HPLC-UV analytical validation of a method for quantification of progesterone in *ex vivo* trans-corneal and trans-scleral diffusion studies

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1. Introduction

Progesterone (PG), pregn-4-ene-3, 20-dione (Figure 1), is a C-21 steroid hormone which has been shown to inhibit apoptosis and inflammation [1,2] and hence it may have therapeutic potential for the treatment of various neurodegenerative diseases. Its safety and viability have been demonstrated in several models of traumatic brain injury [2]. Although it has been shown that PG can reduce free radical damage after traumatic brain injury in rats, its chemical structure does not resemble that of typical antioxidants [3].

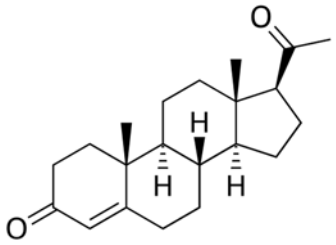

Figure 1: Structure of progesterone (PG).

Retinitis pigmentosa (RP) is a heterogeneous group of genetic retina degenerative diseases affecting photoreceptor cells. RP is the most frequent cause of hereditary blindness [4]. RP causes the death of the photoreceptor cells and begins affecting rod cells [5]. As the number rod photoreceptors decrease, tunnel vision and nyctalopia (night blindness) ensue. As the disease progresses, cone cells suffer significant reduction in acuity of the central vision and even full blindness, when the disease is advanced. It has been shown that PG has a positive effect in the treatment of RP after being administered orally, either alone or with oil as a carrier [3].

Chemically PG is a highly hydrophobic sex steroid hormone (log P = 3.9) with very low solubility in water at room temperature (7-10 µg/mL) [6]. PG dissolves slowly and incompletely in gastrointestinal fluids, which together with its low solubility and rapid liver metabolism, limits its oral bioavailability. All this, taken together pose a significant problem for the developing of oral formulations of the drug [7]. PG's low solubility also hinders the therapeutic application of PG in ocular pathologies.

Diffusion of topically applied molecules at the ocular level is very low, due to the inherent anatomical and physiological barriers present in the eye [8]. Ocular bioavailability of drug diffusion after ocular application represents less than 5% and in some cases can be as low as 1% [9–11]. Topical drug administration remains the preferred route of administration over intracameral or intravitreal injections for the treatment of eye diseases such as RP. Ease of application and high patient compliance justify this preference. Analytical methods for the determination of PG by HPLC with UV detection have been previously described [12–15] but the PG concentrations detected are relatively high. In ex vivo ocular diffusion studies, some contamination from biological material in the samples is unavoidable and may interfere with chromatographic detection of PG. Additionally, the methods previously described identify

several molecules in addition to PG which resulted in lengthy procedures. We therefore needed a method free from interferences, with lower limits of detection and quantification and preferably shorter retention times which would result in an optimized method for HPLC determination of PG.

The aims of this study were to validate a method to determine PG in ocular *ex vivo* studies. but it becomes necessary to adapt these methods to our specific situation, and to assess the feasibility of ocular administration of PG by determination of its diffusion through cornea and sclera. For this, *ex vivo* trans-corneal and trans-scleral permeation of PG was determined.

2. Materials and methods

2.1. Materials

Progesterone (PubChem: 5994): methyl-β-cyclodextrin complex (85.2 mg of progesterone per gram), was obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetonitrile, ultrapure water, NaOH (sodium hydroxide) and NaCl (sodium chloride) were obtained from Análisis Vínicos, S.L. (Tomelloso, Spain).

2.2. Preparation of Standard Solutions

Stock solutions of PG (1 mg/mL) were prepared in Phosphate Buffer Solution (PBS) (pH 7.4; 150 mM). Six standard solutions for PG (0.5, 1, 5, 10, 50 and 100 µg/mL) were prepared by further dilution of the stock solution in isotonic buffer. These standard fresh solutions were used for the calibration curves.

2.3. Instrumentation and chromatographic conditions

HPLC was performed using a Waters system equipped with a quaternary pump (Waters 1525), a UV/VIS diode-array detector (Waters 2707) and an automatic injector fitted with 50 µL sample loop (Waters 2998 Plus). Computerized data acquisition and treatment were performed with the Breeze2® software. The chromatographic separation of the analytes was performed at room temperature (25 ± 2ºC) using a Waters Sunfire C18 (150 x 4.6 mm) reverse-phase column packed with 5 µm silica particles. The mobile phase consisted of a mixture of acetonitrile (ACN) and pure water 80:20 (v/v), pH 7.4. The mobile phase was filtered through a 0.45 µm ester cellulose membrane filter DURAPORE® (Millipore Corporate, Billerica, MA, USA). Samples were eluted at a flow rate of 1.0 mL/min and absorbance was measured at 240 nm.

