Confocal Raman spectroscopy

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Confocal Raman spectroscopy: Evaluation of a non-invasive technique for the detection of topically applied ketorolac tromethamine *in vitro* and *in vivo*

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ABSTRACT

Current information about the pharmacokinetics of an ocular drug can only be achieved by invasive sampling. However, confocal Raman spectroscopy bears the potential to quantify drug concentrations non-invasively. In this project, we evaluated the detection and quantification of ocular ketorolac tromethamine levels with confocal Raman spectroscopy after topical administration.

Confocal Raman spectroscopy and high-performance liquid chromatography (HPLC) were compared in terms of sensitivity of detection. Enucleated pig eyes were treated with different concentrations of ketorolac. Hereafter, ketorolac concentrations in the aqueous humor of pig eyes were analyzed by confocal Raman spectroscopy and HPLC.

Subsequently, twelve rabbits were treated with Acular[™] for four weeks. At several time points, ketorolac concentrations in aqueous humor of the rabbits were measured by confocal Raman spectroscopy followed by drawing an aqueous humor sample for HPLC analysis.

In ketorolac treated pig eyes, both *ex vivo* Raman spectroscopy as well as HPLC were able to detect ketorolac in a broad concentration range. However, *in vivo* confocal Raman spectroscopy in rabbits was unable to detect ketorolac in contrast to HPLC.

To conclude, confocal Raman spectroscopy has the capacity to detect ketorolac tromethamine *in vitro*, but currently lacks sensitivity for *in vivo* detection.

1. Introduction

Ocular pharmacokinetic studies investigate time- and dose dependent behavior of ophthalmic drugs. These studies are important to detect the maximum drug concentration (C_{max}), the time to reach C_{max} (T_{max}), half-life, and clearance of the drug. Based on those parameters, a dosage regimen can be created (DeVane and Jusko, 1982). Evaluation of a pharmacokinetic profile should include assessment of systemic exposure (i.e., blood, plasma or serum levels) as well the distribution

and levels in ocular tissues (e.g., cornea, iris, aqueous humor). Currently, the assessment of ocular pharmacokinetics is using tissues or fluids in a destructive test which comprises chemical pre-treatment followed by high-performance liquid chromatography (HPLC) (Buckley and Ryder, 2017). Besides extensive processing time, also sampling has been a challenge as ocular tissues or fluids cannot be harvested without interfering with the anatomical integrity of the eye (e.g., during intraocular surgery). Due to the limited accessibility of samples from humans, and the destructiveness of the method, pharmacokinetic

Abbreviations: BAK, Benzalkonium chloride; BSS, Buffered saline solution; f80, collimation lens with a focus length of 80 mm; CCD, cooled charge-coupled device; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; IR, infrared; f60, lens with a focus length of 60 mm; C_{max} , maximum drug concentration; NIR, near-infrared; NIRS, near-infrared spectroscopy; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate buffered saline; T_{max} , time to reach the maximum drug concentration

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research is relying on animal experiments using rabbits, dogs, pigs, or monkeys (Schoenwald, 1990), because the eyes of these animals show similarities to human eyes (Kompella et al., 2010). However, animal experiments have been widely criticized for both, ethical and economical reasons (Hornof et al., 2005).

A non-invasive pharmacokinetic assessment technique could resolve these issues. A technique that is potentially suitable for non-invasive detection of ocular pharmacokinetics is Raman spectroscopy. Raman spectroscopy identifies molecules, based on the specific inelastic scattering properties of their rotational and vibrational modes (Raman and Krishnan, 1928; Bauer, 1999; Erckens, 2001). This technique enables real-time detection of molecules without pre-processing and damaging tissue. As such the number of animals and its associated costs needed for ocular pharmacokinetic studies can be reduced, once a calibration model has been acquired. Compared to infrared (IR) spectroscopy, Raman spectroscopy bears the advantage that molecules do not have to possess a permanent dipole moment and therefore more molecules can be detected. Furthermore, Raman spectroscopy is not affected by aqueous samples, whereas IR is absorbed intensively by water. Near-infrared (NIR) spectroscopy (NIRS) is, similar to Raman spectroscopy, not affected by aqueous samples. However, absorption bands tend to overlap in NIRS, which results in less accessible molecule-specific information, providing a lower specificity compared to Raman spectroscopy (Gowen et al., 2015).

