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Antifibrotic Drug Delivery for Glaucoma Treatment – Determination of Josamycin in vivo

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Abstract: Antifibrotic treatment during glaucoma surgery leads to an improved outcome for patients. Drug delivery systems for placement under the subconjuctiva after surgery made of cyclodextrins and crosslinked with Epichlorohydin were loaded with Josamycin and tested in vivo. After subconjunctival application in three New Zealand White rabbits, blood samples were taken by the means of Mitra devices at defined time points. Determination of blood levels of Josamycin was performed using liquid chromatography mass spectrometry. Detection of Josamycin was achieved during 48 h suggesting a sustained release over the course of this study.

Keywords: Drug delivery, antifibrotic, glaucoma surgery, mass spectrometry, in vivo application

1 Introduction

1.1 Glaucoma Treatment

Glaucoma is one of the main causes of blindness worldwide with a global prevalence for open-angle glaucoma of 2% [1]. As the intraocular pressure is the main risk factor for the glaucomatous optic nerve atrophy, reduction of intraocular pressure represents the standard practice of care by means of drugs or surgery with or without intraocular stents.

The gold standard of glaucoma surgery is the so-called trabeculectomy. During this surgery an artificial outflow is formed from the anterior chamber into the subconjunctival space to obtain the drainage of aqueous humour. Fibrosis, as a main complication, may occur later on and lead to a closure of that outflow channel. For prevention of fibrotic events usually antifibrotic drugs, such as mitomycin C or 5-Fluorouracil are applied.

1.2 Josamycin – a Formulation of an Antifibrotic Drug?

Josamycin (JM) is a 16-membered ring macrolide that has been used as an antibiotic since the 1970s. Stahnke et al. found that Josamycin also exhibits antifibrotic effects on tenon fibroblasts by suppressing proliferation and fibrosis [2,3]. We decided to test JM as a drug for trabeculectomy together with the development of a drug delivery system (DDS) do ensure a sustained release. To formulate JM in such a manner, we used γ-cyclodextrine (CD). This cyclic carbohydrate is known to bind to JM by forming a molecular binding pocket. CDs were crosslinked by Epichlorohydrin (EPI) to form gel like DDS that were loaded with JM. To answer the question, if these DDS are able to show a sustained drug release in vivo, they were applied subconjunctivally during a trabeculectomy in New Zealand White rabbits. Blood samples were taken at defined time points for determination of blood levels of JM by the means of liquid chromatography mass spectrometry (LCMS), to record the first data on JM, delivered subconjunctivally by a DDS.

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2 Materials and Methods

2.1 Materials

Josamycin (MW: 827.99 g/mol) was purchased from Chemodex (Switzerland). γ-Cyclodextrin was purchased from Wacker Chemie AG (MW: 1297.12 g/mol, 98%, Germany). Epichlorohydrin (EPI), was purchased from Sigma-Aldrich (Germany). Acetonitril, water, methanol and ethanol (all LCMS grade) were purchased from Carl Roth (Germany). Formic acid (LCMS grade) was bought from Fisher Chemicals, Germany. Rabbit Blood was purchased from Innovative Research (USA). MITRA microsampling devices (see Figure 1) were purchased from Neoteryx (Australia). Centrifuge filters with a MWCO of 10000 Da were purchased from Merck Millipore (Germany).

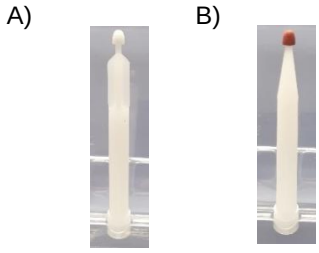

Figure 1: Mitra Device A) prior and B) after sampling,

2.2 Preparation of DDS and in vivo application

Josamycin loaded CD hydrogels were manufactured as described elsewhere [5]. As therein described, CD was crosslinked with EPI. Resulting CD hydrogels were washed twice with DI water at 37°C for 1 h and overnight. A drying step overnight at room temperature followed. Hydrogels were sterilized by the means of γ-radiation and loaded by swelling in a JM solution (c = 13 mg/mL) for 24 h to a final concentration of 110 µg per DDS. The resulting DDS were implanted subconjunctivally during surgery into three New Zeeland White rabbits (Granja San Bernardo (Spain)) at AnaPath Research S.A.U. (Barcelona) with one DDS per animal. Rabbits were 10 to 11 weeks old and approximately 2-2.5 kg in weight. Duplicate capillary blood samples (10 µL) were obtained by direct puncture into the ear using a lancet and collected using MITRA sticks at time points: 0, 2, 4, 6, 8, 24, and 48h. For each time point samples were taken from n=3 animals, except for 24 h where samples were taken from n=2 animals.

Sticks were allowed to dry on air and were stored prior to processing at -20°C.

2.3 Preparation of Calibration samples

160 µL of Rabbit Blood was spiked with JM dissolved in ethanol to final concentrations of 1, 2.5, 5, 10, 25 and 50 ng/mL for loading onto MITRA tips. To load the MITRA tip, it was gently touched to the surface of the blood, ensuring that the tip was not fully submerged. Blood was allowed to permeate through the entire tip until visibly saturated (completely stained, see Figure 1 B)). They were allowed to dry in air and stored at -20°C.

