DS was examined and quantified using an HPLC system with a pump (Model LC-10A, Shimadzu, Japan), a UV detector (Model SPD-10A, Shimadzu) at 276 nm, and a Diamonsil C18 column (5 μm, 150 mm × 4.6 mm, Dikma Technologies, China). The column was eluted with a freshly prepared solution containing a mixture of methanol, water and acetic acid (80:20:2, v:v:v). The flow rate was 1.0 mL/min and column temperature was 30 °C. The retention time of DS was 6.89 min. For the analysis of diclofenac in the tears, this method was evaluated through intra-day and inter-day analysis of precision and accuracy. Precisions were less than 5.41%, and all the relative recoveries were evaluated to be 94.55–96.3%. The limit of detection and lower limit of quantitation were 25 ng/ml and 100 ng/ml, respectively. The linear regression equation for DS in the tears of rabbits ranging from 0.1 μg/ml to 50 μg/ml was $A = 53219C + 516$ ($r = 0.999$). All samples' concentration was in the concentration range.

2.7. Eye irritancy evaluation

The potential ocular irritancy and/or damaging effects of F3 was evaluated according to the Draize test (Wilhelmus, 2001). Albino New Zealand rabbits weighing 2.0–2.5 kg, obtained from Animal Center of China Pharmaceutical University, were used to study the acute ocular tolerance to LDH nanohybrid dispersion. All animals were healthy and free of clinically observable abnormalities. All animal treatments followed the recommendation of the Regulations for the Administration of Affairs Concerning Experimental Animals.

The congestion, swelling, and discharge of the conjunctiva were graded on a scale from 0 to 3, 0 to 4, and 0 to 3, respectively. Iris hyperemia and corneal opacity were graded on a scale from 0 to 4. Four rabbits, each received a single-dose of 50 μl of 2% PVP K30–diclofenac–LDH eye drop (F3) in the right eye. The contralateral eye was used as control and received 50 μl of saline solution. At 1 h, 2 h, 4 h, 24 h, 48 h and 72 h, the ocular tissues were evaluated according to the Draize eye scoring criteria above. For repeated administration, F3 and the control were applied to the eyes repeatedly three times every day for one week. At the end of the treatment, five observations at 6 h, 24 h, 48 h, 72 h and 7d were carried out to evaluate the ocular tissues. Methylene blue staining was used to evaluate the corneal integrity, which allows an accurate determination of the extent of epithelial damage because of its poor diffusion through the stroma.

3. Results and discussion

3.1. The influences of pH and solvents on the characteristics of LDHs

According to the acid–base property of LDHs, LDHs can be divided into two types, acid LDHs and basic LDHs (Weir and Kydd, 1998). Layer compositions of acid LDHs are stable in solution at pH values of about 3–7, e.g. Zn–Al and Ni–Al. Acid LDHs are often synthesized by direct coprecipitation, or by anion exchange reactions involving the nitrate or chloride form of the LDH in a neutral or mildly acidic aqueous medium. Since $Mg(OH)_2$ is fairly soluble at pH 8 and below, basic LDHs are often synthesized above pH 8, e.g. Mg–Al or Mg–Ga LDHs. Considering that the suitable pH range of formulations for ocular drug delivery is 5.0–9.0 and pH of the commercially available diclofenac ophthalmic solution is between 7.0 and 7.3 (Ahuja et al., 2006), we selected acid Zn–Al–LDH as a carrier for ocular drug delivery. In our experiments, pH of Zn–Al–NO_3–LDH (H_2O) and Mg–Al–NO_3–LDH (H_2O) was 7.2 ± 0.2 and 9.3 ± 0.6, respectively. Well-dispersed transparent dispersions of Zn–Al-NO_3–LDH were prepared with coprecipitation method and were stable at least for three months at room temperature. All particle sizes of LDHs were smaller than 200 nm. It seems that LDH particles with an average size between 40 and 300 nm that are individually separated in the stable suspensions are more desirable for these applications (Xu et al., 2006). For blank LDHs, the particle size of Zn–Al–NO_3–LDH (H_2O) and Mg–Al–NO_3–LDH (H_2O) was 132 ± 9 nm and 68 ± 4 nm, respectively, which may be attributed to the larger ionic radius of Zn^{2+} (0.125 nm) than that of Mg^{2+} (0.072 nm). Because of insertion of diclofenac in the interlayer region, in most conditions the particle size of LDHs loaded with diclofenac was larger than that of blank LDHs. In order to obtain nanocarriers with better characteristics for ocular delivery, the influence of different reaction solvents on the particle size and drug loading of diclofenac–Zn–Al–NO_3–LDHs was studied. Water, 10% EtOH water solution and 2% PEG400 water solution had been used in the synthesis of diclofenac–Zn–Al–NO_3–LDH. The particle size of diclofenac–Zn–Al–NO_3–LDH (2% PEG400) was 135 ± 11 nm, which was almost the same as that of blank Zn–Al–NO_3–LDH (H_2O). Actually, 2% PEG 2000 and 2% PEG 4000 water solutions have been used for the reaction solvents and the particle sizes of diclofenac–Zn–Al–NO_3–LDHs were all above 3000 nm. There is almost no report about the influence of different reaction mixed

