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Determination of Three Corticosteroids in the Biologic Matrix of Vitreous Humor by HPLC-tandem Mass Spectrometry: Method Development and Validation

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\textbf{ABSTRACT}

\textbf{Purpose}: To develop a simple, specific, and rapid method to determine corticosteroid concentrations in vitreous humor.

\textbf{Methods}: An analytical method based on high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS) with a simple extraction procedure was developed. New Zealand albino rabbits ($n=54$) received a single (0.1 mL) intravitreal injection of dexamethasone (DXM, 0.1 mg), methylprednisolone (MP, 2 mg), or triamcinolone acetonide (TA, 10 mg). Eyes were enucleated and mean vitreous steroid levels were quantified at 12 h and 1, 2, 3, 7, and 14 days.

\textbf{Results}: Corticosteroids were extracted from the vitreous with acetonitrile, and TA was extracted with ethyl acetate, yielding high protein precipitation and clean solution samples. Vitreous samples were analyzed by isocratic HPLC-MS with mobile phase comprising acetonitrile and 2 mM ammonium formate buffer in water, pH 3.5. The linear range was 50–100,000 ng/g with a lower quantification limit of 45 ng/g for DXM and MP, and 50 ng/g for TA. Vitreous levels of DXM and MP were not detectable 14 days post-administration. Vitreous levels of TA were positive and stable throughout the study in both injected and control eyes.

\textbf{Conclusions}: The HPLC-MS analytical method is an alternative to HPLC-MS/MS methods, sensitive enough for identifying and quantifying steroids in vitreous humor at a therapeutic dosage scale.

\section*{Introduction}

Ocular pathologies affecting the posterior segment of the eye are a major cause of blindness in developed countries and are becoming more prevalent due to the increase in population longevity. Successful therapies for diseases affecting the back of the eye require the delivery and maintenance of effective concentrations of the active substance at the intraocular target site. To increase local drug concentrations in the inner tissues of the eye and limit the risk of systemic adverse effects through topical and systemic pathways, intravitreal injections are applied clinically to treat different ophthalmic pathologies.\textsuperscript{1}

Corticosteroids may reduce the concentration of inflammatory cytokines and growth factors, and also decrease vascular permeability.\textsuperscript{2} A single intravitreal injection of triamcinolone acetonide (TA) can stabilize the blood–retinal barrier and inhibit angiogenesis by different mechanisms, making it a useful alternative treatment for several eye diseases or disturbances that present with macular edema (such as diabetic retinopathy, retinal vein occlusion, ocular surgery, or inflammation) and are resistant to treatment with topical eye drops.\textsuperscript{2}

Dexamethasone (DXM) is a corticosteroid with proven efficacy for treating both anterior- and posterior-segment ocular diseases. This anti-inflammatory steroid is more potent than TA and intravitreal administration of DXM has a lower risk than TA of inducing ocular hypertension and cataracts.\textsuperscript{3} The short half-life of DXM (3.5 h), however, reduces its clinical application.\textsuperscript{4}

Ophthalmologists have evaluated the effect of subconjunctively administered methylprednisolone (MP) on the recovery of blood aqueous barrier permeability following uncomplicated cataract surgery. While administration of MP did not demonstrate superiority to standard postoperative steroid eye drop treatment with regard to intraocular inflammation and intraocular pressure after uncomplicated phacoemulsification surgery, it might be considered for treating individuals with compliance issues.\textsuperscript{5}

Various methods have been used to analyze corticosteroid levels, such as immunoassays and gas chromatography,\textsuperscript{6} but high-pressure liquid chromatography (HPLC) is the preferred technique in the vast majority of published papers and pharmacopoeia due to its many advantages.\textsuperscript{7} Traditional detectors (ultraviolet, fluorescence, etc.) are used, but their lack of specificity and lower sensitivity make their application difficult for biologic samples. Mass spectrometry (MS) detectors can overcome the disadvantages of traditional detectors, and...
tandem MS (MS/MS) detectors have recently gained popularity due to their higher sensitivity and selectivity. The need for time-consuming purification steps and the higher costs associated with the use of these systems, however, limit their use in research or specialty laboratories.

