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Research Article

Preparation, Characterization, and *in vivo* Pharmacokinetics of Innovative Mixed Polymeric Nanomicelles Coated with Hyaluronic Acid as a Retinal Brimonidine-Carrier System

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Abstract

Background: Due to the limited ocular bioavailability, local ocular injections and systemic delivery were used instead of topical application. These alternative approaches were accompanied by numerous disadvantages. ***Objectives***: Create and test mixed polymeric nanomicelles coated with hyaluronic acid as a topical drug carrier for targeted delivery to the vitreous fluid and retina, overcoming the limitations of eye drops. ***Methods***: The thin-film hydration process was utilized to create nanomicelles, and their physical properties were studied. Colored, pigmented, healthy albino rabbits were employed *in vivo* experiment. Following anesthesia, 35µl of brimonidine nanomicelles and Alphagan® were administered topically. Samples from the vitreous and retina were collected for RP-HPLC analysis. ***Results***: The nanomicelles' physical qualities made them appropriate as a carrier system for the vitreous fluid. They were spherical with a clear appearance and a pharmacological concentration of 97.11%. The particle size range was 134.2nm, with a PDI of 0.2824. The drug entrapment effectiveness was 62.69%, whereas the surface tension was 39.18 mN/m. Their vitreous pharmacokinetics Cmax, Tmax, and AUC0-t were 28.1ng/µl, 2.0min, and 1268.6ng/µl*min, respectively, compared to 5.0ng/µl, 8.0min, and 129.32ng/µl*min for Alphagan eye drops. The retinal pharmacokinetics were 64.9ng/µl, 17.0min, and 18688.04ng/µl*min, respectively, compared to 31.6ng/µl, 30min, and 6377.67ng/µl*min with Alphagan eye drops. ***Conclusions***: Brimonidine nanomicelles coated with hyaluronic acid could be an effective topical drug-carrier method for delivering medications to the vitreous and retina, with higher bioavailability than eye drops.

Keywords: Brimonidine, Nanomicelle, Ocular bioavailability, Ophthalmic delivery, Retinal targeting.

تحضير وتوصيف والحركية الدوائية في الجسم الحي للنانوميسيلات البوليمرية المختلطة المبتكرة المغلفة بحمض الهيالورونيك كنظام ناقل للبريمونيدين في الشبكية

الخلاصة

الخلفية: يتم استخدام الحقن الموضعي للعين و الجهازي كبديل للاعطاء الموضعي وذلك بسبب انخفاض التوافر الحيوي للدواء في العين. و ارتبطت هذه الطرق البديلة بالعديد من العيوب. **الهدف**: تحضير وتقييم المذيلات النانونية البوليمرية المختلطة و المغلفة بحمض الهيالورونيك كحامل موضعي للدواء للسائل الزجاجي والشبكية. **الطرق**: تم استخدام طريقة ترطيب الغشاء الرقيق لإعداد المذيلات النانوية، وتم تحديد خصائصها الفيزيائية. تم استخدام الأرانب الصبغية الملونة و الخالية من أمراض العين والتشوهات للدراسة الحية. بعد التخدير، تم وضع 35 ميكرولتر من المذيلات النانوية للبريمونيدين وألفاجان® موضعيًا وتم أخذ عينات من السائل الزجاجي والشبكية وتحليلها باستخدام HPLC. **النتائج**: كانت الخصائص الفيزيائية للمذيلات النانوية المحضرة مناسبة كنظام حامل للسائل الزجاجي. كانت كروية الشكل وشفافة ومحتوى الدواء يساوي 97.11٪. كان نطاق حجم الجسيمات 134.2 نانومتر مع معامل تشتت قدره 0.2824. كانت النسبة المئوية لكفاءة حجز الدواء 62.69٪ ، بينما كان التوتر السطحي 39.18ملينيوتن/متر. وقد وجد أن حركية الدواء في الشبكية بما في ذلك Cmax وTmax وAUC0-t كانت (64.9نانوغرام/مايكرولتر و17 دقيقة و18688.04) نانوغرام/مايكرولتر*دقيقة على التوالي مقارنة بـ 31.6 نانوغرام/مايكرولتر و30 دقيقة و6377.67 نانوغرام/مايكرولتر*دقيقة لقطرة العين الفاجان®. **الاستنتاجات**: يمكن أن تكون المذيلات النانوية للبريمونيدين و المغطاة بحمض الهيالورونيك نظامًا مناسبًا لحمل الدواء الموضعي وتوصيل حمولتها الى الأجزاء الخلفية من العين، وخاصة الجسم الزجاجي والشبكية مع تحسين التوافر الحيوي للعين مقارنة بقطرة العين.