2.4. Validation

The method was evaluated in terms of specificity, linearity, precision, accuracy, limit of detection, limit of quantification and robustness. The analytical method was validated with six different concentrations of progesterone (0.5, 1, 5, 10, 50, 100 µg/mL). Four aliquots were assayed to determine within-day reproducibility. Analysis was repeated on three different days to determine between-day validation.

Calibration curves were obtained by the least squares linear regression of the peak area as a function of PG concentration. The linearity of the calibration curves was tested by statistical comparison of the slopes with zero and the correlation coefficient with 1. Accuracy expresses the closeness of agreement between a calculated value and the accepted reference value (true value) and it is calculated as the relative error of known concentration solutions. To be considered acceptable, measures should fall within ± 10% for all concentrations [16]. The precision of the analytical method provides information about the random error and corresponds with the closeness of agreement between a series of measurements obtained from multiple analyses of the same homogeneous sample under prescribed conditions. It is measured as the relative standard deviation [RSD (%)] of the areas analyzed for each concentration. The RSD is considered acceptable when it is lower than 10% at all analyzed concentrations [17,18]. In order to determine the specificity of the method, absence of interference was evaluated by analyzing 10 blank samples from the same isotonic buffer used to prepare the standard solutions for calibration [PBS, pH 7.4; 150 mM].

The limit of detection (LOD), the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified, was expressed as the concentration that yielded a peak area equal to three times that of baseline noise at the retention time of the drug. The limit of quantification (LOQ) was the smallest quantity of a substance in a sample that could be quantitatively determined with well-defined

accuracy under the experimental conditions. Both limits were determined chromatographically for the drug by examining of the signal to noise at rate the lowest concentration [17,18].

Assuming a normal distribution of measured concentration values, LOD and LOQ were calculated from the residual standard deviation of the regression data according to the criteria LOD=3.3x(S.D./b) and LOQ=10x(S.D./b) where S.D. is the residual standard deviation and b is the slope of the linear regression equation. [17,18]

The robustness of a method is the ability to remain unaffected by small changes in operating conditions. To assess robustness (i.e. the ability of a method to remain unaffected by small changes in operating conditions), experimental conditions were purposely altered at three different levels and chromatographic response and retention times were evaluated as described elsewhere [19]. Briefly, in order to study their effect, factors were altered one at a time as follows: a) the detector wavelength was varied by 2 nm (238 nm and 242 nm); b) the composition of the mobile phase at buffer solution and ACN ratio were varied to 82:18 (v/v) and 78:22 (v/v); c) the pH of the mobile phase was modified by 0.2 units (7.2 and 7.6 buffer pH) and d) the flow rate of mobile phase by 0.1 units (0.9 and 1.1 mL/min).

2.5. Stability of Drugs in Solution

Seven groups of a concentration sample (50 µg/mL) of PG in cyclodextrins were stored in different conditions to determine the stability of the compound in aqueous solution. Firstly, these solutions were injected, and quantified while being protected from light. They were then divided into aliquots and stored in darkness under different conditions: in a freezer (-80 ºC), refrigerator (4 ºC), laboratory incubator (37 ºC) and at room temperature (25 ± 2 ºC). Other samples of the solution were stored at room temperature but exposed to light. All samples were assayed 3, 7 and 17 days after preparation to determine the residual PG concentration.

2.6. Ex vivo Ocular Diffusion of Progesterone.

The validated HPLC method was used to quantify progesterone in the samples obtained from *ex vivo* ocular diffusion studies. These experiments were carried using all-glass Franz diffusion cells purchased from DISA (Milan, Italy) with a diffusion area of 0.567 cm^2. Corneas and scleras from rabbits' eyes where used as membranes to separate the donor and receptor compartments (Figure 2). Whole eyes from two-month-old hybrid albino rabbits of either sex were obtained immediately after sacrifice. Eyeballs were rinsed in saline solution to remove any trace of blood, after which the adherent muscle was removed with scissors. Fresh excised corneas and scleras were obtained by cutting along the sclera-limbo junction and the individual excised tissue was used for diffusion studies [20,21]. The experimental protocol was approved by the Ethical Committee of University CEU Cardenal Herrera (Ref. 2011/010) and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (Ref. No. 2017/VSC/PEA/00192). Prior to sacrifice, animals were housed, fed, and handled according to current animal welfare principles (Spanish Royal Decree 1201/2005, (BOE 2005)).