Various HPLC-MS and HPLC-MS/MS methods have been developed to identify and quantify corticosteroids in biologic matrixes such as urine, plasma, and vitreous humor. Most of these methods, however, are based on the use of expensive HPLC-MS/MS techniques that require several sample purification steps to achieve satisfactory results.

Although intravitreal corticosteroids are routinely applied on a large scale to treat retinal diseases, the purpose of this study was to develop and validate a simple, specific, and rapid method for determining corticosteroid concentrations in the vitreous humor using HPLC-MS, and to test this method in vivo after intravitreal administration of three steroids: TA, DXM, or MP. Here we describe a method based on readily accessible HPLC-MS instrumentation coupled with a simple extraction method that could be an alternative solution for analyzing DXM, MP, TA, and even betamethasone (BM) in the vitreous humor.

Materials and methods

Reagents and standard solutions

DXM, BM, MP, and TA were purchased from Sigma-Aldrich (Sigma-Aldrich Corp, St. Louis, MO, USA) with purity higher than 97%. Formic acid, ammonium formate, and sodium formate were also obtained from Sigma-Aldrich (+99% spectroscopy grade). Acetonitrile (ACN), methanol, and ethyl acetate (AcOEt) were purchased from Scharlab (HPLC-MS grade, Barcelona, Spain).

Stock standard solutions of DXM, BM, MP, and TA (1000 µg/mL) were prepared by dissolving 10 mg of the drug in 10 mL of ACN (DXM, BM, MP) or methanol (TA), and storing at 4°C. Working standard solutions (concentrations ranging from 100 to 100,000 ng/mL) were prepared daily by serial dilution of stock solutions with ACN (DXM and MP) or methanol (TA).

Calibration standards for the vitreous were prepared by adding different volumes of drug working standard solutions to drug-free vitreous (0.5 g) to obtain concentrations ranging from 50 to 100,000 ng/g vitreous. The mixtures were vortexed for 30 s prior to normal sample preparation (vide infra).

Extraction conditions and sample preparation

DXM samples

Vitreous (0.5 g) was placed in a 10 mL screw cap glass centrifuge vial and then 5 mL ACN and 100 µL MP solution (2000 ng/mL, internal standard) were added. The mixture was successively vortexed for 60 s, sonicated for 5 min, vortexed for another 60 s, and finally centrifuged for 10 min at 4000 rpm. The supernatant was collected and filtered through a 0.22 µm syringe filter before being evaporated under vacuum at room temperature. The dried extract was reconstituted in 200 µL mobile phase and analyzed by HPLC-MS.

MP samples

The samples were prepared in the same way as those of DXM but using BM solution (2000 ng/mL) as the internal standard.

TA samples

Vitreous (0.5 g) was placed in a 10 mL screw cap glass centrifuge vial and then 2.5 mL ACN, 2.5 mL AcOEt, and 100 µL BM solution (2000 ng/mL, internal standard) were added. The mixture was successively vortexed for 60 s, sonicated for 5 min, vortexed for another 60 s, and finally centrifuged for 10 min at 4000 rpm. The supernatant was collected and filtered through a 0.22 µm syringe filter before being evaporated under vacuum at room temperature. The dried extract was reconstituted in 200 µL mobile phase and analyzed by HPLC-MS.

Chromatographic instrumentation and conditions

Sample analysis

Samples were analyzed using a Waters 2990 HPLC system (Waters Cromatografia, Barcelona, Spain) coupled to a Waters ZQ4000 MS detector (Waters Cromatografia, Barcelona, Spain) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved using a Phenomenex Kinetex C18 (Phenomenex, Torrance CA, USA) (2.6 µm, 75 × 4.6 mm) column at 40°C. The mobile phase comprised a mixture of ACN and 2 mM ammonium formate buffer in water, adjusted to pH 3.5 with formic acid and doped with sodium formate (0.2 mM; 35:65). Samples (10 µL) were injected and eluted at a flow rate of 0.2 mL/min.