Since Raman spectroscopy is a scattering technique, fiber-optics and remote sampling can be used. Samples can be measured directly in a glass container or in case of pharmaceuticals, samples can be measured in original sachets. Because of a high spatial resolution, components from complex samples can be identified (e.g., cell-media components from a commercial recombinant-protein manufacturing process) (Li et al., 2010). However, due to the weak nature of Raman scattering (1 in 10^9 or 10^{10} (Long, 2004)), a higher sensitivity of Raman spectroscopy requires higher power lasers as excitation source. Therefore, Raman spectroscopy is very suitable for chemical applications; however, the photo-thermal effects in light-tissue interaction could cause concerns *in vivo* if the wrong wavelength is selected as emitting source. If the local temperature reaches up to 43.0°C hyperthermia will occur. A temperature up to 70°C could lead to tissue coagulation and welding. Higher temperatures (above 100°C) cause vaporization and (above 300°C – 450°C) carbonization (Yun and Kwok, 2017). Another challenge for Raman spectroscopy is interference from fluorescence. Biological samples often emit fluorescence signals in the same wavelength range as Raman signals (Buckley and Ryder, 2017). Finally, data processing of Raman spectroscopy is a challenging task. Since there is no well-accepted standard procedure in data processing for bio-spectroscopy yet (Byrne et al., 2016), each (animal) model needs a specific calibration model. In this model it is important to remove interferences such as background from the substrate. Several technical approaches have been developed to meet these challenges for the biological applications of Raman spectroscopy (Byrne et al., 2016; Willemse-Erix et al., 2009).

As shown by our group, many ophthalmic drugs have very specific Raman fingerprint patterns (patterns specific for a drug-molecule) (Elshout et al., 2011). Bauer et al. demonstrated that confocal Raman spectroscopy can be used for pharmacokinetic detection of Dorzolamide ophthalmic solution in tear-film, and corneas of living rabbits (Bauer et al., 1999). Another study showed the detection of glucose levels in aqueous humor in rabbits (Borchert et al., 1999) and human samples (Pelletier et al., 2005). Sideroudi et al. showed that Raman spectroscopy is of interest to test drug concentrations in an artificial anterior chamber model using ciprofloxacin as target drug (Sideroudi et al., 2007). Ganciclovir, ceftazidime and amphotericin B have been detected with Raman spectroscopy *in vitro* after injecting the drugs into the anterior chamber of rabbit eyes (Byrne et al., 2016; Hosseini et al., 2003). Although these studies demonstrate the potential of Raman spectroscopy, (animal) models are often not representative of the clinical situation. For example, the injection of drugs in the anterior

chamber results in far too high drug concentrations in the aqueous humor and a limited distribution through the tissues (Schoenwald, 1990).

In this study, we designed and performed *in vitro* and *in vivo* animal experiments to detect ketorolac tromethamine, a non-steroidal anti-inflammatory drug (NSAID), using Raman spectroscopy and confirm our findings by high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Materials

Ketorolac trometamine was purchased from MSN laboratories (Telangana, India), ketorolac 0.5% ophthalmic solution (Acular™) was purchased from Allergan (Dublin, Ireland), Methocel® 2% was purchased from OmniVision (Santa Clara, CA, United States), and sterile buffered saline solution (BSS), 0.9% NaCl solution with a pH 7.4 was purchased from B. Braun (Melsungen AG, Germany). Ethylenediaminetetraacetic acid (EDTA) (#E5134), benzalkonium chloride (BAK) (#B-1383), methanol (#34860), and Brand® cuvettes (#7592-00) were purchased from Sigma-Aldrich (MO, United States). Ketamine was provided by Alfasan (Woerden, the Netherlands), midazolam by Actavis (Dublin, Ireland), and MINIMS® Oxybuprocaine hydrochloride by Bausch & Lomb Pharma (Brussels, Belgium). Insulin syringes (BD Micro-Fine™) were bought from Becton Dickinson (NJ, United States). MilliQ water and phosphate buffered saline (PBS) (pH of 7.4) were freshly produced. Freshly enucleated eyes were kindly provided by “Slachthuis Kerkrade Holding” (Kerkrade, the Netherlands).

2.2. Sample preparation

Freshly enucleated eyes from the domestic pig (*Sus Scrofa Domestica*) were obtained from an abattoir and transported to the laboratory on ice. Before use, the pig eyes were inspected using a stereo microscope (Olympus SZX9, Tokyo, Japan). Only eyes with clear corneas and without visible corneal epithelial defects (i.e. epithelial damage) were used in the experiment. After removal of excess surrounding tissue, the eyes were washed in PBS. Within 3 h after enucleation, the pig eyes were submerged in 15 mL of a dilution of ketorolac tromethamine in PBS in a 50 mL centrifugal tube. The following ketorolac concentrations were used to submerge the pig eyes: 0.05%, 0.1%, 0.125%, 0.25%, 0.5%, 1.0%, 1.25%, 2.5%, and 5.0%. As negative control, PBS was used, and as positive control Acular™ was used. Three eyes were used per concentration. The pig eyes were stored for about 24 h in the dark at 4°C and were measured by Raman spectroscopy. After the Raman measurement, 100 μL to 150 μL aqueous humor was collected using an insulin syringe and the ketorolac concentration was investigated using Raman spectroscopy and HPLC.