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INTRODUCTION

The way drugs move from topical ophthalmic preparations to the eye is through the cornea or non-corneal (conjunctival and scleral) pathways [1]. These pathways were linked to low (≤5%) ocular bioavailability [2] for both hydrophilic and hydrophobic drugs because of the barriers in the eye, especially for the cornea [3]. Corneal barriers include physiological barriers (tears, blinking, nasolacrimal drainage, and low residence time) and anatomical barriers (three cell layers: lipophilic epithelium, hydrophilic stroma, and

lipophilic endothelium) [4]. Therefore, we must use alternative routes for drugs to reach the posterior segments of the eye, such as the vitreous and the retina. These include systemic treatments, which may have adverse drug effects, and local ophthalmic injections, which are costly, invasive, and may involve patient noncompliance and infection risk. Additionally, these injections require specialist clinics and medical staff for administration [5]. Different delivery systems and dosage forms were available for ophthalmic preparations, including liquids (drops, suspension, and emulsion), semisolids (ointments and gels), solids (injectable powders, inserts, and contact lenses), mixed (in situ gel), and nanostructured carriers (liposomes, niosomes, nanomicelles, nanoemulsions, nanosuspensions, polymeric nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers, cubosomes, nanocrystals, dendrimers, bilosomes, olaminosomes) [6]. Therefore, researchers have focused on the latest drug carrier delivery systems, known as nano-drug carriers, primarily for topical preparations. These systems aim to surmount the barriers previously mentioned and enhance ocular bioavailability, particularly in the posterior eye segments [7]. Additionally, normal nanomicelles, primarily polymeric ones, can serve as an alternative hydrophobic drug delivery carrier system for the vitreous and retina. This is because of their small size, hydrophilic outer shell, and high physical stability, which stems from their low critical micelle concentration [8]. Amphiphilic molecules like soluplus (a grafted copolymer) and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGs) (natural surfactant) can be used to prepare mixed polymeric nanomicelles with superior properties owing to their biocompatibility, biodegradability, safety, and the solubilizing effect for hydrophobic drugs [9-12]. The pathways for their delivery to the vitreous fluid would be through the non-corneal aqueous pores of the sclera (about 350 nm in diameter) [13]. Adding hyaluronic acid as a coating molecule will have many benefits because it is biocompatible, biodegradable, and adheres to mucosa. It will also help target the retinal Cluster Determinant (CD44) cell surface receptors [14]. The FDA approved brimonidine, a selective -2 adrenergic agonist receptor, for glaucoma in 1966 (Alphagan® eye drop) to lower intraocular pressure. Furthermore, research has revealed that brimonidine exhibits a neuroprotective effect in the retina and optic nerve cells, potentially mitigating the development of complications that may require invasive treatments like surgery or laser procedures [15]. It is a quinoxaline (organic) hydrophobic molecule with a molecular weight of 292.135 g/mol. It has two pKa values, 7.78 and 8.32 (alkaline characteristics), and a polar surface area of 62.2 Å^2. Its water solubility was 0.154 mg/ml with log P equal to 1.37. The ocular half-life was 3 hours [16]. The goal of this study was to create and improve a way to apply brimonidine polymeric nanomicelles topically to the back parts of the eye (the vitreous and retina) more quickly than with regular eye drops.