Mass spectrometry

The mass spectrometer was operated in ESI positive mode. Ionization was optimized by direct infusion (10 µL/min) of the drugs dissolved in the mobile phase. The analyses were performed in the 200–900 Da scan range. The ionization conditions that produced comparable ionizations for all drugs were 4000 V (capillary) and 70 V (cone). Data analysis was performed with the Waters MassLynx software. Retention times of the analytes are shown in Table 1.

Analytical method validation

Linearity, precision and accuracy, recovery, matrix effects

Analytical method validation was carried out according to ICH guidelines. Linearity was assessed by seven-point calibration curves in rabbit vitreous, in triplicate, on 3 consecutive

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Ret (min)</th>
<th>Molar mass (g/mol)</th>
<th>Identification ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-α-Methylprednisolone (Betamethasone)</td>
<td>10.0</td>
<td>374</td>
<td>315, 316, 317, 318, 319</td>
</tr>
<tr>
<td>Prednisolone (Betamethasone)</td>
<td>11.4</td>
<td>392</td>
<td>315, 316, 317, 318, 319</td>
</tr>
<tr>
<td>Triamcinolone (Betamethasone)</td>
<td>14.7</td>
<td>434</td>
<td>435, 436, 437, 438, 439</td>
</tr>
</tbody>
</table>

*Rt = 11.1 min; MW = 392; identification ions: m/z = 393 [M+H]+, 415 [M+Na]+.*
days. The curves were constructed by plotting the peak area ratio of the drug to the internal standard (IS) versus the analyte concentration over a range between 50 and 100,000 ng/g. The curves were evaluated by residuals and fitted by weighted linear regression ($1/X^2$ weighting factor). The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined by the method based on the standard deviation of the slope and response. Thus, LLOQ was established as the lowest concentration on the calibration curve at which precision and accuracy were within 20%, by analyses of three replicates. In addition, the analyte response at this concentration was at least 10 times the baseline noise. The quantification parameters are listed in Table 2.

To evaluate the precision and accuracy of the method, quality control samples at three concentration levels (50, 5000, and 100,000 ng/g) of drug were analyzed in three replicates on three different days. Intra-day run and inter-day run precision were calculated and expressed as the relative standard deviation (RSD), while accuracy was expressed as the relative error (RE).

Recovery of DXM, MP, and TA was determined by comparing the peak areas of extracted vitreous samples with those of direct injected standards at the same concentration. Recovery was evaluated by analyzing three replicates at low, middle, and high concentration levels. Recovery of the IS was determined in the same way as the standard working concentration.

We evaluated the matrix effect to determine if ion suppression or enhancement, due to the co-elution of matrix components, occurred. The peak areas of drug and IS from the spiked samples after extraction were compared with those of the standard solutions in the mobile phase, at the same concentrations.

The selectivity of the method was evaluated comparing the extracted blank vitreous of healthy specimens with that of drug-spiked specimens at the LLOQ.

The stability of corticoids in vitreous was tested by analyzing the concentration after four freeze–thaw cycles before analysis. QC samples stored at –30°C were analyzed at monthly intervals for 10 months, and processed samples stored at 4°C for 24 h were also analyzed. In all cases, the corticoids were found to be stable as long as the concentration measured was higher than 90% of the initial value.

### Animal study: In vivo determination

#### Animals

Female New Zealand albino rabbits weighing between 2 and 3.5 kg were used in this study. All the animals were handled according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609/EEC. Animal care was performed in compliance with the ARVO Statement for the Use of Animals in Experimental Procedures and Other Scientific Purposes. All procedures were performed according to the Project License P1/2/02285 approved by the in-house Ethics Committee for Animal Experiments for the University of Zaragoza, Spain. The animals were singly housed in metabolic cages in a light-controlled room (12 h/12 h dark/light cycle) at 20 ± 2°C with a relative humidity of 40%–70%. Diet and water were available ad libitum and clinical observations were performed daily.