solvents on the particle size of LDHs to date. Gardner et al. (2001) have used methanol, ethanol, propanol and butanol as a single reaction solvent to prepare LDHs. The XRD line widths of Mg_3–Al–LDH products prepared in alcohols were found to decrease with increasing chain length of the alcohol, suggesting that the size of the fundamental particle increases with decreasing acidity of the alcohol. In their experiments with high pH, alkoxide-intercalated derivatives of LDHs had been formed, and in our reaction media with pH 7–8, it was impossible to form alkoxide-intercalated LDH compositions. The exact mechanism about influence of different reaction mixed solvents on the particle size of LDHs is unclear. One presumption is the interaction between hydroxyl group(s) of EtOH or PEG 400 and LDHs due to hydrogen bonds, which will lead to the surrounding of alcohol to newborn LDHs and inhibition of the particle aggregation of LDHs (Wang et al., 2006). On the other hand, viscosity of the reaction medium was enhanced with the increase of the chain length of an alcohol, resulting in formation of larger particle size of LDHs. Therefore, a suitable chain length of an alcohol in the reaction medium may be beneficial for formation of nano-size particles of LDH.

In addition, zeta potential of diclofenac–Zn–Al–NO_3–LDH (H_2O), diclofenac–Zn–Al–NO_3–LDH (10% EtOH) and diclofenac–Zn–Al–NO_3–LDH (2% PEG400) was 28.1 ± 0.4, 29.7 ± 0.6 and 27.3 ± 0.9 mV, respectively. The positive zeta potential of LDH particles is attributed to the structural positive charge and the electric double layer on the LDH surface (Xu et al., 2008). Drug loading of above LDHs was 30.4 ± 0.9, 32.2 ± 1.2 and 35.3 ± 0.7, respectively. Because diclofenac–Zn–Al–NO_3–LDH (2% PEG400) had the highest drug loading, most of the following researches focused on it.

3.2. Configuration model of diclofenac molecule intercalated in LDH interlayers

Successful intercalation of diclofenac into the LDH host is demonstrated by the XRD analysis of the nanocomposites in our previous report (Wang et al., 2009), where the interlayer distance of (0 0 3) peak increases from 0.795 nm in the case of blank LDH to 2.348 nm for diclofenac–Zn–Al–NO_3–LDH (2% PEG400). In order to understand the structure configuration of the nanocomposites, the molecule of diclofenac was optimized by the PM3 method in the Gaussian 03 software. The largest dimension of the drug molecule, taking into account the dimensions of the molecule and the van der Waals radii of the "external" atoms, is 1.006 nm. Considering that thickness of the LDH layer is 0.48 nm (Whilton et al., 1997), the gallery height of diclofenac–Zn–Al–NO_3–LDH (2% PEG400) was calculated as 1.868 nm. The largest dimension of diclofenac is markedly smaller than the gallery height experimentally measured for our samples, and thus even a completely perpendicular orientation would not account for the experimental results. Thus, formation of bilayers should be assumed.