METHODS

Materials

Brimonidine was purchased from Anshi Pharmaceutical Co., Ltd., China; Soluplus® from BASF SE, Germany; Tocophersolan from Henan Guange Biotechnology Co., Ltd., China; hyaluronic acid from Xi'an Sowu Biotech Co., Ltd., China; and Alphagan® 0.2% eye drops were purchased from the market. All other chemicals and reagents obtained are of analytical grade.

Nanomicelle preparation and In-vitro characterization

Soluplus (59.4 mg/ml), tocophersolan (2.64 mg/ml), and brimonidine (1.98 mg/ml) were mixed with methanol using the Premium Hotplate Stirrer (Witeg Labortechnik. GmbH) and heated and stirred at 50±0.5 ºC and 600 rpm. Then, the mixture was put in a Rotavapor® R-300a (Buchi, GmbH) and set to 50±0.5 ºC, 244 mbar, and 100 rpm to get rid of all the organic solvent and form a thin film. The thin film was left overnight for complete dryness, and then deionized water was used as the hydration solvent for nanomicelle preparation under heat and stirring (55±0.5 ºC, 600 rpm) [17]. Hyaluronic acid, 0.1%, was sprinkled over the formed nanomicelles at room temperature and stirred at high speed (1000 rpm) for 30 min. The hyaluronic acid-coated soluplus-tocophersolan mixed polymeric brimonidine (HST-BR) nanomicelles formula was put in the fridge (4±2ºC) for at least 24 hours before it was analyzed and characterized [18]. It was used to check the nanomicelles' physical properties, such as their shape, the amount of drug they contained after being filtered (0.22 µm Sterifil® Syringe Filter, Microlab, China), their particle size, the polydispersity index (PDI) (Malvern Zetasizer, UK), their surface tension (du Noüy ring method using Sigma 703D Attention-Force Tensiometers, Biolin Scientific, Gothenburg, Sweden) [20], and a field emission scanning electron microscope (FESEM) (Inspect F50, FEI Company, Netherlands) [21].

Animals and housing conditions

Pigmented-eye albino rabbits [22] weighing 1.1-1.7 kg of both sexes were housed and handled in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research [23], as well as the Research Ethics Committee for Experimental Investigation of the College of Pharmacy at the University of Baghdad, Iraq, under the protocol number RECAUBCP312024W. The rabbits were housed in individual cages for three days under controlled temperature and humidity, with a 12-hour light/dark cycle and free access to laboratory food and water [24].

The rabbits were anesthetized with xylazine + ketamine (10 mg + 50 mg/kg body weight) intraperitoneally [25] and then euthanized with an anesthetic overdose administered intramuscularly [26].

In vivo study

The animals were separated into four groups (three animals in each sample session), with two additional animals serving as controls (not treated). After anesthesia, a single dose of 35 µl of brimonidine nanomicelles was administered in the lower cul-de-sac of the rabbit's right eye. Group 1 vitreous (30 animals, 10 sampling time) and group 2 retina (36 animals, 12 sampling time) were analyzed, while Alphagan® 0.2% group 3 vitreous (24 animals, 8 sampling time) and group 4 retina (33 animals, 11 sampling time) were analyzed. At a predetermined time (2, 5, 10, 15, 30, 45, and 60 minutes, and 2, 4, 6, 12, 24, and 48 hours), vitreous samples were collected immediately under anesthesia for groups 1 and 3, and the eyeballs were removed and dissected immediately after scarification for groups 3 and 4, from which the retina was extracted. The investigation was conducted duplicate, with all materials collected and frozen until HPLC analysis [27,28]. The pharmacokinetic parameters Cmax, Tmax, and AUC_{0-t} were computed using the non-compartmental analytical tool PK-Solver [29].

