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ORIGINAL ARTICLE

Lipid emulsions as a potential delivery system for ocular use of azithromycin

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Abstract

Objective: To obtain stable positively charged Azithromycin (AZI) emulsions with a mean droplet size of 120 nm for the treatment of eye diseases. *Methods*: The emulsions were obtained by using a suitable homogenization process. The physical stability was monitored by measuring the particle size, zeta potential, and visible appearance. The drug entrapment efficiency was measured by both ultracentrifugation and ultrafiltration methods. Compared with a phosphate solution of AZI, the stability profiles of AZI in lipid emulsions at various pH values were monitored by high-performance liquid chromatography. A pharmacokinetic study was performed to determine the drug levels in rabbit tear fluid using Ultra-performance liquid Chromatography–mass spectrometry. *Results*: Almost all the AZI in the lipid emulsion was distributed in the oil phase and small unilamellar liposomes without contact with water, thereby avoiding hydrolysis. The elimination of the AZI lipid emulsions in tear fluid was consistent with the basic linear pharmacokinetic characteristics. The $AUC_{0\text{-}t}$ of the AZI lipid emulsion (1%, w/v) and aqueous solution drops (1%, w/v) was 1873.58 ± 156.87 and 1082.46 ± 179.06 μgh/ml respectively. *Conclusions*: This study clearly describes a new formulation of AZI lipid emulsion for ocular administration, and lipid emulsions are promising vehicles for ophthalmic drug delivery.

Key words: *Azithromycin; lipid emulsions; pharmacokinetics; rabbit tear fluid; stability; UPLC–MS*

Introduction

Azithromycin (AZI) (Figure 1), known as a synthetic macrocyclic lactone antibiotic, is a long-acting derivative of erythromycin. AZI has been incorporated into many dosage forms such as tablets, capsules, granules, infusion solutions, suspensions, syrups, and injections. It has a unique pharmacokinetic profile that allows rapid distribution to tissues, sustained high tissue levels, good acid stability, and enhanced absorption compared with erythromycin. On account of its potent efficacy against chlamydia and bacteria, large oral doses of AZI have been used to treat ocular infection and inflammation. However, entry of the drug into the systemic circulation might produce undesirable systemic side effects and reduce its biological availability[1,2]. A number of researchers have sought to develop an effective topical method of administration. Accordingly, ocular administration is one approach to increase the beneficial action of drugs and reduce their systemic adverse effects.

Ocular diseases are mainly treated topically by application of drug solutions administered as eye drops. These conventional dosage forms account for nearly 90% of the currently accessible marketed formulations, mainly due to their simplicity and convenience[3]. Drugs are commonly applied to the eye for a localized action on the surface or in the interior of the eye[4]. However, extensive precorneal loss is caused by the defense mechanisms of the eye, such as lachrymation, reflex blinking, and increased drainage. Only 5% of the applied drug in eye drops penetrates the cornea and reaches the intraocular tissues with the rest of the dose undergoing drainage via the nasolacrimal duct from tear fluid, which results in loss of drug into the systemic circulation and the associated risk of side effects. In addition, drugs liable to hydrolysis in aqueous solutions will be degraded, thereby losing their antibacterial activity.

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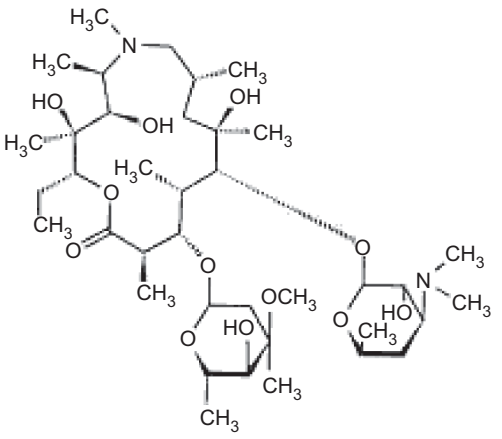

Figure 1. Structure of azithromycin (AZI).

Several drug delivery carriers have been employed to overcome such delivery challenges, including the use of thermosetting in situ gelling polymer-based systems, nanoparticles, liposomes, and micellar solutions. In this context, lipid emulsions offer a promising alternative. In the last decade, the parenteral o/w emulsion, as far as current parenteral nutrients are concerned, have been well accepted as intravenous delivery systems for their ability to incorporate water-insoluble drugs and to reduce the side effects of a variety of potent drugs[5,6]. Lipid submicron emulsions are receiving increasing attention as colloidal drug vehicles for various potential therapeutic applications. Several studies are in progress to develop submicron emulsions not only for parenteral but also for ocular delivery or other topical applications[7]. The main advantage of such systems is the potential to increase the solubility and bioavailability of drugs as well as to reduce the local irritation. They have been investigated and are now being exploited commercially as delivery system to improve the ocular bioavailability of some drugs[8–10]. The first anionic lipid emulsion containing cyclosporine A 0.05% (Restasise, Allergan, Irvine, CA, USA) for the treatment of chronic dry eye disease was approved for clinical use by the Food and Drug Administration (FDA) in December 2002, and is now available in the United States[11]. Furthermore, an over-the-counter product, Refresh Enduraw, a non-medicated anionic emulsion has been exploited in the United States for eye lubricating purposes in patients suffering from moderate-to-severe dry eye syndrome[12]. AZI eyedrops, AzaSite, approved by the FDA were marketed in the United States in 2007. Compared with other antibiotic agents used to treat bacterial conjunctivitis, AzaSite has smaller number of administration. However, AzaSite is an aqueous solution and AZI is also in aqueous media, so lipid emulsions have more potential to overcome the following questions regarding: (1) chemical instability because of hydrolysis; (2) low bioavailability of the eyedrops; and (3) eye irritation on account of exposure to the drug.

