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Methazolamide Calcium Phosphate Nanoparticles in an Ocular Delivery System

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A new system for the local delivery of methazolamide to the eye has been developed based on calcium phosphate (CaP) nanoparticles. The methazolamide loaded CaP nanoparticles were prepared through the formation of an inorganic core of CaP and further adsorption of the methazolamide. The maximum loading of methazolamide studied using UV-vis spectrophotometry was about 0.2% (w/w). The drug-loaded particles had a negative surface charge at about −30 mV while their mean particle diameter was estimated to be 256.4 nm. *In vitro* release studies demonstrated diffusion-controlled release of methazolamide from the CaP nanoparticles over a period of 4 h. *In vivo* studies indicated that the intraocular pressure (IOP)-lowering effect of the inorganic nanoparticle eye drops lasted for 18 h, which was significantly better than the effect of 1% brinzolamide eye drops (6 h). Physical stability studies indicated that the preparation was stable for 6 months at 40℃. These findings suggested that methazolamide bound to CaP nanoparticles might be useful in the local treatment of glaucoma.

Key words——methazolamide; calcium phosphate nanoparticle; ocular delivery system; sustained release

INTRODUCTION

Carbonic anhydrase inhibitors (CAIs) have been used to treat glaucoma due to their ability to lower intraocular pressure (IOP) by reducing aqueous humour formation. Although IOP can be controlled by oral administration of CAIs, systemic side effects severely limit this mode of therapy, but topical administration directly to the eye could reduce the side effects.[1] However, a drug molecule should possess both hydrophilic and lipophilic characteristics to ensure it is both soluble and has the ability to cross membranes.[2]

Methazolamide (MTA), a carbonic anhydrase inhibitor, is practically insoluble in water and in the aqueous tear fluid and this significantly limits its ocular bioavailability. Until now, there have been only two MTA topical administration formulations (MTA gel and MTA cyclodextrin eye drop solutions).[3,4] MTA gel was shown to increase the contact time and to lower the IOP in rabbits and humans; however, the gel has been precluded due to a higher incidence of bulbar infection and follicular conjunctivitis.[5] MTA cyclodextrin eye drop solutions offered numerous advantages over the conventional therapeutic approach, yet there were still tissue irritation and toxicological implications caused by penetration enhancers.[6]

Calcium phosphates (CaP) are of high relevance in materials science, biology, and medicine because they constitute the inorganic part of human hard tissue, *i.e.*, bones and teeth.[7,8] They have shown promise for drug delivery applications due to their proven biocompatibility and non-toxic degradation products.[9,10] Their small size mean that CaP nanoparticles are readily transportable through small capillaries and are readily taken up by cells, thereby permitting accumulation in target sites.[11] The use of biodegradable materials for nanoparticle preparation allows for the sustained release of drug within a target site over an extended period of time.[12] CaP nanoparticles as novel non-viral vectors for gene delivery have been generally used in *in vitro*[13,14] and *in vivo* applications.[15] They offer a promising avenue to fulfill the need for an ophthalmic drug delivery system.

In this paper we report a simple method to adsorb methazolamide on CaP nanoparticles and a more detailed physicochemical characterization including the *in vitro* and *in vivo* studies of these nanoparticles. Both *in vitro* and *in vivo* studies indicated that the methazolamide adsorbed on CaP nanoparticles had a better sustained release character than free methazolamide did.

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EXPERIMENTAL

Materials and Reagents Methazolamide, of 99 % purity, was purchased from AOYIPOLLEN (Hangzhou, China). One percent brinzolamide eye drops (AZOPT) were purchased from ALCON (UK). The Spectra/Por dialysis membrane, 10 kDa to 12 kDa molecular weight cutoff, was obtained from INALCO (USA). All other chemicals used were of pharmaceutical or special analytical grade.