A solution of PG in β-cyclodextrins (102 µg/mL) was prepared in an isotonic buffer [PBS, pH 7.4] and 0.5 mL of this solution was placed in the donor compartment. The receptor chamber was filled with propylenglycol:water (40:60 v/v) pH 7.4 (4.2 mL) [22], the receptor chambers were submerged in a water bath at 37ºC and stirred by a rotating magnet placed inside the cell to prevent boundary layer effects. Samples (200 µL) were manually obtained from the receptor chamber at 30, 60, 90, 120, 180 min, and an identical volume of pre-warmed fresh medium was added. The amount of PG in each sample was quantified by HPLC. At the end of the *ex vivo* ocular diffusion studies, the amount of drug retained in each cornea and sclera was extracted by placing them in a solution of acetonitrile: water (80:20) for 12 hours. PG concentration was measured by the HPLC method described previously.

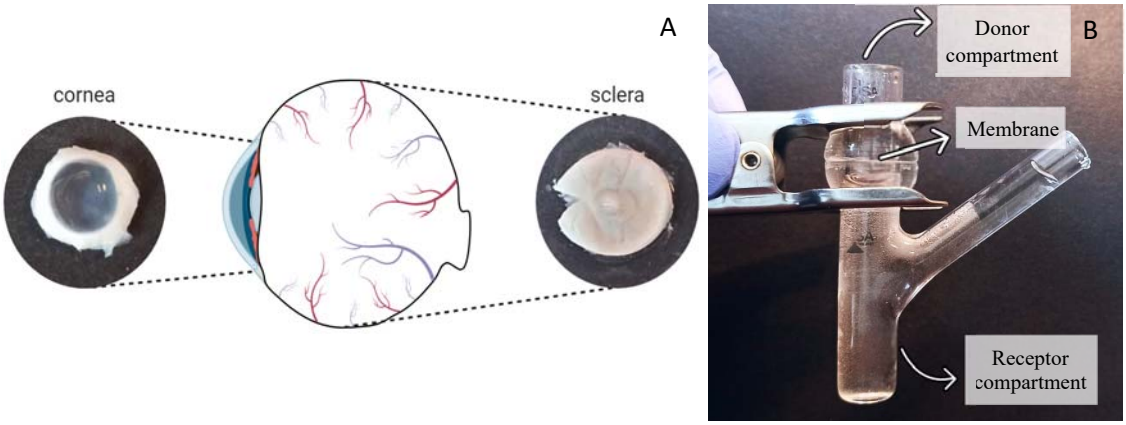

Figure 2. Diagram of an eye with photographs of the dissected cornea and sclera from the eye of a rabbit (A). Franz cell used in ocular *ex vivo* diffusion experiments (B).

2.7. Statistical analysis

Statistical analysis was performed using the Mann-Whitney test between the values obtained for both membranes at each of the ocular diffusion study times. The confidence level was 95%. The same test was also used to evaluate differences in accumulated amount between the different membranes. ANOVA was used to detect statistical comparison of the slopes with zero and the correlation coefficient with 1 in regression curves. In order to assess the robustness of the assay a one-way ANOVA, which would be followed if appropriate by the Scheffé post hoc test, was used to compare the effect of these variations on the detector response or on the retention time (depending on the change applied).

3. Results and discussion

3.1. Validation method

The solubility of PG in isotonic buffer solution [pH 7.6, 25°C] is 7 µg /mL [23]. Given its log P value, it can be catalogued as practically insoluble in water, thus PG enclosed in cyclodextrins was used to ensure adequate solubility. An analytical method for quantifying PG in samples from *ex vivo* corneal or scleral permeation experiments needs to be highly specific; as such, samples usually contain endogenous compounds released from the eye. Furthermore, the method must be rather sensitive because the frequently low concentrations of PG in the collected samples.

Various HPLC methods that rely on UV-vis detection for PG determination have been described [14,15]. In order to identify which method is more sensitive and specific and to adapt it to the detection of PG in samples from *ex vivo* experiments, we have made modifications to avoid having overlapping peaks from biological material present in the samples.