#### In vivo administration

Fifty-four rabbits were included and randomly divided into three groups (n = 18/group). Each rabbit received an intravitreal injection of a single dose of steroid (DXM, 0.1 mg/0.1 mL; MP, 2 mg/0.1 mL; or TA, 10 mg/0.1 mL) with a 25-gauge needle in the superior–temporal quadrant of the right eye, approximately 3–5 mm posterior to the limbus, using a surgical microscope to visualize the injection site. Before the intravitreal injection, aqueous humor paracentesis with a 30-gauge needle was performed to avoid an increase in intraocular pressure. After the injection, the absence of backflow was verified in all cases by 30 s compression of the injection site with a cotton swab. Left eyes were not injected and served as controls.

All injections were performed under general anesthesia and aseptic conditions. The animals were anaesthetized intramuscularly with a mixture of ketamine hydrochloride (25 mg/kg, Ketolar 50®), Pfizer, Madrid, Spain) and medetomidine (0.5 mg/kg, Domtor®, Esteve, Madrid, Spain). Topical anesthesia in eye drops with tetracaine chloride (1 mg/mL) and oxibuprocaine chloride (4 mg/mL) (Colircusí Anestésico Doble®, Alcon Cusí SA, Barcelona, Spain) was used, and povidone-iodine solution (5%) was applied to the ocular surface for antisepsis before and after the intravitreal injections. All injections were performed by the same ophthalmologist.

#### Sampling schedule

Animals were humanely killed with a rapid intravenous bolus injection of sodium pentobarbital (30 mg/kg). Eyes were enucleated and the vitreous humor specimens were obtained by eye dissection. Translucent vitreous samples of ~600 µL were collected from the injected and contralateral eyes at 12 h and 1, 2, 3, 7, and 14 days; and immediately snap-frozen and stored at –20°C.

#### Pharmacokinetic data analysis

All values are expressed as mean ± standard deviation (mean ± SD). Mean vitreous concentration values ($C_{\text{vitreous}}$) of samples collected after intravitreal injection of TA, DXM, and MP were taken to perform the pharmacokinetic analysis.

Experimental data were evaluated for the best fit and were consequently modeled according to a one-compartment model using Microsoft Excel’s “PKSolver Add-in” (Albuquerque, NM, USA) in which equations describing $C_{\text{vitreous}}$ as a function of...
time are used. The following equations were used to describe the time course–concentration in vitreous humor after administration:

\[ C_{\text{vitreous}} = C_0 e^{-K_e t} \]

where \( C_{\text{vitreous}} \) is the vitreous concentration, \( C_0 \) is the concentration in vitreous at time \( t_0 \), \( K_e \) is the vitreous elimination rate constant which is derived from the half-life of the drug \( (K_e = 0.693/\text{t}_{1/2}) \) and \( C_{\text{vitreous}} \) is the drug clearance from the vitreous. The vitreous clearance was calculated according to the following equation:

\[ Cl_{\text{vitreous}} = \frac{\text{Dose}}{\text{AUC}} \]

where the Dose was different for each group (10 mg TA, 2 mg MP, and 0.1 mg DXM) and \( \text{AUC}_{0-\infty} \) is the area under the curve estimated by the linear-trapezoidal method from the experimental vitreous concentrations, in which the area from the last concentration point (\( t_{\text{last}} = \text{day 14} \)) to infinity (\( \infty \)) was calculated as \( C_{\text{last}}/K_e \).