Animals Male and female New Zealand albino rabbits, weighing 2.5–3.0 kg, were provided by the Animal Experimental Center of Nanjing Medical University. The animals, housed in standard cages in an air-conditioned and light-controlled room at 25±1 °C and 70±5% relative humidity, were given a standard pellet diet and were provided with water *ad libitum*. All studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication no.92–93, revised in 1985). The local ethics committees for animal experimentation approved all experiments.

Preparation of CaP Nanoparticles Calcium phosphate nanoparticles were prepared of calcium chloride, dibasic sodium phosphate and sodium citrate. Mixing was accomplished by slowly adding 7.5 ml of 12.5 mM calcium chloride solution and 7.5 ml of 12.5 mM dibasic sodium phosphate solution into 1.5 ml of 15.6 mM sodium citrate solution at 4°C by continuous stirring (2000 rpm) for 48 h. The mixture was sonicated (frequency 22 kHz; power 120 W) for 30 min, the dispersion was filtered and was kept at −4°C for further use.[16,17]

Preparation of Methazolamide CaP Nanoparticles Methazolamide was adsorbed on CaP nanoparticles by the following method: 50 mg MTA was dissolved in 10 ml absolute alcohol; 1.25 ml CaP nanoparticle suspensions (20 mg/ml) were centrifuged (AllegraTM X22, Beckman Coulter, USA) at 8500 rpm for 15 min at 4°C. The precipitation was added to the MTA-absolute alcohol solution, and then dispersed by water bath sonication (frequency 22 kHz; power 120 W) for 2 h. The mixture was stirred (125 rpm, 2 h) at room temperature (24°C) and centrifuged (8500 rpm, 15 min) at 4°C. The precipitation was dispersed by 5 ml distilled water, then 0.01% (w/v) benzalkonium bromide was added as bacteriostatic agent.[17]

Determination of Loading Efficiency The percentage of drug loaded was calculated after dissolving the prepared methazolamide CaP nanoparticles in 0.01 M HCl. The concentration of MTA in 0.01 M HCl was estimated spectrophotometrically at λ_{max}= 290 nm. The loading efficiency expressed as loading percentage was calculated through the following equation:[18]

$$\text{Loading efficiency percentage} = \frac{\textit{loading drug}}{\textit{total drug}} \times 100\%$$

Particle Size and Zeta Potential Measurements Particle size of the prepared methazolamide CaP nanoparticles was measured with a laser light scattering method using a submicron particle analyzer (N4 Plus, Beckman Coulter, Germany). The measurement was performed in triplicate, and the median size and range of distribution were obtained. The surface charge of particles was also measured using a zeta potential analyzer (Delsa 440SX, Beckman Coulter, Germany).

Characterization of Methazolamide CaP Nanoparticles The X-ray diffraction (XRD) patterns of the samples were obtained using a Rigaku D/max (2500xt/PC, Japan) under a condition of 40 kV, 20 mA and Cu Kα radiation. The samples were run for 1h in the 2 theta (θ) range of 3–85°. Fourier transform infrared (FTIR) spectra were obtained at room temperature (24°C) using a spectrometer (Tensor 27, Bruker, Germany) in the spectral range between 400 and 4000 cm^{-1}.

Stability Studies According to Chinese pharmacopoeia (CP),[19] stability studies were performed to investigate the pH, osmotic pressure, content and related substances of MTA CaP nanoparticles that may have developed in long-term storage. The preparations were separately stored for 10 days at 60°C (high-temperature test; sampling at 0, 5, 10 days), for 10 days at 4500 Lx±500 Lx (highlight test; sampling at 0, 5, 10 days), and for 6 months at 40°C and 75% R. H. (accelerated rest; sampling at 0, 1, 2, 3, 6 months). The content was tested by UV-visible spectrophotometer at λ_{max}=290 nm. Related substances were tested by HPLC (mobile phase: methanol/water 30/70; rate: 1.0 ml/min; temperature: 40°C; 290 nm).

