Journal of Chromatography A, 1519 (2017) 83–90

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Full length article

Quantitation of lanosterol in the vitreous humor of rabbits after ocular administration of lanosterol/thermogel formulation by ultra high performance liquid chromatography–tandem mass spectrometry with the electrospray ionization mode

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ARTICLE INFO

Article history:
Received 22 December 2016
Received in revised form 18 August 2017
Accepted 24 August 2017
Available online 1 September 2017

Keywords:
UPLC–ESI-MS/MS
Lanosterol/thermogel formulation
Vitreous humor
Quantitation

ABSTRACT

Cataracts are the most common cause of blindness worldwide affecting tens of millions of people. Here, we report a simple, rapid, sensitive and specific method by ultra performance liquid chromatography–tandem mass spectrometry with the electrospray ionization mode (UPLC–ESI-MS/MS) for quantitation of lanosterol, a possible effective drug for cataracts, in the vitreous humor of rabbits after ocular administration. The injected lanosterol was prepared by dispersing lanosterol molecules into the poly-(DL-lactic acid-*co*-glycolic acid)–poly(ethylene glycol)–poly-(DL-lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) thermogel solution. The analyte and internal standard (IS, panaxadiol) were extracted by the simple protein precipitation with methanol. The chromatographic separation used an Agilent RRHD SB-C_{18} column with a methanol mobile phase containing 50 mM of ammonium acetate aqueous solution (with 0.1% formic acid) (95:5, v/v). The protonated analyte was quantitated in positive ionization by multiple reaction monitoring (MRM) with a mass spectrometer. The mass transitions m/z 443.5 → 235 and m/z 461 → 127 were used to measure the analyte and IS, respectively. The assay exhibited a linear dynamic range of 1–1250 ng mL^{-1} for lanosterol in vitreous samples. The lower limit of quantitation (LLOQ) was 1 ng mL^{-1} with a relative standard deviation (RSD) of less than 15%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. A run time of 5 min per sample offered a throughput of more than 200 samples per day. This validated method was used to analyze vitreous samples of New Zealand white rabbits for pharmacokinetic studies. The results provided useful information on pharmacological action mechanism of lanosterol and were meaningful for cataract treatment among the elderly population.

1. Introduction

Cataracts cause lens opacity, visual impairment, and poor image quality. They are the most common cause of blindness worldwide affecting tens of millions of people [1]. Currently, the only treatment is surgical removal of cataractous lenses; the surgery costs cause great economic burdens as the elderly population continues to increase worldwide. Moreover, there is great chance of post surgery complications including posterior capsule rupture, nuclear lens material drop in the posterior segment, rhegmatogenous retinal detachment, etc. [2,3]. Therefore, there is a great need for alternative pharmaceutical treatment for this age-related disease.

Studies have shown that cataracts are mainly caused by crystallin aggregation [4]. Recently, the amphipathic molecule lanosterol was reported to reverse the accumulation of the protein inside the lens and thus alleviate cataract severity [5], which brings

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http://dx.doi.org/10.1016/j.chroma.2017.08.081
0021-9673/

hope for the discovery of effective drugs for cataract. The report showed effective results in opacified dog lenses *in vivo* using injections of lanosterol loaded in lipid-polymer hybrid nanoparticles to the vitreous body every 3 days for over 6 weeks combined with daily eye drops. However, these consecutive injections can cause conjunctiva and sclera scarring and endophthalmitis with poor patient compliance. Therefore, it is highly desirable to develop new ophthalmic formulations and drug delivery systems to decrease medication times, improve patient compliance, and sustain therapeutic effect.

Biodegradable hydrogels based on an amphiphilic triblock copolymer of poly- (DL-lactic acid-*co*-glycolic acid)–poly(ethylene glycol)–poly-(DL-lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) have gained increased attention as a carrier material (thermogel) for drug delivery [6–10]. Drugs can be incorporated into the thermogel solution at low temperatures, and the corresponding formulation can be injected into the *in vivo* target site. This then rapidly turns into a gel when contacting with physiological heat at 37 °C to act as a slow release drug depot [11,12]. Thermogels can also be used for sustained drug delivery after intra-vitreal injection to treat posterior segment disease of the eyes [9,13]. In this paper, lanosterol/thermogel formulations were prepared by dispersing lanosterol molecules into a thermogel solution. Then the lanosterol/thermogel formulation was injected into the vitreous cavity to investigate *in vivo* drug release.

The detection of lanosterol has been reported in the literatures [14–17]. For instance, a LC–MS/MS method has been developed to determine lanosterol and its major metabolite [14]. However, atmospheric pressure photoionization (APPI), which is less applied in routine analysis, must be used because steroids are lipophilic and do not exhibit moieties for typical protonation on electrospray ionization (ESI). The sensitivity is insufficient for *in vivo* analysis (LLOQ = 5 ng/mL). Another study used a LC–ESI-MS/MS method to analyze sterol profiles in human serum [17]. To improve the detection sensitivity of sterol on ESI, the sample was derivatized, but the pretreatment method is cumbersome versus the direct protein precipitation which is commonly used.