The chromatographic method devised by Maliwal *et al*. (2009) consists of a Linchrocart C18 column (4.0 x 250 mm) with a 5 µm pore, a mobile phase of methanol and water 80:20 (v/v), a flow-rate of 1 mL/min, a wavelength of 254 nm, and an injection volume of 20 µL. These researchers reported a retention time of 6.39 min for PG. The chromatographic method reported by Wilson (2009) uses a Waters µBondapak C18 column (3.9 x 300 mm) with a 10 µm particle size, a mobile phase of ACN and water 50:50 (v/v), a flow-rate of 1 mL/min monitored at 270 nm, and an injection volume of 40 µL. Using this approach, the authors reported a retention time of 20.7 min. Our method consists of a Waters Sunfire C18 (150 x 4.6 mm) with a 5 µm pore, a mobile phase of ACN and water, 80:20 (v/v), at pH 7.4, an injection volume of 50 µL a flow-rate of 1 mL/min. and monitoring the signal at 240 nm. In our method, PG spectra displayed a maximum peak of absorbance at 240 nm. We selected this for detection, as there were not interferences from the biological samples at the molecule retention time at this wavelength, which proved to be sensitive and specific enough to analyze PG at all the concentrations found in our samples.

Table 1. Summary of validated progesterone detection methods between Maliwal *et al*. (2009), Wilson (2009) and our method. The flow rate is 1mL/min in all of them.

Method	Column	Mobile phase	Wavelength	Injection volume	Retention time	Range µg/mL	LOD µg/mL	LOQ µg/mL

Maliwal *et al.*(2009)	Linchrocart C18, (250 X 4.0 mm), 5 µm pore	Methanol : water 80:20 (v/v)	254 nm	20 µL	6.39 min	Not reported	Not reported	Not reported
Wilson (2009)	Waters µBondapak C18, (300 X 3.9 mm), 10 µm pore	ACN : water 50:50 (v/v)	270 nm	40 µL	20.7 min	32.2–161.0	0.8	1.6
Our method	Waters Sunfire C18, (150 x 4.6 mm), 5 µm pore	ACN : water 80:20 (v/v)	240 nm	50 µL	3.42 min	0.5-100	0.42	1.26

Using the method described here retention time for PG was found to be 3.42 min (Figure 4) in contrast to retention times of 20.7 min and 6.39 min reported by Wilson and Maliwal *et al.* respectively [14,15], as shown in table 1.

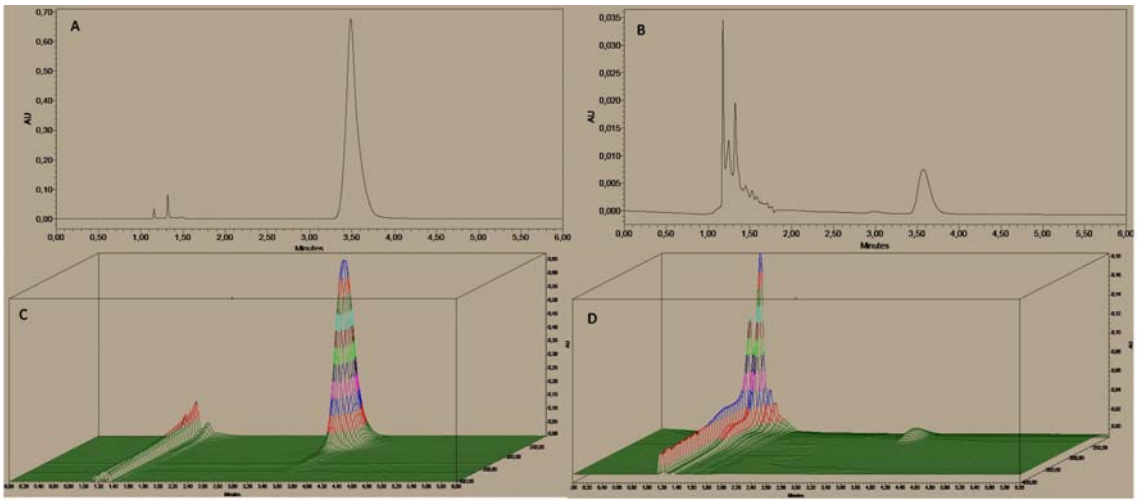

Figure 3. 2D chromatograms of 50 µg/mL PG standard solution (A) and sample obtained from ex vivo ocular diffusion experiments (B) at 240 nm. 3D chromatograms of 50 µg/mL PG standard solution (C) and sample obtained from ex vivo ocular diffusion experiments (D) for all wavelengths assayed (z-axis).