Release of MTA from CaP Nanoparticle Preparation *in Vitro* The methazolamide CaP nanoparticle suspension, equivalent to 1 mg methazolamide, was placed in a filter bag with a molecular weight cutoff 10 kDa to 12 kDa and dialyzed against 100 ml of release buffer (PBS pH 7.4).[20] The dialysis

process was performed at 37 °C. The stir bar was adjusted to rotate at a constant speed (50 rpm). Samples were collected at 5 min, 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h (5 ml/time), filtered through a 0.22 μm millipore filter, then assessed spectrophotometrically for drug content at $\lambda_{max}=290$ nm.

***In Vivo* Studies** two-one hundredths percent (w/v) methazolamide CaP nanoparticles were tested for the intraocular pressure (IOP)-lowering activity on the unrestricted, conscious rabbits, and the data were compared with that of plain nanoparticles, brinzolamide eye drops (1%, w/v) and normal sodium chloride (0.9%, w/v). The rabbits were randomly divided into 4 groups, each consisting of 5 rabbits: Group I received methazolamide CaP nanoparticles; Group II received plain nanoparticles; Groups III and IV were administered brinzolamide eye drops (1%, w/v) and normal sodium chloride (0.9%, w/v), respectively.[21] The resting IOP level was determined in both eyes as the mean of IOP measurements taken every 30 min over a 2-h period before administration of the test solutions to the eye. Two drops of the test solutions were administered to both eyes at time 0 and the IOP was measured at 1-h intervals after administration.[21] All measurements were made during the same hour on each day, and were done by the same investigator with the same tonometer (YJI, Suchou minren, China).

RESULTS AND DISCUSSION

Preparation and Loading Efficiency The preparation of methazolamide CaP nanoparticles was optimized. Improving the content of the nanoparticles could increase the loading efficiency, but the osmotic pressure would go beyond the acceptable range. The nanoparticles would be precipitated sufficiently when centrifuged at 8500 rpm for 15 min. Stirring at 120–220 rpm made no difference in loading efficiency, but stirring for 2 hours achieved the highest loading efficiency which was about 0.2%. It was clearly observed that 4 μg of methazolamide per mg of calcium phosphate could be easily loaded in the nanoparticles.

Particle Size and Zeta Potential Measurements Particle size and zeta potential of three batches of methazolamide CaP nanoparticles were examined. Particle size was a major issue considered by formulation scientists when formulating dispersed systems, especially those intended for ocular administration. For ocular administration, irritation and tear wash out may occur on administration of large-sized particles, since smaller particles were better tolerated.[22] The particle diameter of methazolamide CaP nanoparticles was estimated to be 256.4±31.1 nm. In general, particles could be dispersed stably when the absolute value of zeta potential was about 30 mV due to the electric repulsion between particles.[23] The zeta potential of obtained methazolamide CaP nanoparticles was −30.4±2.2 mV, which demonstrated that the nanoparticle dispersion prepared in this study was a physically stable system.

Characterization of Complex Formation Both XRD and FTIR were used to confirm the complexations of methazolamide with CaP nanoparticles. The X-ray powder diffraction patterns of methazolamide, calcium phosphate nanoparticles and their complexations in comparison with the physical mixtures are shown in Fig. 1 (A–D). XRD confirmed that the three samples (A, B, C) exhibited the $Ca_{10}(PO_4)_6(OH)_2$ phase (highest intensity peak at $2\theta=31.8°$).[15] The pattern of MTA exhibited a series of intense peaks, which was indicative of its crystalline character. The pattern of the physical mixtures was composed of the superposition of the spectra of each single component. In the case of MTA CaP nanoparticles the crystallinity of the drug's character was totally lost. It was assumed that the doped MTA inhibited the crystallization of the entrapping matrix.