Orientation has been proposed for intercalation of naproxen, an anion with molecular dimensions close to those of diclofenac. The anion forms a bilayer with the carboxylate groups pointing towards the hydroxide layers and the aromatic rings are in the middle of the gallery (Del Arco et al., 2004). Molecular dimensions of an anion of 4′-chlorostilbenecarboxylic (CSC) acid is similar with that of DS and the hydrotalcite complex involving CSC revealed the CSC molecules to be intercalated as a double layer, the tilt angle of the molecular plane being ca. 39.4° (Sasai et al., 1999). Though the experimental techniques used here do not permit a conclusive determination of the orientation of the anions in the interlayer. According to the PXRD data recorded and the literature information summarized above, we can propose a tilt bilayer is formed in the interlayer, as depicted in Fig. 1. The carboxylate groups of diclofenac molecules point towards the brucite-like layers with electrostatic

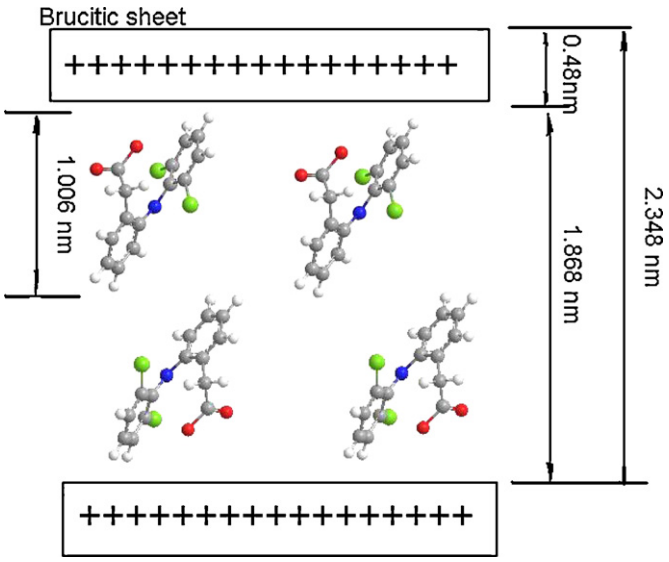

Fig. 1. Sketch of the possible location of diclofenac in the interlayer space of Zn–Al–NO_3–LDHs.

interactions. Because one diclofenac molecule consists of two aromatic cycles linked by an amine group and each aromatic cycle has one or two substitutes. The molecule cannot have a flat configuration due to the hindrance of the aromatic cycles as shown in Fig. 1. One aromatic ring of a diclofenac molecule may be parallel with the same aromatic ring of the other diclofenac molecule, forming π bonds of delocalization. Electrostatic interactions and forming π bonds of delocalization may make the diclofenac-intercalated Zn–Al–NO_3–LDH nanocomposite system more stable.

Additionally, the interlayer distance, the gallery height and the bilayer model of diclofenac–Zn–Al–NO_3–LDH nanocomposites in our experiments are very similar with those of diclofenac–Mg–Al–Cl–LDH nanocomposites reported by Ambrogi et al. (2002). On the contrary, a monolayer molecule of diclofenac in the diclofenac–Mg–Al–CO_3–LDH nanocomposites was suggested by Dupin et al. (2004).

3.3. Determination of diclofenac–LDHs formula

3.3.1. Thermogravimetric analysis

The TG analysis for DS and diclofenac–LDH (H_2O) is shown in Fig. 2. The TG curve of diclofenac–LDH shows four weight loss steps, (1) 7.33% from 50 to 100 °C, (2) 8.11% from 100 to 200 °C, (3) 20.51% from 200 to about 280 °C, and (4) about 20% from 280 to 650 °C. In the range of up to 200 °C, at least two endothermic events are detected, which can be associated with the loss of adsorbed and coordinated water, separately (Arizaga et al., 2008). Therefore the second weight loss of water has been used to determine the amount of interlayer water in this sample. Compared to the TG curve of DS, it is found that the third step is probably attributable to the oxidative degradation of the intercalated drug anions. The last step may be due to dehydroxylation of the LDH matrix and burning of DS.

3.3.2. Elemental analysis

Results of elemental chemical analysis for C, H, N, Zn, and Al for diclofenac–Zn–Al–NO_3–LDH (H_2O) are given in Table 1. The Zn/Al molar ratio in sample is close to the value of 2 in the starting solution. Assuming all nitrogen is in the form of DS, C/N ratio for diclofenac–LDH nanocomposites is smaller than the expected value. According to FTIR spectra of DS, blank LDH, DS + LDH physical mixture and diclofenac–LDH nanocomposites reported previously (Wang et al., 2009), strong bands at 1384 cm^{-1} in the spectra of