2.3. Raman spectroscopy set-up

Fig. 1 shows a schematic overview of the used modular confocal Raman spectroscopy system. A diode emitting laser of 785 nm with a continue power of 26 mW (Innovative Photonic Solutions SM 785 nm, Monmouth Junction, NJ, United States) and a 671 nm diode emitting laser with a continue power of 14 mW (Laser Quantum Ignis 671 and SMD 6000, Konstanz, Germany) were used to excite the samples. Raman spectra were recorded with a high-performance Raman module model 2500 (River Diagnostics®, Rotterdam, the Netherlands). This module guides the laser light through a diamond optical fiber, shapes and conditions the beam through a pinhole to the measurement stage. First, the light is sent through a collimation lens with a focus length of 80 mm (f80). In front of the sample, a f80 lens was used when the sample was measured in a cuvette. For the *ex vivo* pig eye experiment, either a long-working-distance microscope objective lens (Jena lens, magnification $\times 25$; numerical aperture = 0.50; focal length = 10 mm;

Fig. 1. Schematic Raman spectroscopy set-up. (A) laser; (B) Raman module, with (C) filter for Raman scattered light; (D) 25 μm pinhole; (E) integrated charge-coupled device (CCD); (F) collimation f80 lens; (G) f60 lens with a Gonio (one-mirror) lens, or objective (Jena lens), or a f80 lens; (H) sample; and (I) computer. Arrows indicate direction of (back-scattered) laser light; dotted arrows indicate direction of Raman-Scattered light. The Raman spectrometer operates in reflectance mode.

Carl Zeiss, Jena, Germany), or a lens with a focus length of 60 mm (f60) in combination with a Gonio lens (Haag-Streit Meridian; CGA1, Köniz, Switzerland) were used. To connect the Gonio lens to the cornea, topically applied Methocel® 2% was used. For the *in vivo* rabbit experiment the f60 lens in combination with a Gonio lens was used. The lens was also connected to the cornea using Methocel® 2%.

In the experiment, the lenses both act to focus the incident light as well as to collect the Raman back-scattered light. As such, the latter is passed back toward the Raman module and projected on a cooled charge-coupled device (CCD) camera (operating temperature -60°C) for signal detection. Raman back-scattered light in the range of 400 relative wavenumbers (cm^{-1}) to 1800 cm^{-1} was detected using the 785 nm laser. For the range from 2400 cm^{-1} to 4000 cm^{-1} , the 671 nm laser was used. The spectral resolution of the measurements was 2 cm^{-1} and the samples were exposed to 3 frames counting 60 s of exposure during the experiment. The system was used in single point modus (not confocal) and location in the sample was determined using the high wave numbers (671 nm laser) (Jongsma et al., 1997). During the *in vivo* rabbit experiment, the exposure was 2 frames of 30 s.

2.4. Identification of ketorolac tromethamine

Multivariate-peak data analysis takes into account all peaks corresponding to the chemical form ketorolac tromethamine. Elshout et al. published the four most intense peaks corresponding to ketorolac tromethamine (1002 cm^{-1} , 1524 cm^{-1} , 1568 cm^{-1} and 1602 cm^{-1}) (Elshout et al., 2011). In our set-up, commercially available ketorolac tromethamine eye drops (Acular™) were compared to each individual ingredient to investigate if the additives in Acular™ interfere with the ketorolac signal in the Raman measurement. Those ingredients were: ketorolac tromethamine (dissolved in PBS, pH 7.4), EDTA (50 mg/mL dissolved in MilliQ water), BAK (50 mg/mL dissolved in MilliQ water). The anesthetics used in the *in vivo* experiment were also individually measured using Raman spectroscopy, those compounds were: ketamine, midazolam and Oxybuprocaine hydrochloride. 75 μL of each sample was pipetted in a Brand® cuvette and measured using Raman spectroscopy.

2.5. Detection of ketorolac tromethamine in aqueous humor of pig eyes

An anterior chamber paracentesis of pig eyes was done to collect 100 μL to 150 μL aqueous humor. After centrifugation (15,000g, 5 min at 4°C to remove proteins) the supernatant was transferred to a cuvette and measured with Raman spectroscopy.

Hereafter, the samples were fivefold diluted using methanol, centrifuged once more (15,000g for 5 min at 4°C) whereafter the supernatant was analyzed by HPLC (Agilent 1260 infinity series with EZchrom software, Agilent inc. Santa Clara, CA, United States). Analysis

was done according to the US Pharmacopeia (US Pharmacopeia, 2018), using an elution time of 20 min and injection volume of 10 μL , and peak UV-detection at 313 nm on a symmetry C18 column ($300\text{ \AA}$, 5 μm , $4.6\text{ mm} \times 250\text{ mm}$; #WAT106151, Waters corp., Milford, MA, United States) with a symmetry C8 VanGuard pre-column ($100\text{ \AA}$, 5 μm , $3.9\text{ mm} \times 5\text{ mm}$, 3/pkg, #186007739, Waters corp., Milford, MA, United States). Ketorolac had a retention time of 10.5 min, has a limit of detection (LOD) of 4 ng/mL, and a limit of quantification (LOQ) of 10 ng/mL.

2.6. In vivo detection of ketorolac tromethamine in rabbits

All animal procedures were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and the Guidelines of the Central Laboratory Animal Facility of Maastricht University. All protocols were approved by the Central Committee for Animal research and were in accordance with the European Guidelines (2010/63/EU).