Based on the above history, a new lipid emulsion of AZI for ocular delivery was investigated in this study. High-pressure homogenization was used to prepare the emulsions. The physical stability of AZI lipid emulsions was evaluated by measuring the particle size, zeta potential, and examining drug entrapment efficiency (EE), whereas the chemical stability was investigated under forced degradation conditions by changing pH values. In addition, animal models were used to evaluate the preparation in vivo and pharmacokinetic data on the experimental AZI lipid emulsion were obtained. The findings obtained show that lipid emulsions are useful as a delivery system for ophthalmic preparations of drugs.

Materials and methods

Materials and animals

The following materials were supplied from the sources in brackets: AZI (Shanghai Sunve Pharmaceutical Co., Ltd., Shanghai, China; chemical purity 94.5%), Clarithromycin (Zhejiang Huayi Pharma Ltd., Zhejiang, China; chemical purity 94.2%), α-tocopherol (Zhejiang Medicine Ltd. Co., Zhejiang, China), medium chain triglyceride (MCT; Lipoid KG, Ludwigshafen, Germany), soybean lecithin (EPIKURON 170, PC72%; Degussa Food Ingredients, Germany), Poloxamer 188 (Pluronic F-68®; BASF AG, Ludwigshafen, Germany), Tween-80 for parenteral use and EL-40 (Shenyu Medicine and Chemical Industry Ltd. Co., Shanghai, China), stearylamine (Beijing Duxin subtle prepn. Plant, Beijing, China), glycerol (Zhejiang Suichang Glycerol Plant, Zhejiang, China), benzalkonium bromide (Shenyang Hongqi Medicine Ltd. Co., Shenyang, China), potassium dihydrogen phosphate (Guangdong Shantou Xilong Chemical Plant, Shantou, China), isopropyl alcohol, acetonitrile, and methanol (Tianjin Concord Technology Ltd. Co., Tianjin, China). All chemicals and reagents used were of analytical or chromatographic grade.

The laboratory animals (New Zealand White rabbits, weighting 2.0–2.5 kg) used in this study were provided by the Animal Experimental Center of Shenyang Pharmaceutical University.

Preparation of AZI ophthalmic lipid emulsions

The emulsions were prepared containing AZI (1%, w/v), MCT (8.5%, w/v), soybean lecithin (1.8%, w/v), stearylamine (0.3%, w/v), and α-tocopherol (0.05%, w/v) as an oil phase, whereas Tween-80 (0.4%, w/v), F-68 (0.4%, w/v), EL-40 (0.3%, w/v), glycerol (2.2%, w/v), EDTA (0.05%, w/v), and benzalkonium bromide (0.003%, w/v)

were dispersed in water to obtain an aqueous phase. The two phases were heated separately in water baths at 75°C until they were uniformly dispersed. The oil phase was then added to the aqueous phase and emulsified in a high shear mixer (ULTRA TURRAX® T18 basic, IKA® WORKS Guangzhou, Germany) at 10,000 rpm to prepare the primary emulsions. Further homogenization was performed using a Niro Soavi NS10012k homogenization apparatus (Via M. da Erba, 29/A-43100 PARMA, Italy), at 70 Mpa for seven cycles. The flow rate of every homogenization cycle was 100 mL/36 seconds. After being adjusted to the appropriate pH, the emulsions were passed through a 0.22-μm membrane filter and transferred to plastic bottles under a nitrogen atmosphere.

Physicochemical character and stability of AZI lipid emulsions

Freeze-thawing procedure

AZI lipid emulsion samples were frozen in a refrigerator at −20°C for 24 hours and then thawed at room temperature prior to the evaluation of their visual appearance and particle size distribution. All the samples were investigated after undergoing five cycles.

Particle size and zeta potential evaluation

The particle size is used as an indicator of physical stability. The emulsion droplet size and its size distribution were determined by means of photon correlation spectroscopy (PCS; dynamic light scattering), using a Nicomp™ 380 Zeta Potential/Particle Sizer (Particle Sizing System, Santa Barbara, CA, USA). The PCS covered the size range from 5 nm to 3 μm and has, therefore, been extensively used for particle size analysis of o/w submicron emulsions[13]. The Nicomp and the Gaussian distribution of particle size were obtained at the same time with intensity-weighting (*z*-average), volume-weighting, and number-weighting while the value of the SD instead of the polydispersity index gave the width of the distribution. Each emulsion sample was diluted to the appropriate concentration with doubly distilled water and measurements were carried out at 25°C. It was verified beforehand that dilution of the samples did not alter the size distribution obtained[14,15].