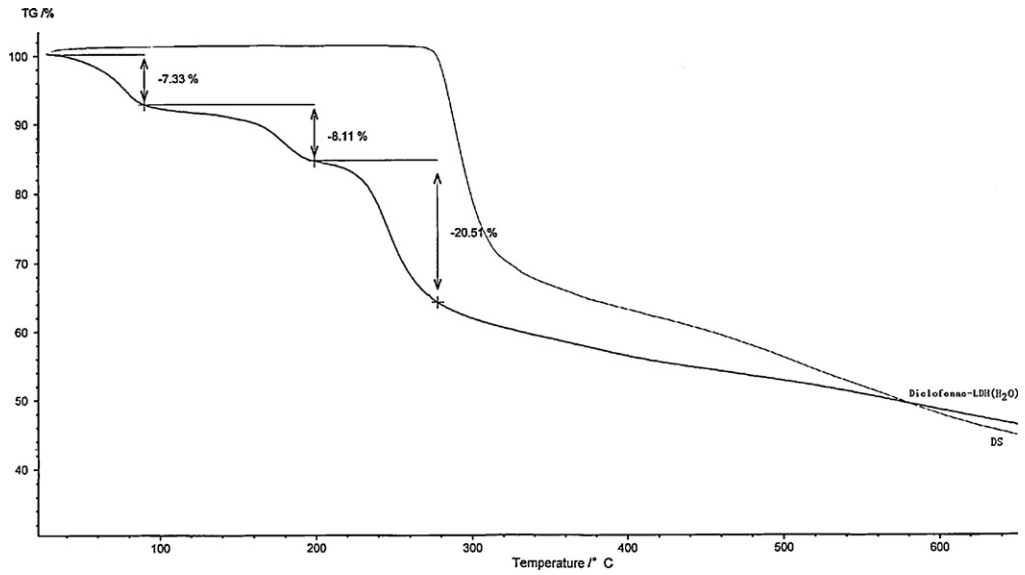

Fig. 2. Thermogravimetric analysis of DS and diclofenac–LDH nanocomposites.

Table 1
Elemental analysis and Chemical composition of samples.

w_{Zn}/%	w_{Al}/%	w_C/%	w_N/%	w_H/%	Chemical formula
22.32	4.42	29.09	2.575	4.415	$[Zn_{0.69}Al_{0.31}(OH)_2](diclofenac)_{0.30}(NO_3)_{0.01}\cdot 0.86H_2O$

blank LDH and DS + LDH physical mixture are due to N–O vibration from the free nitrate ions, and the intensity of this band was significantly reduced in the spectra of diclofenac–LDH nanocomposites. These results show that diclofenac has a higher affinity than nitrate towards the inorganic interlamellae, thus occupies the interlamellae space. However trace amount of nitrate was still in the interlamellae space. From the elemental chemical analysis results, the thermogravimetric studies (for weight loss between 100 °C and 200 °C) and charge balance, the formula can be proposed for diclofenac–LDH nanocomposites as shown in Table 1.

3.4. In vivo precorneal retention evaluation

Bionanocomposites represent an emerging group of biohybrids formed by the assembling of different biopolymers and inorganic solids and can be used as biosensors, packaging materials, superabsorbent materials, etc. (Darder et al., 2008). Preliminary compatibility tests of polymers with LDH dispersion have been conducted (data not shown). 2% (w/v) water-soluble nonionic polymer, such as PVP K30, HPMC, Tween-80, Poloxamer 188, LDH nanocomposites showed good stability. Immediate aggregation of LDH particles was observed when LDH was mixed with water solutions of carbomer, Gellan Gum and CMC-Na, separately. PVP K30 was selected to form a polymer–LDH nanohybrids with better stability for at least two months at room temperature. PVP K30 is widely used as a thickening agent, wetting agent, film forming polymer in ocular formulations (Gilhotra et al., 2009). In view of the fact that the range of the suitable content for PVP K30 used in eye drops is 2–10%, 2% and 10% PVP K30–LDH nanohybrid dispersions were used in our experiments. In addition, accelerated tests (40 °C ± 2 °C/75 ± 5% RH) of different diclofenac–LDH eye drops (F2, F3 and F4) revealed that particle size, pH and drug content of the formulations were almost unchanged for six months.