Twelve New-Zealand White rabbits (weight between 2.0 kg and 2.5 kg upon arrival) were ordered from Envigo (Horst, NL) and housed in group housing, 6 animals per cage, males and females separated. The rabbits had *ad libitum* access to water and received 100 gr. rabbit chow per animal per day. Before the animals were used in experiment, they had one week to acclimatize. The rabbits were treated with 50 μL 0.5% ketorolac tromethamine ophthalmic solution (Acular™) in the lower conjunctival fornix. The contralateral eye was treated with 50 μL BSS. Both treatments were performed three times a day for a total of 28 days, equivalent to a clinically used drop regime.

On day 0, day 7, day 14, day 21, and day 28 the rabbits were measured. All measurements were performed 1 to 3 h after receiving the eye drops. During the examinations, rabbits were anesthetized with ketamine (50 mg/kg) and midazolam (5 mg/kg) intramuscularly. First, a Raman measurement was performed on both eyes, followed by an anterior chamber paracentesis (drawing 50 μL) of the right eye. Before the paracentesis, the eye received additional topical sedation using one drop of 0.4% Oxybuprocaine hydrochloride solution. Aqueous humor samples were frozen on dry ice immediately after sampling and stored in a -80°C freezer until further processing. As a negative control, aqueous humor was drawn (100 μL) from both eyes of seven healthy control animals, after sacrifice. The negative control animals did not receive any topical treatment nor anesthetics. Aqueous humor was drawn within 10 min after sacrificing of the rabbits. A total of thirteen samples was collected (one sample was lost during processing).

Aqueous humor samples were measured with the Raman spectrometer followed by HPLC analysis using protocols as described earlier.

2.7. Pre-processing of the raw acquired Raman spectrum

In order to extract Raman signal from the raw acquired spectrum, it is necessary to pre-process the acquired spectrum (Hosseini et al., 2002). Cosmic ray spikes, randomly generated due to cosmic radiation, were replaced by the average intensity from the neighboring frames. A partial polynomial fitting method combined with the morphology approach of Perez-Pueyo et al. (2010) was used to remove instrumental noise. First, spectra were dissected in different zones. Zones that only contain background fluorescence were used to calculate the polynomial function coefficients. The zone that contains the water-peak (1550 cm^{-1} to 1650 cm^{-1}) was excluded from the fitting calculation. The achieved polynomial function was applied on the full spectrum (400 cm^{-1} to 1700 cm^{-1}) to remove the fluorescence background. Hereafter, the morphology-based approach was applied to eliminate instrumental noise.

In short, our pre-processing procedure are as follows: first, manual cosmic ray removal before any further treatment (Fig. S1-1). Second, averaging of the frames to minimize the fluctuations. Third, applying partial polynomial (5th degree) fitting on the averaged spectrum for subtraction of fluorescence (Fig. S1-3) and fourth, using morphology method to eliminate the instrumental noise (Fig. S1-2). Besides the first step, all procedures are processed by a self-developed MATLAB program (Version 2017b, The Mathworks Inc., Natick, MA, United States). Furthermore, all samples were normalized by dividing ketorolac related peaks by their water-peak (1642 cm^{-1}) (Erckens et al., 1997) correcting for the sample-sample variation.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software inc. La Jolla, CA, United States). All *in vitro* pig eye data were analyzed using 2-way ANOVA multiple comparison tests. The *in vivo* rabbit data was analyzed with paired t-tests. The *in vitro* rabbit data was analyzed using unpaired t-tests because the aqueous humor samples came from different rabbits. All data are shown as mean $\pm$ SD.

3. Results

3.1. Detection of peaks related to ketorolac tromethamine using Raman spectroscopy

Fig. 2 shows the Raman spectrum of Acular™ and PBS. For the multivariate peak analysis, eight high-intensity peaks specific for ketorolac tromethamine were selected: 1002 cm^{-1} , 1282 cm^{-1} , 1348 cm^{-1} , 1432 cm^{-1} , 1472 cm^{-1} , 1524 cm^{-1} , 1568 cm^{-1} , and 1602 cm^{-1} as shown in Fig. 2 (upper spectrum). These peaks were not related to additives such as EDTA or BAK (Fig. S2). The 1602 cm^{-1} peak overlaps partially with the water-peak (1642 cm^{-1} , underlined in both spectra), this peak is not used during further processing. In the following results, the intensity of each individual peak or the average intensity ratio of the seven peaks is plotted and used.

Four background peaks originating from PBS, aqueous humor and cuvette were detected: 930 cm^{-1} , 1120 cm^{-1} , 1448 cm^{-1} and 1642 cm^{-1} (Fig. 2 lower spectrum). These four peaks were identical in PBS and AH (Fig. S4). No peak differences were found between aqueous humor from rabbits and pigs (data not shown).