Results

Analytical method results and validation

Sample preparation

The extraction method was developed by testing ACN and AcOEt as extraction solvents. The results of extraction recovery are shown in Table 4. DXM, MP, and BM were efficiently extracted from vitreous with ACN yielding high protein precipitation and clean solution samples, while TA showed poor solubility in ACN. When extraction was carried out in AcOEt, good extraction of the analytes was achieved, but the extraction method was complicated by the lack of miscibility of the vitreous in the extraction solvent, therefore larger extraction solvent volume and longer times were required to achieve the best results. To solve the problems found in TA extraction, we tested volume and longer times were required to achieve the best extraction solvent, therefore larger extraction solvent volume and longer times were required to achieve the best results. To solve the problems found in TA extraction, we tested volume and longer times were required to achieve the best

MS method

While developing the MS method for extracted vitreous samples, we observed that both \([M+H]^+\) and \([M+Na]^+\) adduct ions were produced in similar amounts; but when analyzing working standard solutions, we only detected the \([M+H]^+\) adduct ion (Figure 1). We evaluated the effect of changing the eluent composition by modifying the amount of ammonium formate or lowering the pH, but these changes produced lower response without any noticeable presence of other cluster ions. That is, we doubled the method sensitivity.

Sample analysis

The chromatographic method was optimized by testing a Phenomenex Luna C\(_{18}\) column (250 × 4.6 mm, 5 µm) and a Phenomenex Kinetex C\(_{18}\) column (75 × 4.6 mm, 2.7 µm). Both columns produced good chromatographic resolution for DXM, MP, and TA using the isocratic method; however, the Kinetex C\(_{18}\) column produced better resolution at shorter elution times. Moreover, BM could not be resolved from DXM in the Luna C\(_{18}\) column, while both compounds could be, at least, partially resolved in the Kinetex C\(_{18}\) column. This is due to the chemical similarity of DXM and BM, which precluded the use of BM as IS for the analysis of DXM. This drawback was solved by using MP as the IS for the analysis of DXM (Figure 2). Corticosteroids were successfully analyzed in vitreous with a sensitivity of ~45 ng/g.

The analyte concentrations were calculated against area ratios of seven-point calibration curves for each analyte–IS couple (Table 1). The spectrometer was operated in single ion monitoring mode. In all cases, the \([M+Na]^+\) adduct ion was selected for quantification purposes because it provided good linearity in the range 50–100,000 ng/g (Table 2). The results are provided in nanograms per gram of vitreous.

Method validation

Standard curves of the analytes in vitreous were linear over the range 50–100,000 ng/g (\( r^2 \geq 0.999 \)). The standard deviation of the slopes of the mean of three standard curves was <5%. The LOD determined by the method based on the standard deviation of the slope and response was ~10 ng/g, except for TA (20 ng/g) and samples of that concentration produced a signal-to-noise ratio (S/N) between 2 and 3, passing the acceptance criterion. The LLOQ determined by the method based on the standard deviation of the slope and response was between 30 and 40 ng/g, but samples of this concentration produced an S/N of 5–7 so, in order to pass the acceptance criterion, LLOQ had to be increased to reach an S/N of at least 10. The LLOQ was found to be ~45 ng/g except for TA (50 ng/g).

The accuracy and precision of three replicates on three consecutive days was assessed and the results are shown in Table 3. The mean values were in the range ±5% of the spiked amount, with a low imprecision as indicated by the low relative error observed (<10%) in both intra- and inter-day analyses.

The absolute recoveries of the analytes and IS from the vitreous were similar, and consistent with mean values of ~100%. The matrix effects were evaluated for the analytes and IS in vitreous, and the concentration values for the analytes spiked in the matrix and in the mobile phase were found to be similar, indicating that the matrix effects were insignificant. The experimental data are listed in Table 4.

The stability of DXM, MP, and TA in vitreous was tested, and the corticoids were found to be stable after four freeze–thaw cycles when stored at ~30°C. QC samples were found to be stable for at least 10 months at ~30°C and 24 h at 4°C. In all cases, the concentration of corticoids in stored samples deviated <10% from the initial concentration.
In vivo determinations and ocular pharmacokinetics

The concentration–time data from each of the three groups are shown in Figure 3. Three animals were used for each sampling time point. We did not attempt to model contralateral vitreous drug concentrations following intravitreal administration of DXM or MP because 83% of the vitreous concentrations were below the LLOQ for the analytical method. Nevertheless, TA was detected in the contralateral eyes of the TA group for up to 14 days post-injection with a peak concentration at ~3 days after administration.