HPLC chromatographic conditions

The chromatographic conditions were like those used by N. K. Karamanos, who used an RP-HPLC Sykam®, Germany, with a Nucleodor LC-18, 5 mm, 250 4.6 mm i.d., stainless steel (Germany), equipped with an RP-18 Gravity precolumn, 3 m particle size, 100 mm length, and 4 mm inner diameter, all from Nucleodor, Düren, Germany [30]. We prepared a standard stock solution of brimonidine (10 mg/100 ml) in the mobile phase of 10% (v/v) acetonitrile in 10 mM triethylamine buffer, pH 3.2, for the validation method at the brimonidine detection wavelength of 248 nm [30].

Validation of HPLC Method

To test chromatographic selectivity and distinguish between brimonidine and sample component peaks, 20 µl of brimonidine standard solution (25 ng/µl) was injected into the HPLC system along with placebo and control blank samples. To ensure linearity, aliquots of brimonidine standard stock solutions (ng/µl) were diluted with the mobile phase. 20 µl samples were then injected into the HPLC system for chromatogram detection and calibration curve building at 248 nm. Brimonidine solutions were injected into the HPLC system three times per day (intra-day) for three days in a row (inter-day) to determine the mean concentration, standard deviation (SD), and percentage of the relative standard deviation (%RSD). To ensure accuracy, a known brimonidine concentration was added to previously examined samples, and the HPLC's % recovery for the new samples was computed [30,31].

Statistical analysis

All value results represent the mean±SE for triplicate studies. ANOVA and t-test were used for statistical analysis [32].

RESULTS

Table 1 and Figure 1 illustrate the *in vitro* characterization of the physical properties of the prepared nanomicelles.

Table 1: *In-vitro* characterizations of the Brimonidine mixed-polymeric nanomicelles coated with hyaluronic acid

Physical characteristics	Results
Drug content (%)	97.11±0.02
Physical appearance	Transparent
Particle size (nm)	134.2±1.2
PDI	0.2824±0.0
EE (%)	62.69±0.01
Surface tension (mN/m)	39.18

Values are expressed as mean±SE. PDI: polydispersity index; EE: entrapment efficiency.

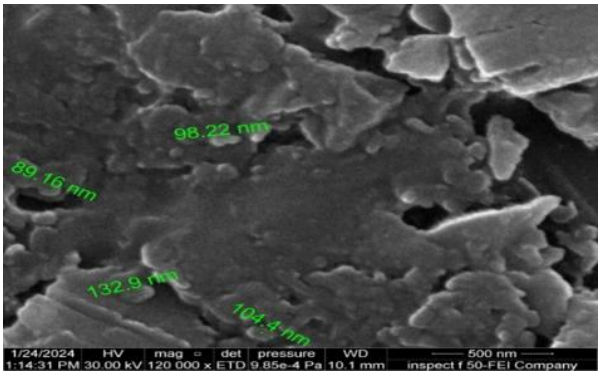

Figure 1: Scanning Electron Microscope image of the brimonidine mixed-polymeric nanomicelles coated with hyaluronic acid.

Figures 2 and 3 illustrate the specificity, the brimonidine indicative chromatogram, and the calibration curve, respectively.