The LOD and LOQ for PG were 0.42 and 1.26 µg/mL respectively in contrast to 0.8 and 1.6 µg/mL reported by Wilson in 2009 (Table 1) [15]. Linearity was obtained in a concentration range of 0.5–100 µg/mL, in contrast to values reported by other authors (32.2-161.0 µg/mL) [15] (Table 1). These modifications of the analytical method allow a larger number of samples from ocular diffusion experiments to be processed faster and more efficiently and also allows PG to be quantified at lower concentrations than those reported by other groups [14,15] without the interferences produced when other detectable molecules from the ocular membranes were present in the samples.

Under these chromatographic conditions, we were able to separate and identify PG from other endogenous compounds present in the eye when our samples were analyzed. Representative chromatograms for standards and samples obtained from our ocular diffusion studies are shown in Figure 4. Ten blank samples were analyzed to investigate the specificity of the method. No interference was detected at the retention time of PG. The method exhibited linearity between the response (y) and the corresponding concentration of PG (x) over the range of concentrations assayed. An average calibration curve was constructed from the results obtained: y = 232554 (±315) x-19079 (±13420). The calibration regression curve together with the 95% confidence interval (CI) is shown in figure 5. The results of the least square linear regression analysis showed a correlation coefficient of $r^2 \geq 0.99999$. The slope of the calibration curve was statistically different from zero, and the intercept was not statistically different from zero. The results of between-day and within-day precision and accuracy are shown in Table 2. It can be seen that calculated values were below 10% in all cases. The highest RSD value (4.98%) was obtained by the 1 µg/mL concentration studied in the within-day condition, hence, being within the percentage limits.

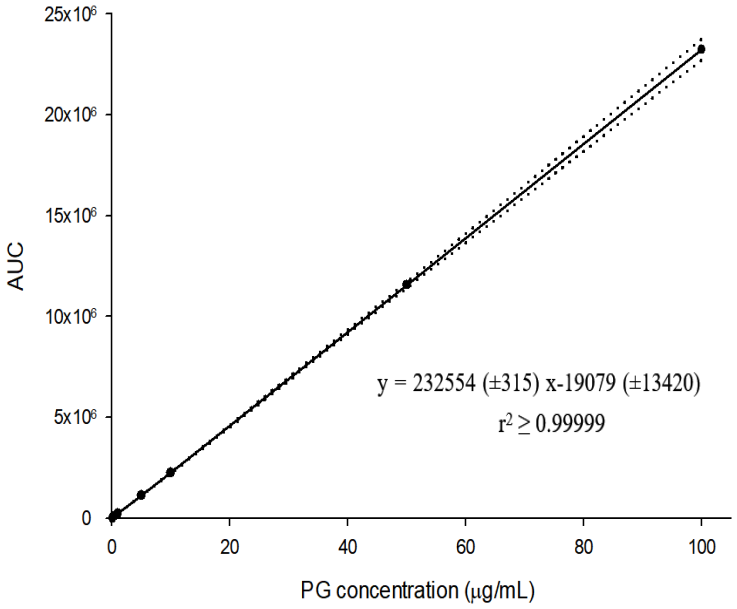

Figure 4. Linear regression of the average calibration curve of PG represented with the 95% confidence intervals (CI).

Table 2. Between- and within-day variability, accuracy and precision of the HPLC method for determining progesterone concentrations in saline-buffered samples.

Real concentration (µg/mL)	Between-day Variability (n=5)			Within-day Variability (n=5)		
	Concentration Found (mean ±SD) (µg/mL)	Accuracy (%)	RSD (%)	Concentration Found (mean ± SD) (µg/mL)	Accuracy (%)	RSD (%)
0.5	0.46 ± 0.01	-7.0	2.71	0.47 ± 0.01	-5.0	1.20
1	0.90 ± 0.01	-9.4	1.14	1.00 ± 0.05	-0.1	4.98
5	4.79 ± 0.12	-4.0	2.55	4.70 ± 0.09	-5.9	1.95
10	9.67 ± 0.13	-3.4	1.39	10.43 ± 0.37	4.4	3.54
50	49.70 ± 0.65	-0.8	1.31	49.42 ± 0.58	-1.2	0.58
100	99.92 ± 2.22	0.1	2.22	100.18 ± 4.30	0.5	4.30

To determine the robustness of the method, experimental conditions were deliberately altered at different levels. Variation of the detector wavelength, composition, pH and flow rate of the mobile phase had no statistically significant effect (ANOVA; $p>0.05$) on the retention time and chromatographic response of the method. The results for the robustness of the PG method are shown in Table 3 and confirm that the modifications carried out did not have an important effect on parameters. Thus, we can confirm that our method is robust.