The vitreous is a transparent, colorless gel with a refractive index of 1.336 that contains salt, sugars, collagens, hyaluronan, opticin and a wide range of proteins. As an unconventional matrix that is hard to obtain, there are few studies and experience which can be referenced for determination of lanosterol in the vitreous humor. Moreover, the PLGA–PEG–PLGA thermogel which was used to deliver lanosterol might interact with lanosterol and some molecules in vitreous humor and effect the accurate measurement of lanosterol. Therefore, it is significative and challenging to detect lanosterol in the vitreous samples. Here, a simple, rapid and reliable UPLC–ESI-MS/MS assay for the quantification of lanosterol in the vitreous humor of rabbits was developed to explore the *in vivo* behavior after ocular administration of lanosterol/thermogel formulations, which can provide helpful information for the clinical application and explanation of pharmacological action mechanism of lanosterol.

2. Experimental

2.1. Chemicals and reagents

Lanosterol (L5768, purity > 98%) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA), and panaxadiol (as internal standard, IS, purity > 98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile, methanol and formic acid were HPLC-grade reagents from Merck Company (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Xylazine hydrochloride was obtained from Jilin Province TAT Animal Pharmaceutical Co. Ltd. (Jilin, China). Diazepam injection was procured from Shanghai Xudong Haipu Pharmaceutical Co. Ltd. (Shanghai, China). Ofloxacin eye ointment was from Shenyang Sinqi Pharmaceutical Co. Ltd. (Shenyang, China). Oxybuprocaine hydrochloride eye drops were purchased from Santen Pharmaceutical Co. Ltd. (Shanghai, China), and phosphate-buffered saline was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The other reagents were of analytical grade and obtained through commercial sources. Chemical structures for lanosterol and IS are shown in Fig. 1.

2.2. Preparation of lanosterol/thermogel formulations

Triblock copolymer of $PLGA_{1700}$–PEG_{1500}–$PLGA_{1700}$ was synthesized and characterized as described previously [18]. The PLGA–PEG–PLGA copolymer (1 g) was dissolved in 3 g of NS to form 25% (w/w) thermogel solution. Then lanosterol (1.6 mg) was added to 4 g of the above thermogel solution and magnetically stirred at 600 rpm and 4 °C for 3 d to form a homogeneous disperse solution to get the lanosterol/thermogel formulations. This thermogel with lanosterol behaves as a liquid (easy to load drug and be injected) below its low critical solution temperature (LCST) whereas it forms hydrogel (delivery and release the loaded drug) when the environmental temperature reaches or exceeds the LCST.

2.3. Animals and study protocol

New Zealand white rabbits with no ocular damage and weighing 2.0–2.5 kg were obtained from the Yin'gen Rabbit House (Shanghai, China). The rabbits were housed under standard conditions (Temperature 25 °C, relative humidity 50%, respectively) in the animal facilities of the EENT Hospital (Fudan University) with free access to food and water. Twelve New Zealand rabbits were divided into two groups (blank group and lanosterol group, six rabbits per group) to carry out the implantation procedures. All experimental protocols, including experiments, transportation, and care of the animals complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Care and Use Committee of Fudan University (Shanghai, China). In this study, mature male New Zealand rabbits were used.

Before implantation, the thermogel solution was sterilized with 0.22-μm filter at low temperature. After general anesthesia with xylazine hydrochloride (40 mg kg^{-1} body weight) and diazepam (1 mg kg^{-1} body weight) and topical anesthesia (0.5% of oxybuprocaine hydrochloride), lanosterol/thermogel formulations (100 μL, 400 μg g^{-1}) were carefully injected into the vitreous cavity in the right eye of lanosterol group using a 28-gauge needle. Ofloxacin eye ointment was applied to the right eye after surgeries to reduce inflammation reaction. All surgeries were performed by one experienced operator. A schematic diagram of the implantation method of lanosterol/thermogel formulations is shown in Fig. 2.

Over 42 days, aliquots (50 μL) of vitreous body solution were withdrawn and collected in test tubes at specific time intervals (1, 4, 7, 14, 21, 28, 35 and 42 d) by inserting a 26-G needle into the vitreous cavity. All samples were stored under −80 °C until measurement by UPLC–MS/MS.

2.4. Equipment and LC–MS/MS conditions

The levels of lanosterol in the vitreous humor were measured by a simple and sensitive UPLC–ESI-MS/MS method. Chromatographic analysis was performed on an Agilent 1290 infinity UPLC system consisting of a binary pump, a surveyor auto-sampling system, and a thermostated column compartment. Chromatographic