Precorneal retention can be used to evaluate the bioadhesion of ophthalmic formulation, and it may provide useful information for prediction of bioavailability in intraocular section. The ocular bioavailability of 2% PVP-K30–diclofenac–LDH nanohybrid dispersion (F3) and 10% PVP K30–diclofenac–LDH nanohybrid dispersion (F4) was evaluated and compared with that of both a free DS saline (F1) and diclofenac–LDH eye drop without PVP K30 (F2). The concentration–time profiles of DS in rabbit tears are shown in Fig. 3. The mean pharmacokinetic parameters are presented in Table 2. Following instillation of F1, the concentrations of diclofenac can be detected by HPLC for only 3 h. However, diclofenac–LDH nanocomposite dispersions provided a significant sustained drug release in the rabbit eyes because the free drug could be detected for up to 6 h. In vitro diclofenac release studies of powders of DS and powders of diclofenac–LDH nanocomposites in deionized H_2O, phosphate buffer solution (pH 6.8) and simulated tear fluid (STF,

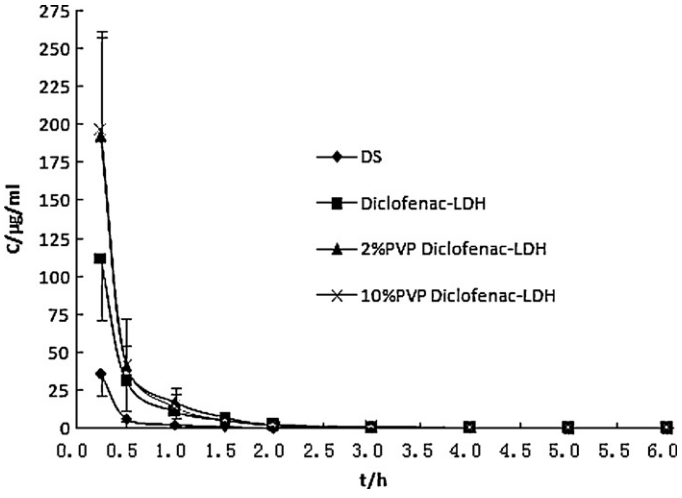

Fig. 3. The concentration–time curves of DS in tear samples following drop instillation of 50 μL of 0.1% (w/v) DS eye drops(♦), diclofenac–LDH dispersion(■), 2% PVP K30 diclofenac–LDH dispersion(▲) and 10% PVP K30 diclofenac–LDH dispersion(×) in rabbit eyes (mean ± SD, $n = 6$).

Table 2
Pharmacokinetics parameters of DS after a single instillation (50 μl) of 0.1% (w/v) DS eye drop or diclofenac–LDH dispersions in the rabbit eyes (mean ± SD, $n = 6$).

Key parameters	DS eye drop (F1)	Diclofenac–LDH (F2)	2% PVP diclofenac–LDH (F3)	10% PVP diclofenac–LDH (F4)
Total time when DS was still detectable (h)	3	6	6	6
C_{max} (μg/ml)	36.25 ± 14.10	112.37 ± 41.20*	192.39 ± 69.85*#	196.70 ± 61.28*#
T_{max} (h)	0.25	0.25	0.25	0.25
AUC_{0-t} (μg/ml h)	9.21 ± 3.46	36.82 ± 17.55*	55.40 ± 21.78*#	53.85 ± 30.69*#

Statistical analysis: *$p < 0.01$ versus F1, #$p < 0.05$ versus F2.

composition: NaCl 0.68 g, $NaHCO_3$ 0.22 g, $CaCl_2 \cdot 2H_2O$ 0.008 g, KCl 0.14 g, and distilled deionized water to 100 mL) were compared in our previous studies (Wang et al., 2009). The release of diclofenac from the powder of DS in deionized H_2O was 98.4% after 5 min, whereas the release of diclofenac from diclofenac–LDH nanocomposites was 8% after 5 min, 13% after 60 min, and 22% after 3 h. In cases of the STF media, there was an early fast release of diclofenac (about 50% release for the initial 30 min) followed by a relatively slower release for diclofenac (about 30% release for the next 150 min). These rapid first and then slow drug release behaviors of LDHs have been observed by Ambrogi et al. (2001). The rate of drug diffusing out of the matrix is controlled by the rigidity of the layers and the diffusion path length. When small species (chloride ion in the STF) exchange for bigger anions (intercalated drug), a consequent decrease of the interlayer distance occurs. This initial exchange of anions of the external part of the crystals usually causes the formation of an external phase with smaller distance, resulting in decrease of the rate of the drug release. These in vitro data indicate that LDHs have delayed the release of diclofenac in STF, which is in agreement with in vivo precorneal retention data.