3.2. Detection of ketorolac tromethamine using the HPLC in aqueous humor of pig eyes

Fig. 3a shows the HPLC quantification of ketorolac tromethamine (concentration curve 0.05% to 1.25%), and the ketorolac concentrations in the aqueous humor of pig eyes that were immersed in a similar concentration range as described earlier. Both solutions demonstrate

Fig. 2. Raman fingerprint spectra of Acular™ (0.5% ketorolac tromethamine ophthalmic solution) and PBS. The structural formula of ketorolac tromethamine is shown in the frame related to Acular™. The numbers above the peak represent the corresponding wavenumbers. Baseline correction is applied using polynomial correction. PBS = phosphate buffered saline, A.U. = arbitrary unit.

linearity with the tested concentrations, R^2 of 0.97 and R^2 of 0.88 respectively. When comparing the ketorolac concentration in Acular™ to the aqueous humor penetrated ketorolac concentration in pig eyes, after submerging for 24 h in Acular™, approximately one fourth of the original ketorolac concentration appeared to have penetrated into the aqueous humor. The concentration in the aqueous humor of the pig eye was significantly lower ($p = 0.0012$) compared to the concentration in which the eye was submerged (Fig. 3b).

3.3. Detection of ketorolac tromethamine using Raman spectroscopy in pig eyes

Fig. 4a provides a detailed overview of the intensity ratios corresponding to ketorolac peaks of Acular™ compared to PBS. All peaks related to ketorolac are about five to one hundred and fifty times higher in Acular™ compared to PBS. After penetration in the aqueous humor of pig eyes, the same ketorolac peaks could be detected, as shown in Fig. 4b. However, no significant difference between the ketorolac and the PBS peak on wavenumber 1348 cm^{-1} was observed.

When the intensity of the peaks is summarized, the aqueous humor penetrated concentration of ketorolac is about one fourth of the original solution (Fig. 4c). As expected, there is no difference between PBS and the aqueous humor penetrated PBS signal. These results demonstrate that Raman spectroscopy is able to detect ketorolac solutions after penetration in the aqueous humor.

Fig. 5 shows a correlation between the Raman signal and HPLC

Fig. 3. HPLC detection and analysis of (a) a dilution curve of ketorolac tromethamine in PBS (submersion solution) (black) and the detection of penetrated ketorolac in aqueous humor (red). The dashed line represents a logarithmic trendline with a R^2 of 0.98 and 0.89 for the black and red points respectively. (b) HPLC analysis of ketorolac tromethamine in Acular™ and the penetrated concentration in aqueous humor after 24 h. Compared using students *t*-test, ** $p = 0.0012$, $n = 3$, AH = aqueous humor, AUC = area under the curve, data is plotted as mean $\pm$ SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

signal from the concentration curve of aqueous humor penetrated ketorolac in pig eyes. This resulted in Pearson's coefficient of 0.89 with a R^2 of 0.79 using correlation on the log-log scale.

Fig. 6 demonstrates the quantitative potential of confocal Raman spectroscopy. Fig. 6a shows a linear relationship between the concentration and the observed signal for three different set-ups. The limit of detection of the ketorolac dilution curve (0.05% to 5.0%) (black line) lies on an intensity ratio of 0.05 ± 0.003 , which is lower than the background signal of aqueous humor detected in a cuvette (red line)

(0.07 ± 0.02). The limit of detection of aqueous humor detected with a Jena lens (green line) or a Gonio lens (blue line) lies however, about nine times higher (0.45 ± 0.03 and 0.34 ± 0.09 , respectively).

Fig. 6b shows the response of the Raman system with three different set-ups. Different intensity ratios when comparing Acular™ to PBS have been observed. When measuring Acular™ and PBS in a cuvette, a clear difference is visible with low background. When the ketorolac concentration in aqueous humor of pig eyes is measured in a cuvette, the intensity ratio of Acular™ is lower, but the background has slightly

Fig. 4. Raman detection of ketorolac in aqueous humor of pig eyes. (a) Peaks corresponding to Acular™ compared to PBS and (b) after penetration in aqueous humor of pig eyes ($n = 3$), row comparison using Tukey's multiple comparisons test. (c) Averaged intensity ratios from Acular™ and PBS as the submerging solution and after aqueous humor penetration in pig eyes. Exposure time 60 s, 3 frames averaged, 785 nm laser. Peaks normalized by dividing the intensity of each peak with the intensity of the peak at $1,642 \text{ cm}^{-1}$, $n = 3$. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. AH, aqueous humor, data is plotted as mean $\pm$ SD.

Fig. 5. Correlation between Raman signal (y-axis) and the HPLC signal (x-axis) of aqueous humor from pig eyes submerged in ketorolac dilutions. The dashed line represents a logarithmic trendline. Pearson's r of 0.89 and a R^2 of 0.79, every dot represents one eye ($n = 3$ per concentration). AUC = area under the curve.

increased. Aqueous humor in the anterior chamber, measured using the Jena lens does not show a difference between an Acular™ submerged pig eye and a PBS treated pig eye due to high background noise. When ketorolac is detected in aqueous humor in the anterior chamber using the Gonio lens, a high background is observed; however, the Gonio lens is capable to distinguish ketorolac from the PBS samples.