The zeta potential is a very useful way of evaluating the stability of any colloidal system. It was also measured with a Nicomp™ 380 Zeta Potential/Particle Sizer employing the electrophoretic light scattering technique and using doubly distilled water as a diluent. Values reported were the mean value for triplicate samples.

Entrapment efficiency

Ultracentrifugation method. The EE of the emulsion was determined by measuring the concentration of AZI in the aqueous layer obtained by ultracentrifugation (UC)[16]. Centrifugation was carried out using a HITACHI ultracentrifugation apparatus, operated at 50,000 rpm (~162,000 × *g*) at 4°C for 2 hours. Polyallomer tubes were used and their bottoms were pricked after centrifugation with a syringe needle to collect the aqueous phase. Concentrations of AZI in both the aqueous layer and the whole emulsion were determined by high-performance liquid chromatography (HPLC) and the drug EE was calculated.

Ultrafiltration method. Ultrafiltration (UF)[17] was performed using VIVASPIN 4 filters (VIVASCIENCE Ltd. Co., Germany) at 810 × *g* for 30 minutes, and these consisted of a filter membrane with a molecular weight cut-off of approximately 10,000 Da. The amount of AZI in the separated aqueous phase was measured by HPLC.

Stability assessment

A series of 10 mg/mL AZI emulsions were prepared by adjusting the pH with 0.1 mol/L HCl or NaOH to pH 4.0–8.5. Identical concentration AZI stock solutions of pH 4.0–8.5 were prepared by mixing AZI with various amounts of 0.025 mol/L buffer solutions (pH 4–6, acetate; pH 6–8.5, phosphate). The stability study was conducted at 80°C and performed in triplicate. All samples were collected at intervals of 0, 15, 30 minutes, 1, 2, 4, 8, 12, 24, and 48 hours for HPLC analysis. The observed degradation rate constants of AZI emulsions and AZI aqueous solutions of various pH values were determined by this method.

Pharmacokinetic studies of AZI lipid emulsion

The pharmacokinetic study performed had two objectives: (1) to compare and evaluate the AZI lipid emulsion (10 mg/mL) and AZI aqueous solutions (10 mg/mL) in vivo (2) to study the elimination characteristics of AZI lipid emulsion in the saccus conjunctiva by administering AZI lipid emulsion of different concentrations (3.0, 5.0, and 10.0 mg/mL) separately. Preparations (30 μL) were administered to the saccus conjunctivae of rabbits using a microsyringe. The tear fluid was collected from non-anesthetized rabbits with disposable 5 μL glass microcapillaries to use its siphon role to adopt tears at predetermined time points (predosing: 5 , 15 minutes, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 hours). No artificial tear secretion stimulation was performed. Immediately after collection, capillaries were evacuated into centrifuge tubes under a gentle stream of nitrogen.

Analytical method for AZI lipid emulsions

High-performance liquid chromatographic analysis

The AZI content of the lipid emulsions was examined and quantified using an HPLC system equipped with a Jasco PU-980 pump (Jasco Int. Co. Ltd., Tokyo, Japan), a Jasco UV-975 detector with a sensitivity of 0.005, and a

C8 analytical column (5 μm, 250 × 4.6 nm^2; KYA TECH Corporation, Tokyo, Japan). The column was eluted with a freshly prepared solution containing a mixture (15:47, v/v) of acetonitrile and potassium dihydrogen phosphate buffer (0.067 mol/L) at pH 4.0, which was adjusted with phosphoric acid. The flow rate was 1.0 mL/min; the column temperature was 35°C; the detection wavelength was 210 nm following the injections of 20 μL AZI standard solutions in isopropyl alcohol and the emulsion samples. AZI emulsions were dissolved in isopropyl alcohol (1:10) to dissolve the oil phase before determination. The regression equation of the calibration curve was $y = 123{,}246x - 9163.2$ ($r^2 = 0.9992$). In the calibration curve, the peak area of AZI was the ordinate and the content was the abscissa. The calibration curve for AZI was linear over the concentration range 0.5–4.0 mg/mL.

UPLC–MS analysis

Liquid chromatography was performed using an ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with an autosampler and a column oven enabling temperature control of the analytical column. An ACQUITY^{TM} UPLC BEH C_{18} column (1.7 μm, 50 × 2.1 mm^2; Waters Corp.) was used and the column temperature was maintained at 35°C. The standard and sample solutions were chromatographed on the ultra-performance liquid chromatography (UPLC) system using a gradient mobile phase consisting of acetonitrile as solvent A and 5 mmol ammonium acetate in water as solvent B. The gradient conditions of the mobile phase were 0 minute 40% A, 0.80 minute 80% A, 1.50 minutes 80% A, and 2.00 minutes 40% A. The flow rate was 0.2 mL/min. For all compounds, the mass spectrometry (MS) was operated in positive ion electrospray ionization mode with selected ion recording. Nitrogen and argon were used as cone and collision gases, respectively. The daughter ions m/z 591 for AZI and m/z 590 for clarithromycin were selected as detecting ions. The optimized ionization conditions were as follows: capillary 3.30 kV, cone 65 kV, source temperature 100°C, and desolvation temperature 400°C. Multipliers were set at 650 V and the dwell time for each transition was 0.2 second. The peak areas of the chromatograms were integrated and the peak area ratios of the analyte/I.S. were calculated. A weight $1/x^2$ linear regression was used to obtain a standard calibration curve: $y = 4.628 \times 10^{-2}x - 1.678 \times 10^{-1}$ ($r^2 = 0.9956$). The lower limit of quantification of AZI was 42.0 ng/mL. The regression equation of the calibration curve was then used to calculate the concentrations of the method validation samples and the unknown samples.