The C_{max} of F1 was 36.25 ± 14.10 μg/ml at 0.25 h. When diclofenac was intercalated into LDHs, the C_{max} was increased to 112.37 ± 41.20 μg/ml at 0.25 h. When 2% and 10% PVP–diclofenac–LDH nanohybrids were administered to the rabbit eyes, the C_{max} of F3 and F4 was 192.39 ± 69.85 and 196.70 ± 61.28, respectively. AUC_{0-t} of F1, F2, F3, F4 was 9.21 ± 3.46, 36.82 ± 17.55, 55.40 ± 21.78 and 53.85 ± 30.69 μg/ml h, respectively. Student's *t*-test between groups was performed using Microsoft Excel, which showed a statistically significant difference between F2 (F3 or F4) and F1 ($P < 0.01$) with regard to the values of AUC_{0-t} and C_{max}. In addition, these two parameters were significantly increased ($P < 0.05$) between F3 and F2 as well as F4 and F2 ($P < 0.05$). Furthermore, we found that, when the concentration of PVP K30 was increased to 10%, AUC_{0-t} and C_{max} of F4 showed no difference with that of F3. These results show that C_{max} and AUC_{0-t} of F2 exhibit 3.1-fold and 4.0-fold increase compared with those of F1, respectively. When 2% PVP K30–LDH nanohybrid dispersion was administered to the rabbit eyes, the C_{max} and AUC_{0-t} were approximately 5.3-fold and 6.0-fold greater than those of F1, respectively. Our data suggest that LDHs may play a major role in the pharmacokinetic behavior of 0.1% DS dispersions. Because cornea and conjunctiva is covered by a thin fluid layer called mucus film (Ludwig, 2005), the primary component of mucus is mucin, a high molecular mass glycoprotein which is negatively charged at physiological pH. Thus, the positively charged LDH can provide a binding force to the eye surface with electrostatic interactions, resulting in slowing down drug elimination by the lachrymal flow and increasing the residence time and concentration of DS in the tear of the rabbits as polymers (Gorle and Gattani, 2009). In addition, it could be promoted by the presence of carbonyl groups and tertiary amine groups of PVP K30 molecules which form hydrogen bonds to the eye surface. Furthermore, this polymer can increase the viscosity of the diclofenac–LDH dispersions, which decreases the lachrymal drainage. This might explain that 2% PVP K30–diclofenac–LDH (F3) could achieve a prolonged precorneal residence time and good bioavailability. Different concentration of PVP K30 showed no difference with pharmacokinetic behavior of 0.1% diclofenac–LDH dispersions. We do not know the exact reasons and a proposed reason is that high concentration polymer can influence (or weaken) the electrostatic interactions between LDHs and mucin.

In order to treat ocular inflammation, diclofenac must penetrate across the cornea to reach therapeutic targets within the globe. The in vitro corneal permeation studies conducted earlier revealed that apparent permeability coefficient of diclofenac was about 1.17×10^{-5} cm/sec for DS solution of pH 7.2 (Ahuja et al., 2006). The higher apparent permeability coefficient and prolonged precorneal retention time of 2% PVP K30–diclofenac–LDH nanohybrid dispersions may facilitate the penetration of drug into the different tissues of the eyes, so an improved distribution of diclofenac in different tissues of the eyes could be expected.

3.5. In vivo tolerance assay

For a novel drug delivery to be proposed as an ophthalmic drug carrier, it is important not only to assay the biopharmaceutical properties but also the ocular tolerability. The ocular tolerability was evaluated following a modified Draize test protocol. The in vivo results showed no sign of obvious irritation or damaging effects to ocular tissues in rabbit eyes. The average scores of the control eyes with single and multi-administration were 0 and 0.75, respectively. The average scores of the right eyes with single and multi-dose test of LDH nanohybrid dispersion were 0 and 1, respectively. No differences of eye irritancy were observed between nanocarrier treated eyes compared to the control. According to the standards of Draize test and taking into consideration that the rabbit eye is more susceptible to irritant substances than the human eye, therefore, diclofenac–LDH nanocomposites are well tolerated by ocular surface structures.