FTIR spectroscopy has also been used to assess the interaction between methazolamide and CaP nanoparticle. FTIR spectra of methazolamide, meth-

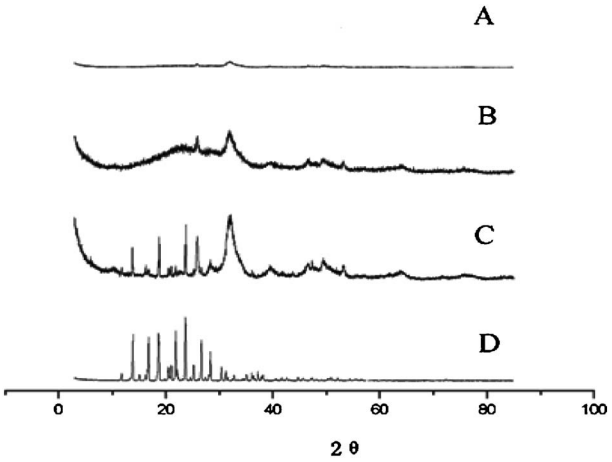

Fig. 1. XRD Patterns of Methazolamide, Calcium Phosphate Nanoparticles, Complexations and Mixtures, Respectively

A: calcium phosphate nanoparticles, B: methazolamide-calcium phosphate nanoparticle complexations, C: physical mixtures of methazolamide and calcium phosphate nanoparticles, D: methazolamide.

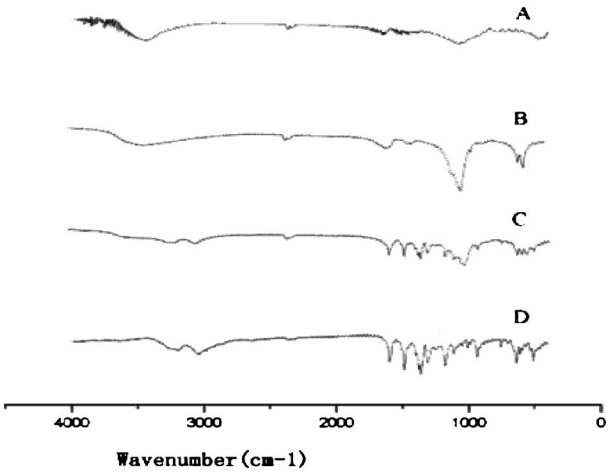

Fig. 2. FTIR Patterns of Methazolamide, Calcium Phosphate Nanoparticles, Complexes and Mixtures, Respectively

A: calcium phosphate nanoparticles, B: methazolamide-calcium phosphate nanoparticle complexation, C: physical mixtures of methazolamide and calcium phosphate nanoparticles, D: methazolamide.

azolamide-calcium phosphate nanoparticle complexations, physical mixtures of methazolamide and calcium phosphate nanoparticle are shown in Fig. 2 (A-D). The spectra (C and D) contained C=O bands at around 1598 cm^{-1}, S=O bands at around both 1349 cm^{-1} and 1162 cm^{-1}, N-H bands at 3200 cm^{-1}, and C-H bands at 3043 cm^{-1}. There was a noticeable loss in methazolamide-calcium phosphate nanoparticle complexation (B). We presumed that methazolamide and calcium phosphate nanoparticles might bind with a hydrogen bond.[24]

Stability Studies The pH (about 6.0), osmotic pressure (0.87–1.21 wt. % NaCl equivalent), content (98.13%–101.33%) and related substances (<0.72 %) of methazolamide calcium phosphate nanoparticles were up to the standard of CP when samples were stored at 60°C for 10 days and at 40°C for 6 months. In a highlight test, however, the related substance (about 3.20%) was out of the permissive range (<1.00%). It was recommended that the preparations should be stored away from light.

***In Vitro* Drug Release Studies** Drug-release experiments were performed in phosphate buffer solution (PBS) (pH 7.4) using the dialysis method. Figure 3 illustrates the cumulative amount of methazolamide released versus time profiles for different drug-containing solutions. For methazolamide eye drops, almost all the methazolamide released immediately after start of the release study. After 30 min over 60% of the MTA bound to CaP nanoparticles