In rabbits, 0.1 mg DXM and 2 mg MP intravitreal injections resulted in no detectable vitreous drug levels at 14 days post-administration. A 10 mg injection of TA produced positive vitreous drug levels at 14 days post-injection in both the administered and control eyes. The time course of the TA concentration in the vitreous of rabbits following intravitreal injection was stable and sustained, whereas the DXM and MP concentrations decreased gradually over time with a similar slope for both groups. TA levels were detected in the vitreous of the contralateral eye following intravitreal injection and although these levels were initially lower than those achieved in the treated eyes for the MP group, were maintained for longer.

The time course of the experimental vitreous values from DXM and MP group could be explained by a one-compartment model with a good correlation between observed and predicted concentrations. The attempt to model vitreous TA levels failed because there were not enough data points at the terminal phase for compartmental model fitting. A non-compartmental model was achievable for contralateral vitreous TA levels. Table 5 summarizes the pharmacokinetic parameters estimated from the model.

Discussion

Topical steroids do not reach intraocular therapeutic concentrations and while periocular injections improve drug delivery to the retina, the short half-life of substances administered via this route requires frequent injections with an increased risk

Figure 1. Representative ESI-positive spectra of 2000 ng/g of (A) DXM, (B) MP, (C) TA, and (D) BM in quality control standards (S), vitreous without sodium formate (V), and vitreous with sodium formate (VNa).
Systemic administration of steroids may be useful, but high doses are required and are associated with severe harmful side effects. Intravitreal injection of steroids provides a powerful therapeutic effect with high local concentrations in the vitreous and hence the retina, but this advantage is offset by several ocular complications (e.g., secondary cataracts and increased intraocular pressure).

TA is a long-term active corticosteroid that was originally approved as a therapeutic agent for arthritis and its use as an intravitreal drug was not originally intended. The appropriate TA dose for intravitreal injection in rabbits is 1.3 mg/0.1 mL, but the high dose of TA used in the present study (10 mg/0.1 mL) was to allow analysis of drug vitreous levels for as long as possible, and because this was the resulting TA concentration obtained using the commercially available drug depot-formulation (40 mg/mL; Trigon®, Squibb Industria Farmacéutica SA, Barcelona, Spain) after sedimentation and removal of the supernatant. Although this method does not eliminate all of the vehicle of TA, previous reports indicated no differences in the vitreous levels of TA (Kenalog 4 mg).

Table 3. Intra- and inter-day precision and accuracy.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC (ng/g)</th>
<th>Intra-day assay</th>
<th>Inter-day assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RE (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>DXM</td>
<td>50</td>
<td>−8.32</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>−1.98</td>
<td>4.07</td>
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<td></td>
<td>100,000</td>
<td>2.56</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>−7.85</td>
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</tr>
<tr>
<td>MP</td>
<td>5000</td>
<td>−1.15</td>
<td>5.07</td>
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<td></td>
<td>100,000</td>
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<td>3.36</td>
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<tr>
<td></td>
<td>50</td>
<td>7.63</td>
<td>4.51</td>
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<td>TA</td>
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<td></td>
<td>100,000</td>
<td>1.92</td>
<td>3.18</td>
</tr>
</tbody>
</table>

Intra- and inter-day precision (RSD) and accuracy (RE) of DXM, MP, and TA in the vitreous humor (n = 3). QC, quality control sample; RSD, relative standard deviation, RE, relative error.