3.4. In vivo detection of ketorolac in rabbit eyes

Fig. 7a shows that the Gonio lens was able to measure aqueous humor in the anterior chamber of living rabbits; however, no difference between treated (Acular™) and non-treated (BSS) rabbit eyes was observed. When the Gonio lens was not properly aligned on the eye, the lens, or the cornea was measured, as shown in Fig. 7b and c respectively. In none of the *in vivo* measurements a significant difference between the treated eye (OD) and the control eye (OS) was observed.

The Raman spectra show the wavenumber (671 nm) signals corresponding to the location in the eye. When measuring specifically in the aqueous humor, a broad peak is visible between 3000 cm^{-1} and 3700 cm^{-1} (Fig. 7a, lowest frame). The lens shows a narrow peak at 2900 cm^{-1} and a broad one between 3000 cm^{-1} and 3700 cm^{-1} (similarly to the peak for aqueous humor) (Fig. 7b lowest frame). The cornea expresses a high peak at 2900 cm^{-1} and a broad peak between 3000 cm^{-1} and 3700 cm^{-1} (Fig. 7c lowest frame).

3.5. Ketorolac tromethamine detection in aqueous humor of rabbits ex vivo

Fig. 8 shows the *ex vivo* detection of the ketorolac concentration in aqueous humor from rabbits, measured in a cuvette by Raman and via HPLC. As shown in Fig. 8a, a significant difference ($p = 0.0017$) was observed between Acular™ treated eyes and non-treated eyes when the ketorolac was measured using Raman spectroscopy. However, due to the large inter-sample variation, no exact concentration could be calculated and linked to individual measured aqueous humor samples measured by our Raman spectroscopic system.

Fig. 8b shows the detection of the concentration ketorolac from aqueous humor of rabbits using HPLC. These results also show significant difference ($p < 0.0001$) between the treated and untreated eyes. When all days are combined, the average drug concentration was $927\text{ ng/mL} \pm 430\text{ ng/mL}$ (mean $\pm$ SD), a maximum detected concentration of 2236 ng/mL and a minimum detected concentration of 63 ng/mL in the treated eyes. The control eyes did not show any signal above noise level when detected with HPLC. No clear correlation between the Raman signal and HPLC concentration could be found with these low concentrations.

4. Discussion

The aim of the study was to evaluate the quantitative use of Raman spectroscopy for the *in vivo* detection of drug levels in the anterior chamber of the eye. Firstly, we needed to optimize the method to analyze our Raman data. The probability of Raman scattering is much

Fig. 6. *In vitro* detection of ketorolac in pig eyes using three different set-ups (aqueous humor in a cuvette, aqueous humor in the anterior chamber with the Jena lens and aqueous humor in the anterior chamber with the Gonio lens) and the control situation (submerging fluid in a cuvette). (a) Dilution series of ketorolac (0.05% to 5.0%) were measured with the Raman spectrometer. Black dots represent the ketorolac tromethamine dilution measured in a cuvette. Red dots represent aqueous humor of pig eyes submerged in corresponding ketorolac tromethamine dilutions after paracentesis measured in a cuvette. The green dots represent measurements on the pig eye, in the anterior chamber with the Jena lens, and the blue dots represent measurement on the eye into the anterior chamber with the Gonio lens. The dashed-lines are drawn as guide to the eyes whereas the solid lines provide the limit of detection. (b) Bar graph with the response of the detection of Acular™ and PBS. The fluids have been measured in a cuvette, in the aqueous humor using a cuvette, or in the eye with the Jena lens, or in the eye with a Gonio lens. Samples are compared using ANOVA multiple comparison tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $p < 0.0001$, $n = 3$ per point. AH = aqueous humor, AC = anterior chamber, data is plotted as mean $\pm$ SD.

Fig. 7. *In vivo* detection of ketorolac in rabbit eyes. (a) Detection in aqueous humor when the Gonio lens is properly aligned on the eye. The asterisk shows the location of focus and the Raman spectrum provides the high wavenumber (671 nm) measurement to assure correct location in the eye. (b) Measurement on the lens or (c) on the cornea, due to misalignment of the Gonio lens. Treated eye received three times a day 50 μ L Acular™, and the control eye was treated with 50 μ L BSS (pH7.4). Paired tests have been executed to test difference between treated and control eyes. Each dot represents one measurement at one rabbit, data is plotted as mean $\pm$ SD.