4. Conclusions

Nanocomposite composed of diclofenac with Zn–Al–NO_3–LDH was synthesized and the suitable particle size and drug loading may be controlled by the reaction pH and solvent. The formula and configuration of the nanocarriers were confirmed by elemental chemical analysis, thermogravimetric analysis and by means of the data analysis of XRD combining with PM3 method in the Gaussian 03 software. It could be supposed that a tilt bilayer of diclofenac molecules was formed in the interlayer. In vivo precorneal retention studies demonstrated that C_{max} and AUC_{0-t} of diclofenac–LDH nanocomposites dispersion exhibit 3.1-fold and 4.0-fold increase compared with those of DS saline, respectively. When 2% PVP K30–diclofenac–LDH nanohybrid dispersion was administered to the rabbit eyes, C_{max} and AUC_{0-t} were approximately 5.3-fold and 6.0-fold greater than that of DS saline, respectively. Our data suggest that the positively charged LDH can provide a binding force to the eye surface with electrostatic interactions, resulting in slowing down drug elimination and increasing the AUC of diclofenac in the tears of the rabbits. The Draize test revealed that diclofenac–LDH nanocomposites are well tolerated by

ocular surface structures. These initial findings demonstrated that a novel ocular drug delivery system, drug–LDH nanocomposites, could be a promising method of improving the bioavailability of drugs in the eyes. In addition, we think long-time stability of LDH nanohybrid dispersion is a challenge because of the aggregate of LDH nanoparticles. Further work to evaluate corneal penetration studies and the tissue distribution of diclofenac is currently ongoing.