Table 4. Recovery and matrix effect.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC (ng/g)</th>
<th>ACN</th>
<th>AcOEt</th>
<th>ACN/AcOEt (50:50)</th>
<th>Mean ± SD</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXM</td>
<td>50</td>
<td>101.11 ± 7.91</td>
<td>98.57 ± 8.30</td>
<td>96.92 ± 8.54</td>
<td>105.05 ± 6.36</td>
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<td></td>
<td>5000</td>
<td>98.25 ± 5.10</td>
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<td></td>
<td>100,000</td>
<td>100.49 ± 2.93</td>
<td>103.65 ± 3.82</td>
<td>98.65 ± 3.51</td>
<td>99.48 ± 7.21</td>
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</tr>
<tr>
<td>BM</td>
<td>50</td>
<td>97.84 ± 7.83</td>
<td>99.88 ± 8.35</td>
<td>96.64 ± 8.60</td>
<td>106.99 ± 8.52</td>
<td>7.96</td>
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<td>5000</td>
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<td>50</td>
<td>80.63 ± 8.34</td>
<td>101.75 ± 7.17</td>
<td>99.01 ± 7.22</td>
<td>108.37 ± 8.96</td>
<td>8.27</td>
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<tr>
<td>TA</td>
<td>50</td>
<td>63.15 ± 7.01</td>
<td>98.50 ± 5.39</td>
<td>96.82 ± 6.35</td>
<td>105.41 ± 6.09</td>
<td>5.78</td>
</tr>
<tr>
<td></td>
<td>100,000</td>
<td>36.47 ± 3.33</td>
<td>97.92 ± 2.70</td>
<td>97.59 ± 2.87</td>
<td>107.90 ± 8.62</td>
<td>7.99</td>
</tr>
</tbody>
</table>

Recovery using different extraction solvents and matrix effect of corticoids in vitreous humor (n = 3). QC, quality control sample; ACN, acetonitrile; AcOEt, ethyl acetate; RSD, relative standard deviation.

Figure 2. Representative HPLC-MS chromatograms of 2000 ng/g concentration of (A) blank vitreous, (B) DXM versus MP, (C) MP versus BM, and (D) TA versus BM.
The TA concentration injected reported vitreous concentrations of TA to Vitreous pharmacokinetic parameters. E. PRIETO ET AL. – On the other hand, MP is an limits its clinical use until a sustained-release Clinically, a Concentration error bars are not visible, they are smaller than the symbols. produced a sustained concentration acute inflammation processes. single dose) after phacoemulsification cataract surgery or recommended dosage for subconjunctival injection (20 mg commercially available ophthalmic solutions used for the treatment of established the possibility of implanting sustained-release inserts in the vitreous body, allowing for therapeutic levels of steroids to remain in the vitreous cavity and retina for more than 6 months. The advantage of these devices is the ability to maintain a stable and sustained concentration of drug with higher therapeutic efficacy, thus reducing the number of injections. The findings of this preliminary study indicate that TA, MP, and DXM can be efficiently extracted from the vitreous humor using the developed analytical method and that these corticosteroid concentrations can be reliably measured after intravitreal injection. Thus, further comparative kinetic investigations of compared with TA preservative-free or in their half-lives (23 and 24 days, respectively). The TA concentration injected was verified before the injection to be 100 mg/mL using HPLC. DXM (1 mg/mL) was administered at a dose of 0.1 mg/0.1 mL because this is the concentration of the commercially available ophthalmic solutions used for the treatment of inflammatory conditions (without regard to the new DXM implants). On the other hand, the dose of MP used in the present study (2 mg/0.1 mL) was selected according to concentration of the commercially available injection solution (Urbason® 20 mg/mL, Sanofi, Barcelona, Spain) and the recommended dosage for subconjunctival injection (20 mg single dose) after phacoemulsification cataract surgery or acute inflammation processes.

In our study, intravitreal injection of 10 mg TA in rabbits produced a sustained concentration–time profile in the vitreous for up to 14 days post-administration. Consistent with these results, Inoue et al. reported vitreous concentrations of TA to be relatively stable for short time intervals, 3–19 days, in human eyes after intravitreal injection; they estimated that intravitreal TA would be detectable for ~4 months. Intravitreal TA delivery exhibited a depot effect that could be explained by the presence of TA crystals appearing in the vitreous that formed whitish lumps that may reduce the dilution rate of the drug and its clearance.