Samples underwent treatment to eliminate tear proteins prior to analysis. Each sample (5 μL) was placed in a centrifuge tube and spiked with 5 μL internal standard solution (0.12 μg/mL clarithromycin acetonitrile solution). After adding 0.5 mL acetonitrile, the tubes were votexed for 3 minutes and centrifuged at 13,000 × *g* for 10 minutes (Shanghai FULGOR Analytical Apparatus Ltd. Co., Shanghai, China). The supernatants were transferred to 700 μL glass vials, and aliquots of 5 μL were injected into the UPLC–MS system for analysis.

Results and discussion

Formulation of AZI lipid emulsions

Despite the similarity with parenteral lipid emulsions, topical ophthalmic lipid emulsions should be formulated with compatible vehicles and additives. The components of the internal and external phases of lipid emulsions should be chosen to produce enhanced solubility and/or stability of the incorporated ocular active drugs. In addition, another aim is to influence the ocular biodistribution or therapeutic index. In our study, the selection of emulsifying agents and their optimum concentrations were investigated.

The oil phase composition, which plays an important role in emulsion formulations, influences the physicochemical properties and the stability of lipid emulsions. The drug concentration of the AZI lipid emulsion was up to 10 mg/mL. Based on experimental data, MCT was an excellent solvent for AZI and 169.7 mg AZI could be dissolved in 1 g MCT when the content of soybean lecithin was not less than 1.2% (w/v) in the formulation. Additionally, the lipid emulsion containing MCT may provide more stable all-in-one admixtures[18] with relatively lower viscosities. Three concentrations of MCT (6.5%, 8.5%, and 10%) (w/v) were chosen to evaluate the EE of the corresponding formulations while maintaining the other components as shown in Formulation IV of Table 1. The drug EEs (determined by the ultrafiltration method) were about 65.0%, 91.0%, and 93.0%, respectively. The explanation for this phenomenon was that when we used 6.5% MCT, about 35.0% AZI in the lipid emulsions is released from the oil phase or small unilamellar liposomes (SUVs) and distributes into the aqueous phase. The lipid emulsion must remain in the low viscosity range, between 2 and 3 cP, since this is considered a suitable viscosity for ocular preparations[19]. Therefore, 8.5% (w/v) MCT was the optimal choice with the initial determined dynamic viscosity 2.85 cP. Since fatty oils are triglycerides, care must be taken to minimize or prevent their oxidation. α-Tocopherol is a good antioxidant and is used to obtain a suitable stabilized lipid emulsion under prolonged storage conditions. After thorough investigation of the stability of the AZI and the oil phase, we chose 0.05% α-tocopherol (w/v) for the AZI lipid emulsion.

Table 1. Effects of different quantities of various emulsifiers on the AZI lipid emulsion.

Formulation	Emulsifiers	Particle size (nm)	Zeta potential (mV)	Physical appearance
I	Soybean lecithin 1.2%, F-68 0.2%	154.7 ± 65.441	−2.56	Emulsion breaking
II	Soybean lecithin 1.8%, F-68 0.2%	150.3 ± 62.372	−6.98	Visible supernatant oil drops
III	Soybean lecithin 1.8%, F-68 0.4%	147.2 ± 55.112	−6.10	Visible supernatant oil drops
IV	Soybean lecithin 1.8%, F-68 0.4%, Tween-80 0.4%	132.8 ± 59.879	−4.19	Unstable after freeze-thawing test
V	Soybean lecithin 1.8%, F-68 0.4%, Tween-80 0.4%, EL-40 0.3%	110.4 ± 35.833	−3.32	Homogenous and stable
VI	Soybean lecithin 1.8%, F-68 0.4%, Tween-80 0.4%, EL-40 0.3%, stearylamine 0.3%	120.4 ± 42.833	26.02	Emulsion with positive charge

Formulations in the table contained (% w/v): AZI 1%, MCT 8.5%, α-tocopherol 0.05%, EDTA 0.05%, glycerin 2.2%, benzalkonium bromide 0.01%, and water for injection to 100%.