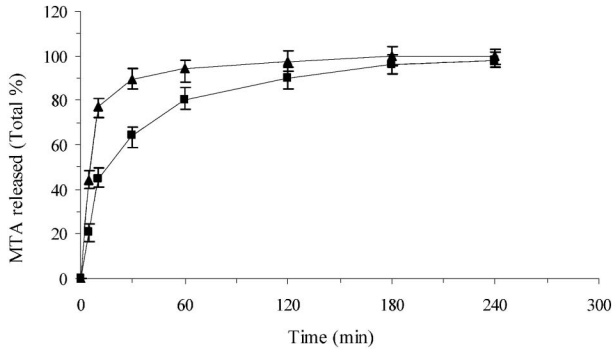

Fig. 3. *In Vitro* Release of Methazolamide from Calcium Phosphate Nanoparticles at pH 7.4 in PBS

(▲): 0.02% methazolamide eye drops, (■): 0.02% methazolamide calcium phosphate nanoparticles. Each symbol represents the mean ±S.D. (n=3).

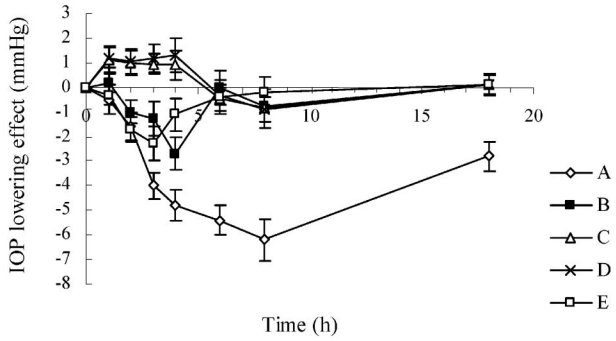

Fig. 4. Lowering of Intraocular Pressure (IOP) after Administration of Two Drops of test Solution to Rabbits

A: 0.02% methazolamide calcium phosphate nanoparticles, B: 1% brinzolamide eye drops, C: normal sodium chloride, D: plain nanoparticles, E: 0.02% methazolamide eye drops. Results are expressed as difference in mmHg from the pre-treatment value. Each value shown is the mean of ten measurements ±S.E.M.

was released in the buffer solution. A total of 99.4% of the original amount of the nanoparticle-bound MTA was released at the end of 240 min. The *in vitro* release results revealed that methazolamide loaded on calcium phosphate could sustain releasing for 4 hours.

***In Vivo* Studies** 0.02% Methazolamide calcium phosphate nanoparticle eye drop solutions had a notable IOP lowering effect in normotensive rabbits (Fig. 4). Despite the fast *in vitro* release rates for the 0.02% methazolamide eye drops (Fig. 3), higher initial *in vivo* pharmacological responses were not observed. Plain nanoparticles and normal sodium chloride showed no effect on lowering the IOP. Methazolamide CaP nanoparticle eye drop solutions produced a significant lowering in IOP compared with 1% brinzolamide eye drops. The maximum IOP

lowering was observed at 4 h after topical administration of brinzolamide (1%) and the duration of the activity was about 6 h. Maximum IOP lowering effect of methazolamide CaP nanoparticles was observed at 2–8 h after drug administration. The duration of the activity was about 18 h. These results might be due to the crossing cell membrane activity of MTA CaP nanoparticles. The release of MTA from nanoparticles would increase its local concentration at the corneal surface. After release from the nanoparticles, drug molecules relied on passive diffusion to cross the corneal barrier.[25] Thus, the longer the contact time at the corneal surface, the higher would be the bioavailability of the drug obtained.[26] Calcium phosphate nanoparticles as drug carriers could change the rate and amount of drug absorption. Hence, a more pronounced sustained reduction in IOP was produced.

CONCLUSION

This study shows that methazolamide calcium phosphate nanoparticles produced a significant and sustained reduction in IOP. It is possible to achieve effective intraocular delivery of poorly water soluble CAI, like methazolamide, from aqueous eye drop formulations containing the drug in calcium phosphate nanoparticles.