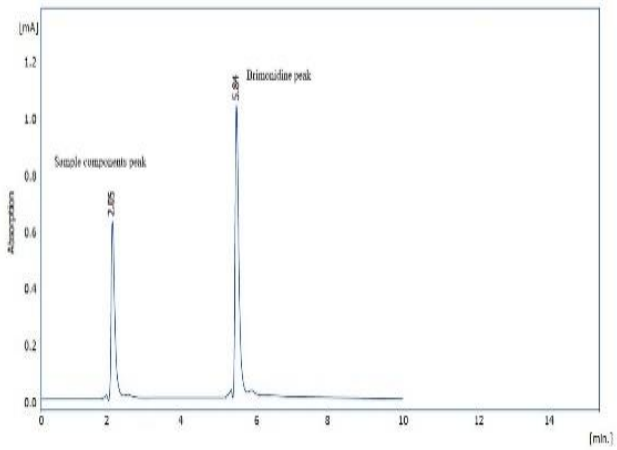

Figure 2: The specificity and the brimonidine indicative chromatogram

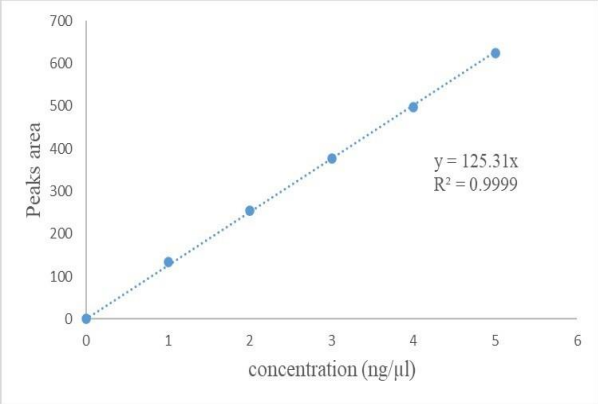

Figure 3: Calibration curve of the brimonidine peaks area versus concentration (ng/µl) in the mobile phase

Tables 2 and 3 display precision and accuracy. The limit of detection was found to be 0.11 ng/µl, while the limit of quantification was 0.35 ng/µl.

Table 2: HPLC-Precision method

Brimonidine (ng/µl)	Intra-day Precision		Inter-day Precision	
	Mean±SD	%RSD	Mean±SD	%RSD
5	625.36±0.48	0.08	625.10±0.14	0.02
15	1875.75±2.67	0.14	1875.52±1.83	0.10
25	3123.23±5.86	0.19	3121.75±4.87	0.16

Table 3: HPLC-Accuracy method

Brimonidine added to 5ng/µl sample	Total theoretical brimonidine (ng/µl)	Mean brimonidine (ng/µl) HPLC	Recovery (%)	RSD (%)
10	15	14.98±0.01	99.87	0.07
15	20	19.97±0.01	99.85	0.05
20	25	24.97±0.01	99.88	0.04

Values are expressed as mean±SD

The brimonidine concentration within the time was measured in the posterior segment of the eye in two compartments, the vitreous fluid and the retina, after a topical application of both the nanomicelles and Alphagan® 0.2%, as shown in Figures 4 and 5.

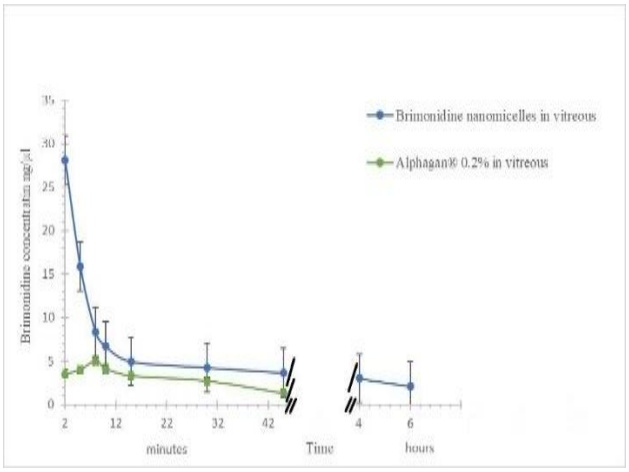

Figure 4: Brimonidine concentration (ng/µL) in the vitreous versus time measured after topical application of 35µl of brimonidine nanomicelles and Alphagan®.