Fig. 8. *Ex vivo* detection of ketorolac tromethamine in aqueous humor from rabbit eyes. General grouped difference between the aqueous humor of treated eyes and control (non-treated) eyes measured with (a) Raman spectroscopy and (b) HPLC. ** $p < 0.01$, **** $p < 0.0001$. Each dot represents one sample from one rabbit, data tested using student *t*-test and is plotted as mean $\pm$ SD, $n_{\text{treated}} = 58$ and $n_{\text{control}} = 13$.

lower than intrinsic fluorescence emission in biological samples. Therefore, in order to extract Raman signal from the raw acquired spectrum (Hosseini et al., 2002). Furthermore, cosmic ray spikes, randomly generated due to cosmic radiation, which affected different wavenumbers each time, needed to be removed (Hosseini et al., 2002; Zhang et al., 2017). For the latter, there are two approaches described in the literature (Zhang et al., 2017; Li and Dai, 2011). In this study, we went for the easiest method, by replacing the intensities of the cosmic peaks

with the average from the neighboring frames (left and right from the ray) as suggested by Zhang et al. (2017).

Several approaches have been proposed to minimize the influence from the background fluorescence (Byrne et al., 2016). The most accepted method for background subtraction is polynomial fitting but as mentioned by Byrne et al. no standardized protocols are available (Byrne et al., 2016). Zhao et al. introduced an automated polynomial background subtraction method for biomedical applications, which could subtract the background fluorescence (Zhao et al., 2007). Zhang

et al. developed an automated method for fluorescence background subtraction named “automatic pre-processing method for Raman imaging data set (APRI)” (Zhang et al., 2017). However, previous methods encountered difficulties when handling spectrums containing instrumental noise. In some *in vivo* experiments, the contribution from instrumental noise is inevitable and cannot be neglected, thus affecting the conventional polynomial methods. Hence, further treatments have been developed to eliminate the instrumental noise. Perez-Pueyo et al. introduced a morphology-based baseline removal method for Raman spectrums (Perez-Pueyo et al., 2010). It employs Tophat filtering using basic operations as dilation and erosion to filter the features beyond or below a pre-set threshold, thereby removing the instrumental noise. In this study, we used a partial polynomial fitting method combined with the morphology approach of Perez-Pueyo et al. to remove instrumental noise (Perez-Pueyo et al., 2010). Hereafter we normalized all samples by dividing ketorolac related peaks by their water-peak (1642 cm^{-1}) (Erckens et al., 1997), thereby correcting for the sample-sample variation. Overall we created a solid method to remove hardware related shifts from Raman data.

After we optimized the analysis for Raman spectroscopy, we first confirmed the four ketorolac tromethamine peaks described by Elshout et al. (2011) and then identified four additional peaks specific for ketorolac. We observed that the 1602 cm^{-1} peak partially overlaps with the water-peak (1642 cm^{-1}); therefore, this peak was ignored during processing.

During the experiment, we examined buffered saline solutions (PBS and BSS), aqueous humor from pig eyes and aqueous humor from rabbit eyes. Although, there are differences between the buffered solutions and aqueous humor, the Raman signal was identical, as shown in supplementary Fig. S4, and also did not affect the HPLC results. In the pig model, a concentration range of dissolved ketorolac was used and Acular™ was used as a positive control, whereas only Acular™ was used in the rabbit model. No differences were found in Raman signal, nor have we observed shifted peaks during the experiments. Although we expected that the Raman signals might be affected due to the additives, we did not observe any interference of the additives on the ketorolac signal (Fig. S2). Neither have we observed influences of the anesthetics on the Raman signal in the *in vivo* experiment (Fig. S5). However, we observed superior penetration of dissolved ketorolac in PBS compared to the commercially available solution (Acular™) (Fig. S6). The pH of both solutions was similar (pH 7.4) but the osmolality differs, i.e. Acular™ displays an osmolality of 290 mOsmol/kg (Allergan Inc., 2012) whereas dissolved ketorolac tromethamine in PBS has a (theoretical) osmolality of 330 mOsmol/kg. This could explain the higher ocular penetration by the dissolved ketorolac solution. Lee et al. also found osmolality to be a relatively large influencer of ocular penetration in studying penetration of topically applied Atenolol (Lee and Lee, 1993). However, in our experiment we used post mortem tissue in which the cellular membranes, and tight junctions between the cells are affected and in which clearance of the drug is hampered. Due to submerging of completely enucleated eyes more scleral diffusion is expected leading to increased intraocular drug concentrations as compared to eye drops (Bertens et al., 2018; Del Amo et al., 2017). Furthermore, the long contact time of 24 h also enhanced the intraocular drug concentration. The detection of ketorolac in the *in vitro* model was also challenging due to corneal haze in the stromal layers after leaving the eyes in the buffered solutions (Fig. S3), high background signals were detected which disturbed the Raman measurements. This resulted in high background noise signals that were more than three times higher (when normalized), compared to regular cuvette measurements (Fig. 6b).

The *in vivo* animal model represents a realistic clinical situation of an eye drop scheme. The detection of the ketorolac concentration is in line with previously published data. Ling et al. found a $C_{\max}$ of 1905 ng/mL (Ling and Combs, 1987). Although we were not looking for a $C_{\max}$, our highest detected concentration was 2236 ng/mL. The mean ketorolac concentration in our experiment was $927\text{ ng/mL} \pm 430\text{ ng/mL}$,

whereas an average concentration of $1079\text{ ng/mL} \pm 882\text{ ng/mL}$ is found in human eyes, when instilling Acular™ eye drops four times a day, two days pre-surgery (Bucci and Waterbury, 2008).