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References

Agnihotri, S.M., Vavia, P.R., 2009. Diclofenac-loaded biopolymeric nanosuspensions for ophthalmic application. Nanomedicine 5, 90–95.
Ahuja, M., Dhake, A.S., Majumdar, D.K., 2006. Effect of formulation factors on in-vitro permeation of diclofenac from experimental and marketed aqueous eye drops through excised goat cornea. Yakugaku Zasshi 126, 1369–1375.
Ambrogi, V., Fardella, G., Grandolini, G., Perioli, L., 2001. Intercalation compounds of hydrotalcite-like anionic clays with antiinflammatory agents. I. Intercalation and in vitro release of ibuprofen. Int. J. Pharm. 220, 23–32.
Ambrogi, V., Fardella, G., Grandolini, G., Perioli, L., Tiralti, M.C., 2002. Intercalation compounds of hydrotalcite-like anionic clays with anti-inflammatory agents. II. Uptake of diclofenac for a controlled release formulation. AAPS PharmSciTech 3, 1–6.
Arizaga, G.G., Mangrich, A.S., da Costa Gardolinski, J.E., Wypych, F., 2008. Chemical modification of zinc hydroxide nitrate and Zn–Al-layered double hydroxide with dicarboxylic acids. J. Colloid. Interface Sci. 320, 168–176.
Darder, M., Aranda, P., Ruiz, A.I., Fernandes, F.M., Ruiz-Hitzky, E., 2008. Design and preparation of bionanocomposites based on layered solids with functional and structural properties. Mater. Sci. Technol. 24, 1100–1110.
De Campos, A.M., Sánchez, A., Alonso, M.J., 2001. Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. Int. J. Pharm. 224, 159–168.
Del Arco, M., Gutiérrez, S., Martín, C., Rives, V., Rocha, J., 2004. Synthesis and characterization of layered double hydroxides (LDH) intercalated with non-steroidal anti-inflammatory drugs (NSAID). J. Solid State Chem. 177, 3954–3962.
Dupin, J.C., Martinez, H., Guimon, C., Dumitriu, E., Fechete, I., 2004. Intercalation compounds of Mg–Al layered double hydroxides with dichlophenac: different methods of preparation and physico-chemical characterization. Appl. Clay Sci. 27, 95–106.
Fresta, M., Panico, A.M., Bucolo, C., Giannavola, C., Puglisi, G., 1999. Characterization and in vivo ocular absorption of liposome-encapsulated acyclovir. J. Pharm. Pharmacol. 51, 565–576.
Gardner, E., Huntoon, K.M., Pinnavaia, T.J., 2001. Direct synthesis of alkoxide-intercalated derivatives of hydrotalcite-like layered double hydroxides: precursors for the formation of colloidal layered double hydroxide suspensions and transparent thin films. Adv. Mater. 13, 1263–1266.
Gaudana, R., Jwala, J., Boddu, S.H., Mitra, A.K., 2009. Recent perspectives in ocular drug delivery. Pharm. Res. 26, 1197–1216.
Gilhotra, R.M., Gilhotra, N., Mishra, D.N., 2009. Piroxicam bioadhesive ocular inserts: physicochemical characterization and evaluation in prostaglandin-induced inflammation. Curr. Eye Res. 34, 1065–1073.
Gorle, A.P., Gattani, S.G., 2009. Design and evaluation of polymeric ocular drug delivery system. Chem. Pharm. Bull. (Tokyo) 57, 914–919.
Ladewig, K., Niebert, M., Xu, Z.P., Gray, P.P., Lu, G.Q.M., 2010. Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. Biomaterials 31, 1821–1829.
Li, B., He, J., Evans, D.G., Duan, X., 2004. Enteric-coated layered double hydroxides as a controlled release drug delivery system. Int. J. Pharm. 287, 89–95.
Li, N., Zhuang, C., Wang, M., Sun, X., Nie, S., Pan, W., 2009. Liposome coated with low molecular weight chitosan and its potential use in ocular drug delivery. Int. J. Pharm. 379, 131–138.
Li, Y., Liu, D., Ai, H., Chang, Q., Liu, D., Xia, Y., Liu, S., Peng, N., Xi, Z., Yang, X., 2010. Biological evaluation of layered double hydroxides as efficient drug vehicles. Nanotechnology 21, 105101.
Ludwig, A., 2005. The use of mucoadhesive polymers in ocular drug delivery. Adv. Drug Deliv. Rev. 57, 1595–1639.
Qin, L., Xue, M., Wang, W., Zhu, R., Wang, S., Sun, J., Zhang, R., Sun, X., 2010. The in vitro and in vivo anti-tumor effect of layered double hydroxides nanoparticles as delivery for podophyllotoxin. Int. J. Pharm. 388, 223–230.
Sasai, R., Shinya, N., Shichi, T., Takagi, K., Gekekko, K., 1999. Molecular alignment and photodimerization of 4-chloro-4-stilbenecarboxylic acid in hydrotalcite clays: bilayer formation in the interlayers. Langmuir 15, 413–418.
Shen, J., Wang, Y., Ping, Q., Xiao, Y., Huang, X., 2009. Mucoadhesive effect of thiolated PEG stearate and its modified NLC for ocular drug delivery. J. Control. Release 137, 217–223.
Valls, R., Vega, E., Garcia, M.L., Egea, M.A., Valls, J.O., 2008. Transcorneal permeation in a corneal device of non-steroidal anti-inflammatory drugs in drug delivery systems. Open Med. Chem. J. 22, 66–71.
Wang, J.F., Du, B.Z., Kang, Z.Q., 2006. A study of preparation of nano-layered double hydroxides by differfnt reaction media. Bas. Sci. J. Tex. Univer. 19, 58–61.
Wang, Y.J., Cao, F., Ping, Q.N., 2009. Preparation and characterization of diclofenac sodium layered double hydroxide nanohybrids. J. China Pharma. Univ. 40, 321–326.
Weir, M.R., Kydd, R.A., 1998. Synthesis of heteropolyoxometalate-pillared Mg/Al, Mg/Ga, and Zn/Al layered double hydroxides via LDH-hydroxide precursors. Inorg. Chem. 37, 5619–5624.
Whilton, N.T., Vickers, P.J., Mann, S., 1997. Bioinorganic clays: synthesis and characterization of amino- and polyamino acid intercalated layered double hydroxides. J. Mater. Chem. 7, 1623–1629.
Wilhelmus, K.R., 2001. The Draize eye test. Surv. Ophthalmol. 45, 493–515.
Xu, Z.P., Jin, Y., Liu, S., Hao, Z.P., Lu, G.Q., 2008. Surface charging of layered double hydroxides during dynamic interactions of anions at the interfaces. J. Colloid. Interface Sci. 326, 522–529.
Xu, Z.P., Stevenson, G., Lu, C.Q., Lu, G.Q., 2006. Dispersion and size control of layered double hydroxide nanoparticles in aqueous solutions. J. Phys. Chem. B 110, 16923–16929.
Zhang, J.P., Wang, Q., Xie, X.L., Li, X., Wang, A.Q., 2010. Preparation and swelling properties of pH-sensitive sodium alginate/layered double hydroxides hybrid beads for controlled release of diclofenac sodium. J. Biomed. Mater. Res. B: Appl. Biomater. 92, 205–214.