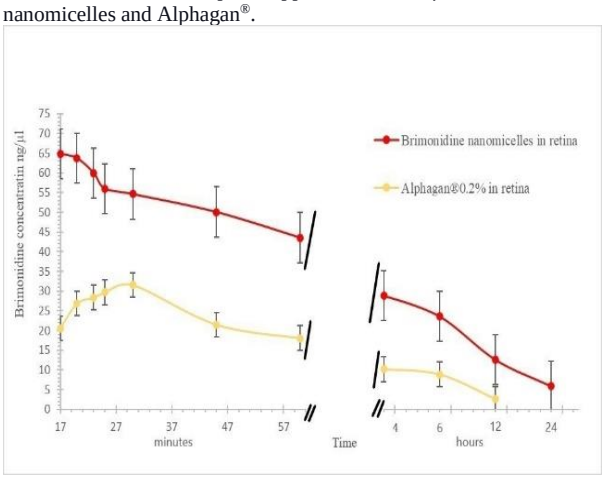

Figure 5: Brimonidine concentration (ng/µL) in the retina versus time measured after topical application of 35µl of both brimonidine nanomicelles and Alphagan®.

The most recent samples were taken when the brimonidine concentration was below the limit of detection for the HPLC method used. This happened after 12 and 48 hours for the nanomicelle in the vitreous and the retina, and after 4 and 24 hours for Alphagan® 0.2%. Table 4 illustrates the pharmacokinetic parameters.

Table 4: The C_{max}, T_{max}, and AUC_{0-t} in the vitreous and retina after topical application of 35µl of both brimonidine nanomicelles and Alphagan®

Pharmacokinetic parameters	Brimonidine Nanomicelles		Alphagan 0.2%	
	Vitreous	Retina	Vitreous	Retina
C_{max} (ng/µl)	28.12±3.6	64.9±2.5	5.0±0.49	31.6±4.0
T_{max} (min)	2±0.0	17±0.0	8±0.0	30±0.0
AUC_{0-t} (ng/µl*min)	1268.63±65.43	18688.04±520.4	129.32±3.2	6377.67±315.88

Values are expressed as mean±SE,

DISCUSSION

The physical properties of the prepared nanomicelles prepared were suitable for targeting the posterior segments of the eye. The drug content value for the filtered formula was within the accepted range (±15%). The particles' size and shape, along with their outer layer that attracts water, made it easy for them to pass through the non-corneal pathway, which is made up of 350 nm-wide scleral aqueous pores, to reach the vitreous [13]. The surface tension was a little lower than that of human tear film (≈43–46 mN/m) [33], which shows that the nanomicelles are compatible with tears and have the potential to increase the precorneal residence time. This is made more likely by the presence of hyaluronic acid coating molecules [34,35]. Although the entrapment efficiency (EE) percentage was not particularly high, the appearance was homogenous with an acceptable PDI.

This could be attributed to the drug's different solubilization positions within the nanomicelles due to its polarity and hydrophobicity. This suggests that the drug may be in the palisade layer of the nanomicelles, which is the layer between the initial carbons of the hydrophobic tail and the hydrophilic head, in addition to the hydrophobic core [36]. The validation of the results demonstrated the specificity, accuracy, and precision of the HPLC method, along with the chromatographic conditions used to analyze the brimonidine concentration in both the rabbit's vitreous and retina. The tested pharmacokinetic parameters showed that the nanomicelles had significantly higher brimonidine concentrations (p<0.001) than Alphagan® in both the vitreous and retina. They also had higher ocular bioavailability, as shown by the AUC_{0-t}, which was many folds higher (9.81 for the vitreous and 2.93 for the retina) than Alphagan®. This might be due to the physical properties of the nanomicelles [37] and the hyaluronic acid coating (hyaluronate), which targeted the posterior part of the eye [38].

Conclusion

The mixed soluplus-tocophersolan nanomicelles, which are coated with hyaluronic acid, work well as a carrier system for hydrophobic drugs. They make it easier for drugs to reach the vitreous and target retinal cells through the non-corneal pathway (scleral aqueous pores), which improves the bioavailability of the drug in the eye. Additionally, as a topical ophthalmic product, it may provide an alternative to systemic and local ophthalmic injections for drug delivery to the posterior segments of the eye.

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Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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