Conversely, the DXM and MP concentrations decreased gradually over time after administration with similar rates of decrease for both groups (\(K_e\) 5.48 and 1.46 day\(^{-1}\), respectively). There were no significant differences in the distribution volume values (\(V_{ss}\) 57.55 and 51.82 g, respectively) of both molecules, although drug elimination from the vitreous revealed substantial differences between the two steroids (\(Cl_{vitreous}\) 315.29 and 75.54 g/day, for DXM and MP, respectively). Therefore, DXM exhibited a shorter half-life (\(t_{1/2} = 3\) h) than MP (\(t_{1/2} = 11\) h) in the present study. No vitreous drug levels were detected at 14 days after the intravitreal injection of 0.1 mg DXM or 2 mg MP. Moreover, 83% of vitreous concentration values in the contralateral eyes were below the LLOQ for both groups. DXM is an anti-inflammatory steroid that is more potent than TA; however, its short half-life (3.5 h) limits its clinical use until a sustained-release DXM device is developed. On the other hand, MP is an intermediate-term active corticosteroid that is less potent than DXM and with a similar half-life (2–3 h), and is preferably used to treat acute inflammatory processes due to its fast onset of effect.

TA is almost insoluble in water and remains in the vitreous cavity longer than the other corticosteroids and the vitreous cavity has increasingly been used as a reservoir of drugs for the direct treatment of intraocular diseases. Clinically, a longer duration may be of benefit for treating chronic eye diseases and therefore different research groups have established the possibility of implanting sustained-release inserts in the vitreous body, allowing for therapeutic levels of steroids to remain in the vitreous cavity and retina for more than 6 months. The advantage of these devices is the ability to maintain a stable and sustained concentration of drug with higher therapeutic efficacy, thus reducing the number of injections.

The findings of this preliminary study indicate that TA, MP, and DXM can be efficiently extracted from the vitreous humor using the developed analytical method and that these corticosteroid concentrations can be reliably measured after intravitreal injection. Thus, further comparative kinetic investigations of

Table 5. Vitreous pharmacokinetic parameters.

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Units</th>
<th>TA_contralateral</th>
<th>DXM</th>
<th>MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_0)</td>
<td>ng/g</td>
<td>391.70</td>
<td>1737.71</td>
<td>38,597.81</td>
</tr>
<tr>
<td>(K_e)</td>
<td>Day(^{-1})</td>
<td>–</td>
<td>5.48</td>
<td>1.46</td>
</tr>
<tr>
<td>(t_{1/2})</td>
<td>Day</td>
<td>13.23</td>
<td>0.13</td>
<td>0.48</td>
</tr>
<tr>
<td>(V_{ss})</td>
<td>g</td>
<td>12,914.41</td>
<td>57.55</td>
<td>51.82</td>
</tr>
<tr>
<td>(Cl)</td>
<td>g/day</td>
<td>676.52</td>
<td>315.29</td>
<td>75.54</td>
</tr>
<tr>
<td>(AUC_{0-\infty})</td>
<td>ng/g/day</td>
<td>14,781.57</td>
<td>317.17</td>
<td>26,475.56</td>
</tr>
</tbody>
</table>

Vitreous pharmacokinetic parameters following intravitreal administration of 10 mg triamcinolone acetonide (TA), 0.1 mg dexamethasone (DXM), and 2 mg methylprednisolone (MP).

Not enough data points for compartmental model fitting. A non-compartmental model was achievable for contralateral vitreous TA levels.

\(t_{1/2}\) value was determined by calculation of the lambda Z parameter (0.05 day\(^{-1}\)).
conventional steroid formulations (suspensions and solutions) and sustained-release formulations can be performed using this accessible analytical method.

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**Declaration of interest**
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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