It is well known that emulsifiers are another essential component of lipid emulsions. In the case of the AZI lipid emulsion, lecithin was selected as the main emulsifier because of its biocompatibility. However, a single emulsifier of soybean lecithin is not sufficient to produce submicron-sized emulsion droplets or to maintain the stability of the emulsion. This is because the phospholipid monolayer interfacial film is not stable enough and only stable at an alkaline pH. Therefore, additional emulsifiers were included in the AZI lipid emulsion. In this work, the concentrations of each emulsifier used, including soybean lecithin, F-68, Tween-80, EL-40, and stearylamine, were investigated. Table 1 shows the effect of various surfactants. The physical stability of each emulsion was evaluated by measuring the particle size, zeta potential, and examining the physical appearance. As shown in the Table 1, 1.2% soybean lecithin was not able to adequately strengthen and uniformly disperse in the emulsifier interfacial film, so Formulation I could not produce a good emulsification effect and the emulsion broke down. When the quantity of soybean lecithin and F-68 was increased, Formulations II and III displayed a better physical stability because the higher concentration of soybean lecithin emulsified the oil phase and F-68 stabilized the lipid emulsion through strong steric repulsion[20]. However, that was still not enough. An even stronger effect was observed when Tween-80 was used as a co-emulsifier, and this was demonstrated by the fact that there were no visible supernatant oil drops present in Formulation IV with a suitable mean particle size of approximately 130 nm. This was because Tween-80 reduced the oil/water interfacial tension and the mean size of the oil droplets[17]. This proves that the incorporation of Tween-80 is important. Nevertheless, Formulation VI with a suitable mean particle size of approximately 130 nm was not stable enough for the freeze-thawing test. This result suggested that the formulation could not remain stable for a long enough period and oil drops were formed after a few months. Therefore, other emulsifiers with a better emulsifying effect or capable of adjusting the charge need to be added. The concrete effects of EL-40 and stearylamine used in AZI lipid emulsions are illustrated in the following discussion.

Eventually, the quantity of all surfactants was kept under 5% in order to avoid the risk of ocular intolerance. The final formulation contained (% w/v) AZI 1%, MCT 8.5%, soybean lecithin 1.8%, F-68 0.4%, Tween-80 0.4%, EL-40 0.3%, stearylamine 0.3%, α-tocopherol 0.05%, EDTA 0.05%, glycerin 2.2%, and benzalkonium bromide 0.003%.

Evaluation of physicochemical characteristics

Effect of freeze-thawing

Stability is an important concern for lipid emulsions. The small droplet size of emulsions confers stability against sedimentation (or creaming), because the Brownian motion and, consequently, the diffusion rate are higher than the sedimentation (or creaming) rate induced by gravity. Sedimentation and creaming of the emulsions during long-term storage may increase the size to a mean diameter of over 5 μm. For this reason, measurement of particle size is the most important parameter in emulsion stability studies, and this is normally tested using accelerated testing conditions[21], such as freezing-thaw cycles.

As shown in Figure 2, the droplet size of lipid emulsions before freeze-thawing test normally lies in the range 110–140 nm. The physical stability differences between five formulations were examined during the accelerated test. The appearance of the formulation without EL-40 was viscous and opaque without any opalescence after freeze–thaw testing. Coalescence was reflected in a particle size increase of the formulation without EL-40. The mean diameter after freeze-thawing increased significantly in comparison with the initial particle size. Droplets as large as 370 nm in diameter were present. Also, oily drops could be seen on the surface after a few days at

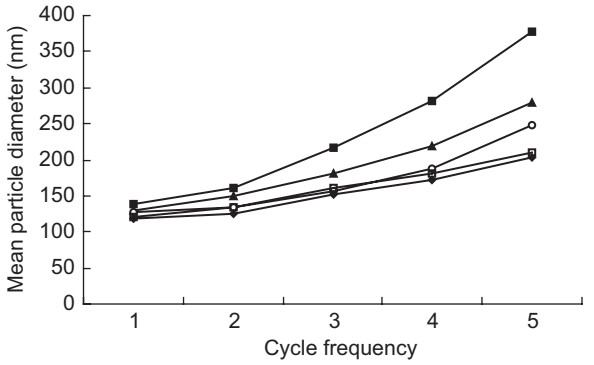

Figure 2. Change in particle size after the freeze-thawing of AZI lipid emulsions containing different amounts of EL-40 (other components are the same as in Formulation IV of Table 1 above): (■), 0% EL-40; (▲), 0.1% EL-40; (○), 0.2% EL-40; (□), 0.3% EL-40; (♦), 0.4% EL-40.

room temperature. It might be that freeze-thawing caused changes at the droplet interface boundary leading to a marked increase in particle size. The coalescence was slower in the formulations containing 0.1% or 0.2% EL-40, in which there was an increase in measured droplet size immediately, in spite of the satisfactory initial particle size. The process was slowest in emulsions prepared using 0.3% or 0.4% EL-40 where no evident destabilization occurred. The concentration of EL-40 above 0.3% proved to be suitable. The change in the mean particle size would be negligible in the freezing-thaw cycles.

Based on the results of formulations using 0.3% or 0.4% EL-40, it appeared that when using at least 0.3% EL-40, the AZI lipid emulsion exhibited a satisfactory stability. Its utility comes from its ability to alter the structure of the interfacial film and, finally, help maintain the stability of the lipid emulsion. However, the amount of EL-40 should be controlled because it has been associated with some adverse toxic effects, such as hypotension and bronchospasm[22]. Therefore, the amount of EL-40 was finally chosen as 0.3% (w/v), as shown in Formulation V of Table 1 above. The LD50 of EL-40 in mice with intravenous injection is more than 12.0 g/kg weight and 0.3% (w/v) EL-40 in the formulation should be secure.