2.4 Sample processing

Each MITRA tip was rehydrated with 40 µL water for 20 min at 37 °C followed by the addition of 160 µL methanol for 15 min at 37 °C. After centrifugation at 13000 rpm for 15 min the supernatant was collected. The extraction was repeated once with 200 µL of methanol. The supernatants were combined and filtered through a centrifuge filter. The filter was washed twice with 100 µL methanol each. The resulting solution was lyophilised overnight and resuspended in 60 µL acetonitrile with 0.1 % formic acid and transferred to the liquid chromatography mass spectrometer for measurements.

2.5 LCMS determination of JM

A Shimadzu LCMS 8030 plus Triple Quad mass spectrometer equipped with a Nexera UHPLC with a kinetex 2.6 µm C18 100 A column, 150 x 2.1 mm (Phenomenex, Germany), and a C18 guard column was used for quantification. LCMS was performed with a gradient method with water/0.1% formic acid as mobile phase A and acetonitrile/0.1% formic acid as mobile phase B at a flow rate of 0.3mL/min.

Three SRM (selected ion monitoring) transitions 828.3>109.1 Da; 828.3>174.1 Da; 828.3>83.1 Da were measured. The transition 828.3>109.1 Da was used for quantification, the two other transitions were used for qualification. Measurements were performed twice for each sample.

3 Results and Discussion

3.1 Calibration

The calibration graph (see Figure 2) was generated from the measurements of the calibration samples. It shows a good linearity in the range of 1 to 50 ng/ml with a coefficient of determination of $R^2 = 0.9606$.

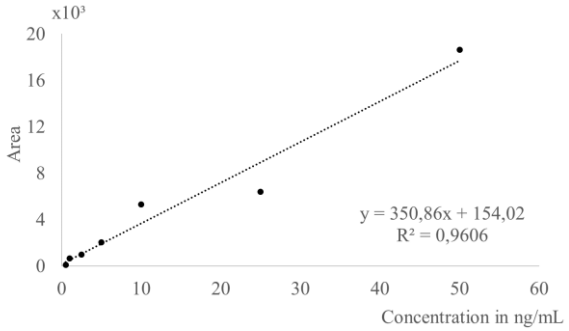

Figure 2: Calibration showing a good linearity in the range of 1 to 50 ng/ml with a coefficient of determination of $R^2 = 0,9606$

3.2 Determination of in vivo blood concentrations of JM

For overall 10 samples at time points of 0, 2, 4, 6, 8h the signal to noise ratio (S/N) was higher than 10, thus being suitable for quantification of JM [4], as shown in Figure 3. For 4 samples at 2, 4, 8 and 48 h the S/N ratio was between 3 and 10 [4], indicating the detection of JM, but not the quantification in blood.

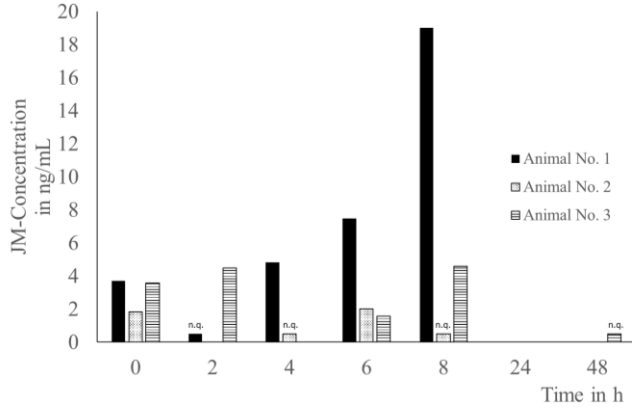

Figure 3: Blood levels of JM from three different animals at defined time points. The mark n.q. (= not quantified) indicates that a signal for JM was detected in the measurement, but it was not suitable for quantification. Absent columns indicate measurements with S/N<3 for JM.

Blood concentration of JM for animal 1 peaked at 8 h, concentrations are given in table 1, whereas no prominent peak concentration was detectable for animal 2 and 3 during the 48 h after DDS implantation. To the best of our knowledge, this is the first study where JM was applied via a DDS made of CD. Since drug release in vivo remains a multifactorial process, and interindividual variance as reported here is often observed in preclinical studies, further experiments are required to investigate potential reasons for differences between animals in terms of drug release.

Table 1: Blood levels of JM from three different animals at time points of 0, 2, 4, 6 and 8h in ng/mL. The term n.q. (= not quantified) indicates that a signal for JM was detected in the measurement, but it was not suitable for quantification. The term „n.d." (= not detected) indicates measurements with S/N<3 for JM.

Animal	0h [ng/mL]	2h [ng/mL]	4h [ng/mL]	6h [ng/mL	8h [ng/mL]
No. 1	3.7	n.q.	4,8	7.5	19,0
No. 2	1.7	n.d.	n.q.	2.0	n.q.
No. 3	3.6	4.49	n.d.	1.6	4.6

4 Conclusion

In this study, it was shown, that JM could be delivered in vivo by a DDS made of CD. It was detected over 48 h, indicating a sustained release from the tested DDS. Moreover, the feasibility of determining blood concentrations of JM using MITRA devices for sampling with subsequent processing and determination by LCMS was shown. With the MITRA devices sequential in vivo sampling can be achieved because of the minimal blood loss enabling the decrease of the number of animals needed, even for highly stacked time points However, some measurements indicate concentrations too small to reach S/N ratios higher than 10. Moreover, signals with S/N-ratios only slightly less than 3 were observed at two samples thus strongly suggesting presence of JM. Analyses using decreased dilutions during sample processing should lead to improved S/N-ratios and thus more quantifiable results. Additionally increasing the number of test animals will also lead to more insights in drug delivery of JM applied by DDS made from CD as shown here.

Author Statement

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