As we know, almost all inorganic nanoparticles are chemically stable. This is an excellent feature so that their physicochemical properties can be kept unchanged during the whole delivery process. Most nanoparticles are believed to accumulate in the cells once they are endocytosed. Therefore, cytotoxicity should be reevaluated when repeated administration is expected.[27]

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REFERENCES

1) Zimmerman T. J., *Ann. Ophthalmol.*, **10**, 509–510 (1978).
2) Loftsson T., Stefansson E., *Drug Dev. Ind. Pharm.*, **23**, 473–481 (1997).
3) Angela C. P., U. K. Patent 2223166 A (1990).
4) Hafrun F., Thorsteinn L., *J. Control. Release*, **44**, 95–99 (1997).
5) Hurvitz L. M., Kaufman, P. L., Robin A. L., Weinreb R. N., Crawford K., Shaw B., *Drugs*, **41**, 514–532 (1991).
6) Kaur I. P., Garg A., Singla A. K., Aggarwal D., *Int. J. Pharm.*, **269**, 1–14 (2004).
7) Weiner S., Wagner H. D., *Annu. Rev. Mater. Sci.*, **28**, 271–298 (1998).
8) Dorozhkin S. V., Epple M., *Angew. Chem. Int. Ed.*, **41**, 3130–4136 (2002).
9) Paul W., Sharma C. P., *J. Biomater. Appl.*, **17**, 253–264 (2003).
10) Pilliar R. M., Filiaggi M. J., Wells J. D., Grynpas M. D., Kandel R. A., *Biomater.*, **22**, 963–972 (2001).
11) Panyam J., Sahoo S. K., Prabha S., Bargar T., Labhasetwar V., *Int. J. Pharm.*, **262**, 1–11 (2003).
12) Sahoo S. K., Labhasetwar V., *Drug Discov. Today*, **8**, 1112–1120 (2003).
13) James R. F. L., Grosveld F. G., "DNA-mediated gene transfer into mammalian cells, Techniques in Molecular Biology," eds. by Walker J. M., Gaastra W., Croom Helm, London and Sydney, 1987, pp. 187–202.
14) Truong-Le V. L., Walsh S. M., Schwabert E., Mao H. Q., Guggino W. B., August J. T., Leon K. W., *Arch. Biochem. Biophys.*, **361**, 47–56 (1999).
15) Indrajit R., Susmita M., Amarnath M., Subho M., *Int. J. Pharm.*, **250**, 25–33 (2003).
16) He Q., Mitchell A., Morcol T., Bell J. D. S., *Clin. Diagn. Lab. Immunol.*, **9**, 1021–1024 (2002).
17) Guan X. H., He Q., Jiang Q., Chinese Patent no. 200510041162.0, (2006).
18) Hathout R. M., Mansour S., Mortada N. D., Guinedi A. S., *AAPS PharmSciTech*, **8**, 1–12 (2007).
19) State Pharmacopoeia Commission of P. R. China, "Pharmacopoeia of the People's Republic of China, Vol. 2," Chemical Industry Press, Beijing, 2005, p. 688.
20) Perugini P., Pavanetto F., *J. Microencapsul.*, **15**, 473–483 (1998).
21) Monem A. S., Ali F. M., Ismail M. W., *Int. J. Pharm.*, **198**, 29–38 (2000).
22) Kaur I. P., Garg A., Singla A. K., Aggarwal D., *Int. J. Pharm.*, **269**, 1–14 (2004).
23) Müller R. H., Jacobs C., Kayser O., *Adv.*

Drug Deliv. Rev., **47**, 3–19 (2001).
24) Kakizawa Y., Furukawa S., Kataoka K., *J. Control. Release*, **97**, 345–356 (2004).
25) Velpandian T., Gupta S. K., Gupta Y. K., Biswas N. R., Agarwal H. C., *J. Microencapsul.*, **16**, 243–250 (1999).
26) El-Gazayerly O. N., Hikal A. H., *Int. J. Pharm.*, **158**, 121–127 (1997).
27) Xu Z. P., Zeng Q. H., Gao Q. L., Yu A. B., *Chem. Eng. Sci.*, **61**, 1027–1040 (2006).