Studies of zeta potential at various pH values and drug content

In this study, positive charges were introduced into the emulsion through the addition of stearylamine (0.3%, w/v). The positive surface potential of the droplets of the present emulsion depended mainly upon the extent of the ionization of stearylamine at the o/w interface. The previous submicron emulsions were based on lecithins that were a mixture of phospholipids of different composition combined with nonionic or anionic emulsifiers. These mixed emulsifiers conferred a negative charge on the emulsified oil droplets, resulting in a high negative zeta potential, which, in turn, prevented droplet coalescence upon random collisions. At present, only a few positively charged o/w emulsions have been developed using a sequential methodology to optimize the o/w emulsion and to obtain the highest positive zeta potential to keep the emulsions stable for a long period[23]. Since epithelial corneal cells exhibit negative charges on their surface, it has been hypothesized that a positively charged emulsion would interact with the corneal cells and exhibit better wettability properties on the cornea[24]. The toxicity of the cationic emulsion was also addressed[25]. However, the overall results[26,27] suggest that novel positively charged emulsions are suitable for ocular application.

The results (Figure 3) show that the phospholipid-stearylamine confers an overall positive charge on the emulsified droplets. It should be noted that the zeta potentials exhibit a tendency to increase under the same conditions by simply changing the drug concentration from 0.3% to 1.0% (w/v). In the case of the present formulation, this could be explained by the fact that adding the basic drug (AZI) contributed a positive charge to the interface, and this would increase the overall surface charge. With increasing AZI content, the ionized form of AZI might be sufficiently water soluble and pass into the aqueous phase at a low pH, thereby increasing the zeta potential to some degree. However, the zeta potentials at different pH values with identical drug concentrations indicate that the pH variation does not significantly affect the surface charge of the emulsions. The explanation for this phenomenon may be that the reduction in pH concomitantly led to a reduction in the ionization of anionic phospholipid components and to an increase in the degree of ionization of the drug and/or stearylamine.

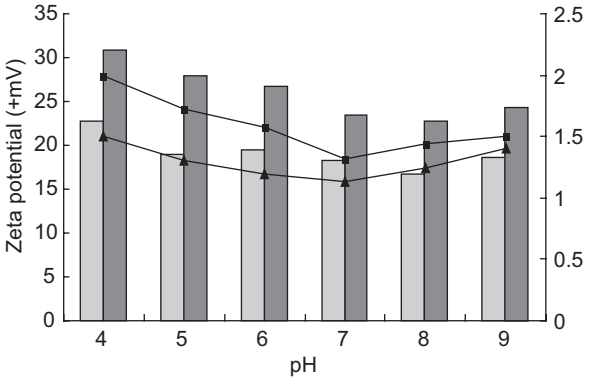

Figure 3. Influence of pH and drug concentration on zeta potentials of AZI lipid emulsions: (▭), 0.3% AZI lipid emulsion; (▬), 1.0% AZI lipid emulsion; (▲), 0.3% AZI lipid emulsion SD of zeta potential; (■), 1.0% AZI lipid emulsion SD of zeta potential.

Entrapment efficiency investigation by UC and UF

As shown in Table 2, a comparison of the drug distribution with increasing soybean lecithin concentration revealed that there was a significant difference in the EE of the two formulations. The EE was higher in the formulation with a higher concentration of the soybean lecithin. The EE of the emulsion with 1.5% (w/v) soybean lecithin by UC was nearly equal to that determined by ultrafiltration, but clearly different results were obtained for the emulsion with 1.8% (w/v) soybean lecithin when the two methods were compared.

The interfacial film is composed of various emulsifying agents and phospholipid plays an important role in maintaining its viscoelasticity. The main factor in increasing the stability of the lipid emulsion is the presence of an interfacial film in the liquid crystalline state, which can incorporate the drug with the oil phase. The AZI concentration of the lipid emulsion was as high as 1.0% (w/v). Not only the oil phase, but also the surfactant layer took up the drug a residence, allowing drug incorporation. The particle size was similar for the two formulations and 1.8% (w/v) soybean lecithin might offer a greater area of surfactant layer, which could provide more sites for the drug compared with 1.5% (w/v) soybean lecithin. For this reason, the EE was increased.

It has been reported that, apart from emulsified oil droplets in the submicron emulsion, SUVs might be formed on account of the excess lecithin, which could contain a considerable quantity of lipophilic drugs[28]. SUVs about 50 nm possessing lecithin bilayer structures have a lipophilic layer, which can dissolve lipophilic drugs like AZI shielding them from the aqueous solvent. Ultracentrifugation is based on different density materials having different sedimentation velocities. Owing to the similar density between SUVs and water, the vesicles containing AZI remain in the aqueous phase and are unable to pass into the upper oil or the oil/water interface layer strata even after a long period of centrifugation. Moreover, UC destroys the structure of lipid emulsions completely followed eventually by the oil phase, the oil/water interface layer, and the aqueous phase. During the course of UC, some drug distributed in the oil phase or oil/water interface layer may be diverted to the SUVs finally passing into the aqueous phase. Consequently, the concentration of AZI present in the aqueous phase was higher and the drug EE of the AZI lipid emulsion was lower, when determined by the UC method.

Table 2. Entrapment efficiency of AZI lipid emulsions determined by two different methods.

