Quantification method for timolol from *in vivo* samples for the development of a new glaucoma drug depot

Abstract: Glaucoma is the second most common cause of blindness. An increased intraocular pressure is the only treatable symptom of glaucoma. Because patients often exhibit a poor therapy adherence, a drug depot consisting of ELA-NCO and hyaluronic acid with timolol was developed to ensure sustained drug release. This drug depot is formed by *in situ* polymerisation after injection into the subconjunctival space.

To test the *in vivo* drug release of timolol in serum and aqueous humour, a liquid chromatography mass spectrometry (LCMS) method was developed and tested using spike- and recovery experiments, and on *in vivo* samples after topical application.

Samples of serum and aqueous humour were taken from New Zealand White rabbits. For topical application, a commercially available formulation of timolol was used. This study presents results concerning the recovery of timolol from spiked samples. Serum and aqueous humour samples were spiked with timolol maleate to a final concentration of 50 ng/mL. Subsequently, the samples were extracted and analysed by LCMS.

External calibration of the developed method showed high linearity. Recovery experiments showed no loss of timolol. Hence, the extraction method is robust and able to recover the whole amount of timolol from aqueous humour and serum.

Keywords: glaucoma, liquid chromatography mass spectrometry, drug delivery, timolol, *in situ* polymerization

1 Introduction

Glaucoma is the second most common cause of blindness with 1-2% affected people in Germany. Ten percent of them will develop blindness or are already blind [1]. Elevated intraocular pressure is the only treatable risk factor associated with primary open angle glaucoma, the most common subtype of the disease. The standard medical treatment is the application of eye drops containing drugs like latanoprost, a prostaglandin analogue, or β-blocking substances like timolol, which decrease the intraocular pressure. Owing to the fact that the illness promotes slowly and without pain, patients often exhibit a poor therapy adherence. Reasons are diverse with difficult application of eyedrops or poor discipline in drug acquisition being very common [2]. Failure of proper administration of eye drops leads to disease progression.

To solve this problem, a glaucoma drug depot consisting of a biodegradable polymer consisting of ELA-NCO and hyaluronic acid [3,4] with timolol maleate as incorporated drug was developed. Via application into the subconjunctival space, a sustained drug release over at least 6 months should be achieved. This would make a topical application obsolete. To test the *in vivo* drug release in serum and aqueous humour, a liquid chromatography mass spectrometry method was developed and tested by spike- and recovery experiments, and on *in vivo* samples after topical application.
2 Materials and methods

2.1 Chemicals

Acetonitril LCMS-grade, methanol (MeOH) HPLC-grade and water LCMS-grade were purchased from Carl Roth, Germany. Formic acid was bought from Fisher Chemicals, Germany. Timolol maleate was purchased from Biomol Germany.

Samples of serum and aqueous humour for recovery experiments were taken from New Zealand White rabbits.

2.2 Sample preparation

For spike and recovery experiments, serum and aqueous humour samples were spiked with timolol maleate to a final concentration of 50 ng/mL.

Afterwards, the samples were treated as follows: 50 µL sample were mixed with 50 µL MeOH. After leaving the samples on ice for 30 minutes, samples were centrifuged with 12000 rpm at room temperature for 30 minutes. The supernatant was withdrawn and the volume was determined. The liquid was pipetted into a HPLC vial and stored at 4°C until LCMS measurements.

2.3 Liquid chromatography mass spectrometry

A Shimadzu LCMS 8030 plus Triple Quad mass spectrometer equipped with a UHPLC Nexera 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. Liquid chromatography was performed with an isocratic method with water/0.1% formic acid: acetonitril/0.1% formic acid (80:20; v:v). Three SRM (selected ion monitoring) transitions 317>261 Da; 317>74 Da; 317>244 Da were measured. The transition 317>261 Da was used for quantification, the two other transitions were used for qualification. External calibration was performed between 0.5 ng/mL and 5000 ng/mL. Measurements were performed twice for each sample.

3 Results

The external calibration was performed from 0.5 ng/mL to 5000 ng/mL and showed excellent linearity (Fig. 1).

![Figure 1: Linearity of the timolol quantification method from 0.5 ng/mL to 5000 ng/mL. Samples were measured in duplicates. The inset shows results for the four lowest concentrations. The coefficient of determination is shown in the upper left corner.](image)

The stability of the drug was tested for 7 days at 15°C and measurements showed no loss of intensity during that time.

After sample preparation of control and spiked samples LCMS measurements were performed. In the control samples no signals were detected for any of the three transitions. In contrast, spiked samples show signals for timolol (Fig. 2).

The analysis of the measured concentration was in good accordance to the prepared concentration (Table 1).

![Figure 2: Chromatograms of the three specific SRM-transitions depicted in the upper left corner of timolol in spiked and control samples taken from the same animal. The colors correspond to the chromatograms. The upper three chromatograms (black, pink, blue) show the detection of timolol at a retention time of 2.30 min in the spiked samples. In the lower three chromatograms (brown, green, dark blue) no signal was observed (control samples).](image)
Table 1: Recovery of timolol from spiked in vivo samples with a resulting concentration of 50 ng/mL. Measured values after sample preparation and extraction are shown.

<table>
<thead>
<tr>
<th>Spiked Timolol content [ng/mL]</th>
<th>Recovery Serum [ng/mL]</th>
<th>Recovery Aqueous Humour [ng/mL]</th>
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<tbody>
<tr>
<td>50</td>
<td>50.75</td>
<td>51.20</td>
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</table>

4 Discussion

The LCMS method developed in this study is suitable to determine the concentration of timolol in a range from 0.5-5000 ng/mL. The extraction method presented here is robust and able to recover the whole amount of timolol from aqueous humour and serum, respectively. Hence, recovery experiments showed no loss of timolol and a good recovery for a concentration of 50 ng/mL. Serum protein binding or degradation processes of timolol could not be observed.

Author Statement

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References

[2] Frech, S., Kreft D., Guthoff, RF., Doblhammer, R., Pharmacoepidemiological assessment and influencing co- factors among primary open-angle glaucoma patients – An observational cohort study, PLOS ONE 2018; 13(1) DOI: 10.1371/journal.pone.0191185