In our Raman system, two different lenses were compared: a Jena lens and a Gonio lens. Since we used a f60 lens in front of the Gonio lens (the Gonio lens itself does not provide any focus power), better focus was achieved compared to use of the Jena lens. The f60 lens has a smaller numerical aperture, which provides a longer integration length. Second, based on the safety point of view, the Gonio lens prevent the laser from direct illumination on the retina, preventing it for the possible light damage. The excitation laser directly illuminates the retina in the Jena lens setup, it limits the common performance improvement methods like raising the laser power or increasing integration time. Besides, we observed a specific drug related difference when we used a Gonio lens. As such, we continued the experiment *in vivo* only with the Gonio lens. *In vivo* the average background signal in the aqueous humor was lower (0.15 ± 0.05) (Fig. 7a, control eye) compared to *in vitro* signal (0.34 ± 0.09 , Gonio lens, PBS) (Fig. 6). Hence, we tend to conclude that the corneal haze was affecting the signal. However, the drug concentration in the aqueous humor was too low to detect using *in vivo* Raman spectroscopy. Another important factor affecting the sensitivity of Raman spectroscopy is the exposure time during the measurement. All *in vitro* samples have been exposed 60 s for 3 frames, whereas the *in vivo* exposure was 30 s with 2 frames. We lowered the exposure time and the number of frames *in vivo* to assure safety of the technique in the rabbits. Because of the limited number of frames the threshold of the intensity ratio (0.15 ± 0.05) was higher *in vivo* (Fig. 7a, control eye) compared to *in vitro* intensity ratio (0.11 ± 0.06) (Fig. 8a, control eye). Besides the lower number of frames, also a shorter exposure time may lead to a decreased Raman signal (Lázaro et al., 2009). Due to the large inter-measurement-variations, the standard deviation in the Raman experiment was too large to clearly quantify the *in vivo* samples. The variation with the HPLC was much smaller resulting in a detection accuracy of nanograms per milliliter. Due to large variations in the *in vivo* fingerprint signals, no correlation could be found using the rabbit samples, whereas there is a clear correlation with Raman spectroscopy and HPLC when measuring higher drug concentrations from the *in vitro* pig eyes (Fig. 7). Overall, multiple factors affect the readout and it is difficult to select one parameter causing low sensitivity with Raman spectroscopy. *In vitro* samples have been centrifuged to remove proteins, which could be a reason for higher signal during the *in vitro* measurements compared to the *in vivo* measurements. Furthermore, the cornea consists of a 500 μm thick stroma, which could scatter or absorb Raman scatter on its way through. Our experiment also shows that the conditions of the cornea (e.g. corneal haze) could affect the Raman signal sensitivity. Finally, temperature might also slightly influence the Raman intensity both for the target components and backgrounds. The cuvette samples and *in vitro* samples were tested at room temperature (about 22°C) while the temperature of *in vivo* measurement is around 35°C in a rabbit eye. It is noticeable that all factors together lower the sensitivity of Raman spectroscopy.

To increase sensitivity in a Raman system, laser power could be increased. The problem however, with increasing laser power in the eye, is the irreversible damage of the photosensitive layers that could occur. In order to protect the eyes, we used a laser power of 26 mW, which is relatively low compared to laser powers which are used on skin (80 mW) (Rangaraju et al., 2018), or on cells or tissue sections (60 mW) (Jonas et al., 2018). For *in vitro* detection of corneal biomarkers an intensity of 300 mW is used (Kaji et al., 2017), and even 1 W is used to create virtual cross-sections of intact eye tissue without dependence on tissue processing (Ammar et al., 2013). According to Marro et al., laser powers up to 100 mW are safe to use on retina organotypic cultures (*in vitro*) (Marro et al., 2014). Besides laser power, the wavelength and the exposure time are of importance for the prevention of tissue damage. In our study, we used ketorolac tromethamine. Other ocular drugs, however, may have a stronger Raman signal and can be easier to detect in

the anterior chamber. All these parameters make Raman spectroscopy a challenging technique. Furthermore, as mentioned by Byrne et al. (2016) there is no common accepted manner to correct Raman data. Due to hardware influences and sample-to-sample variation, every Raman spectrometer needs its own corrections.

5. Conclusion

In this study, we show the value of Raman spectroscopy for the detection of drugs in the anterior chamber of the eye. As expected, the sensitivity and the limit of detection of the HPLC are much higher compared to Raman spectroscopy. However, Raman spectroscopy shows unique potential as a non-invasive technique for real time biomedical analysis. We found good correlation between Raman spectroscopy and HPLC for *in vitro* detection of drugs. Unfortunately, our Raman spectroscopic system is not yet able to detect a clinically relevant dose of ketorolac tromethamine in the anterior chamber of rabbits *in vivo*. More research should be conducted to increase the sensitivity of Raman spectroscopy while still using low, non-damaging, laser powers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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