	Emulsion containing 1.5% (w/v) soybean lecithin	Emulsion containing 1.8% (w/v) soybean lecithin
EE (%) by UC	54.42 ± 1.06	74.17 ± 1.23
EE (%) by UF	59.14 ± 0.74	90.62 ± 0.61

UF has been applied to o/w emulsions[29]. UF is performed by means of a semipermeable membrane with a molecular weight cutoff of approximately 10,000 Da. Accordingly, SUVs are intercepted and do not pass into the aqueous phase. Because only free AZI could be detected in the aqueous phase, when determined by the ultrafiltration method, the drug EE of the AZI lipid emulsion was higher. Nevertheless, the formulation contained 1.5% soybean lecithin, which is less than the amount used, so few SUVs can be formed and no marked difference was observed between the two methods. The drug EE of the AZI lipid emulsion was a little higher by the ultrafiltration method, and this might be due to the adsorbing effect of the semipermeable membrane but was not significant. Therefore, we can conclude that the real drug EE of the AZI lipid emulsion was approximately 90% or slightly lower.

Effect of pH on AZI lipid emulsions and aqueous solutions

The relationship between the observed reaction rate constant in the aqueous phase (k_0) and the emulsion (k_0') can be described using the following first-order degradation kinetic equations:

$$\ln C_{aq} = -k_0 t + \ln C_{0(aq)}$$

$$\ln C = -k_0' t + \ln C_0$$

where $C_{0(aq)}$ and C_0 are the initial concentrations of AZI in the aqueous phase and emulsion, respectively. C_{aq} and C are the residual concentrations of AZI in the aqueous phase and emulsion, respectively. The emulsion and solution plots of ln$C$ (concentration) versus time were linear at all pH values indicating pseudo-first-order degradation kinetics. Degradation rate constants obtained from the slopes of the curves were used to determine the half-lives at different pH values (Table 3). Clear differences could be seen between the drug degradation in emulsion and in solution at the same pH. Table 3 shows that the half-life of AZI in the emulsion at 80°C with the maximum stability was about 5 days, but 1.5 days in aqueous solutions. The first-order rate

Table 3. Half-life ($t_{1/2}$) of 10 mg/mL AZI emulsion and buffers at different pH values at 80°C

pH	$t_{1/2}$ (hour) in buffered solution	pH	$t_{1/2}$ (hour) in AZI lipid emulsion
4.02	6.58 ± 0.51	4.01	92.01 ± 1.36
5.04	21.65 ± 0.63	5.03	121.09 ± 1.48
5.98	37.61 ± 1.74	6.01	121.83 ± 1.94
6.48	31.02 ± 1.08	6.57	120.36 ± 1.85
6.97	10.34 ± 0.84	7.03	100.30 ± 1.20
7.52	7.08 ± 0.41	7.50	77.16 ± 0.65
8.53	2.82 ± 0.23	8.49	71.65 ± 0.24

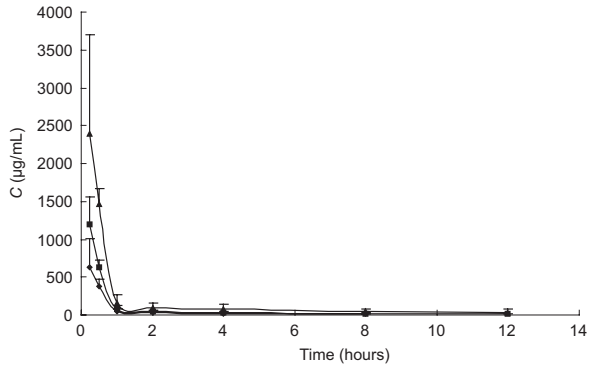

Figure 4. Mean tear fluid concentration-time curve after topical application of AZI lipid emulsion with different contents to rabbit eyes (mean ± SD; $n = 6$) at a dose of 30 μL: (♦), 0.3% AZI lipid emulsion; (■), 0.5% lipid emulsion; (▲), 1.0% AZI lipid emulsion.

constants for degradation of the AZI lipid emulsion at different pH values are plotted in Figure 4. It is clear that the relatively stable pH range of the AZI lipid emulsion extended from pH 5.0 to 7.0, and the chemical stability compared with aqueous solutions was improved significantly at the random pH. This phenomenon was attributed to the fact that biphasic systems, like submicron emulsions, may offer a protective environment for a drug sensitive to degradation in aqueous solutions. Since the lipid emulsion could incorporate AZI into the oil phase, the interfacial surface or the SUVs with proportional EE at different pH values, this reduced AZI hydrolysis by different degrees. In addition, at pH < 5.0, the extent of AZI ionization was increased with reduced EE. At pH > 7.0, lactone is liable to hydrolyze with ring cleavage. As a result, the stability of AZI decreased markedly at extreme pH values. On the whole, compared with aqueous solutions, the AZI lipid emulsion with a suitable pH value (5.0–7.0) displayed excellent chemical stability with only minor chemical degradation of AZI.

Elimination kinetics of AZI lipid emulsion in rabbit saccus conjunctiva

According to the characteristics of different drugs, the AZI lipid emulsion mainly treats superficial corneal and conjunctival bacterial infections. We examined the drug absorption by measuring the AZI concentration in tear fluid. In this experiment, the in vivo evaluation was made by determination of the concentration–time curve of AZI in tear fluid by means of UPLC–MS. Analysis was carried out using a pharmacokinetic program 3p87 and it was produced by the Mathematics Pharmacological Committee of the Chinese Academy of Pharmacology. The main pharmacokinetic parameters calculated by the statistical moment method are shown in Table 4. The biological half-life of drugs has nothing to do with the dose. Furthermore, the AUC_{0-t} was proportional to the increase in dose. Whenever the dose changed, the concentration of the drug at the corresponding time points exhibited a proportional change and this result is seen in Figure 4. Therefore, we were able to conclude that the elimination of the AZI lipid emulsions in tear fluid was consistent with the basic linear pharmacokinetic characteristics. From Figure 5, the curves of the two preparations were similar. The effect of the formulations on the various pharmacokinetic parameters was statistically compared using an independent *t*-test. Significance was assumed at the 0.05 level of probability and two preparations exhibited no statistically significant difference ($n = 6$, $P > 0.05$). For this reason, the lipid emulsion did not significantly change the pharmacokinetics of AZI in vivo. The AUC_{0-t} of the AZI lipid emulsion and aqueous solution drops was 1873.58 ± 156.87 and 1082.46 ± 179.06 μg h/mL, respectively. So, this shows that the lipid emulsions exhibited a 0.73-fold increase in AUC_{0-t} with the aqueous drops. These results could be attributable to the fact that the drug in aqueous preparations in an exposed state could easily be diluted by the tear fluid and quickly discharged from the lacrimal ducts. Furthermore, the viscosity of the solution was low, so the drug was able to be removed from the eyes with the blink reflex, resulting in drug loss. When the drug is incorporated into the core of lipid emulsions, this might reduce the penetration of drug into tissues, in conjunction with a higher tear fluid concentration. These results indicate that the AZI lipid emulsion might be retained in the precorneal area with a higher tear fluid concentration compared with ordinary drops, resulting in improved ocular bioavailability.

Table 4. Pharmacokinetic parameters after topical application of AZI lipid emulsion or aqueous drops ($n = 6$).

	AZI (0.3%) lipid emulsion	AZI (0.5%) lipid emulsion	AZI (1.0%) lipid emulsion	AZI (1.0%) aqueous drops
k (1/hour)	0.42 ± 0.21	0.35 ± 0.03	0.37 ± 0.07	0.36 ± 0.09
$t_{1/2}$ (hour)	2.12 ± 1.03	2.02 ± 0.19	1.96 ± 0.36	1.91 ± 0.44
MRT (hour)	3.06 ± 1.48	2.92 ± 0.27	2.83 ± 0.52	2.77 ± 0.64
AUC_{0-t} (μg hours/mL^{-1})	518.69 ± 382.49	972.06 ± 177.99	1873.58 ± 156.87	1082.46 ± 179.06

Each value represents the mean ± SD.

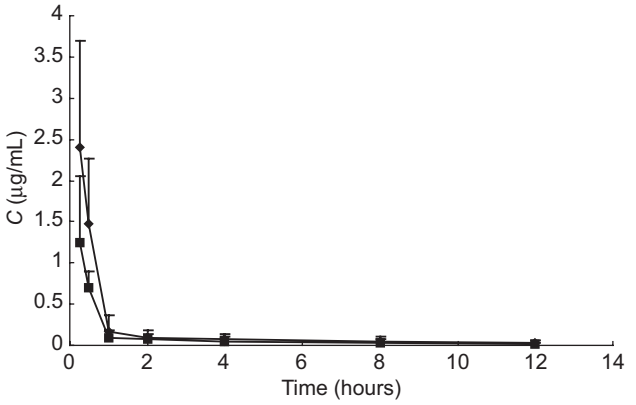

Figure 5. Mean tear fluid concentration-time curve after topical application of lipid emulsion and aqueous solution drops to rabbit eyes (mean ± SD; $n = 6$) at a dose of 30 μL: (♦), 1.0% AZI lipid emulsion; (▲), 1.0% AZI aqueous drops.

Conclusions

This study clearly describes a new formulation of AZI lipid emulsion for ocular administration, which consists of AZI 1%, MCT 8.5%, soybean lecithin 1.8%, F-68 0.4%, Tween-80 0.4%, EL-40 0.3%, stearylamine 0.3%, α-tocopherol 0.05%, EDTA 0.05%, glycerin 2.2%, and benzalkonium bromide 0.003%. The AZI lipid emulsion has a particle size of 124.5 ± 25.6 nm, a positive charge of 23.5 ± 5.5 mV and a drug EE of 90.62 ± 2.41%. The distribution behavior of AZI in the lipid emulsion at a stable pH (5.0–7.0) resulted in improved chemical stability compared with aqueous solutions. Although the pharmacokinetic profiles of the two preparations are similar, apart from the extension of the drug residence time in the conjunctiva, the AZI lipid emulsion also slowed drug release from the delivery system and reduced precorneal drug loss. Therefore, this increased the bioavailability of the drug. In conclusion, we believe that lipid emulsions are promising vehicles for ophthalmic drug delivery.

Declaration of interest: The authors report no conflicts of interest.

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