Table 3. Robustness values of detector and mobile phases. Composition is expressed as percentage of ACN:water (v/v).

Condition		Modification	Area (mean ± SD)	RSD (%)	Retention Time (min) (mean ± SD)
Detector wavelength (nm)		238	6654444 ± 41232	0.62	3.42 ± 0.012
		240	6672351 ± 4262	0.06	3.42 ± 0.010
		242	6642486 ± 43059	0.65	3.41 ± 0.015
Mobile phase	pH	7.2	6654711 ± 27983	0.42	3.41 ± 0.014
		7.4	6649987 ± 3102	0.05	3.41 ± 0.011
		7.6	6661878 ± 19235	0.29	3.41 ± 0.016
	Composition (v/v)	78:22	6620115 ± 19847	0.30	3.42 ± 0.003
		80:20	6646364 ± 36694	0.55	3.41 ± 0.003
		82:18	6679568 ± 21338	0.32	3.42 ± 0.004
	Flow rate ($mL \cdot min^{-1}$)	0.9	6655213 ± 3598	0.05	3.42 ± 0.009
		1.0	6661676 ± 30616	0.46	3.41 ± 0.001

		1.1	6689396 ± 52098	0.78	3.41 ± 0.002

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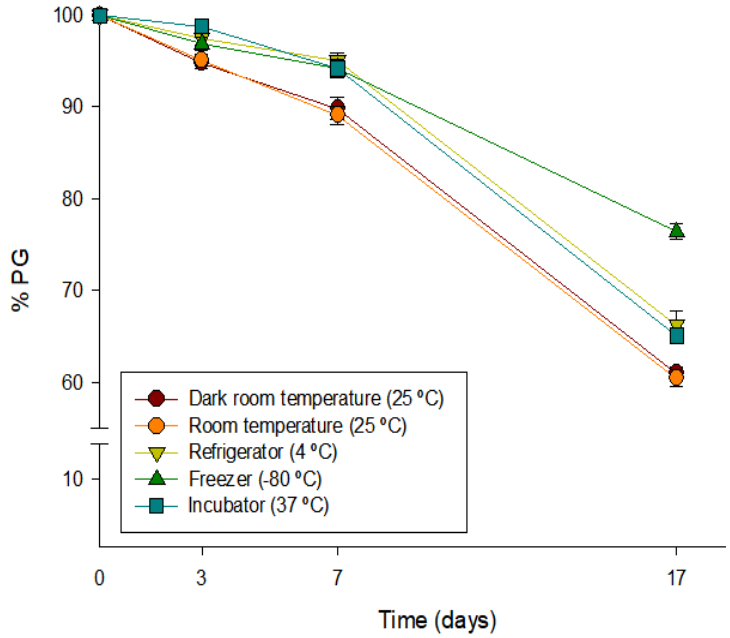
228 3.2. Progesterone Stability in Aqueous Solution

229 The results obtained in the stability test are shown in figure 6 as concentration percentage ± SD
230 *vs.* time (days) for each condition studied. On day 3, none of the samples showed a concentration below
231 90% of the initial concentration, and on day 7 only samples stored in the dark at room temperature and
232 those stored at room temperature under light exposure showed a concentration below 10% of the initial
233 value. The stability results obtained show a significant decrease in PG values after 17 days storage
234 regardless of temperature and light conditions. In all cases the level of PG quantified was between 60 and
235 80% of initial PG values. PG has low stability [24], although it might increase when dissolved in ethanol
236 or in oil microemulsions [24,25]. However, even complexed with cyclodextrin our studies showed low
237 stability of the molecule in a PBS solution (Figure 6). Similar results showing degradation of PG in bovine
238 milk have also been described [26].



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240 **Figure 5.** Stability results obtained for PG in standard solutions over 17 days. Recovery (%) ± SD (%), n=3.

241 *3.3. Ex vivo Ocular Diffusion of Progesterone.*

242 The amounts of PG accumulated in the receptor compartment of a PG solution (initial solution
243 concentration 102 µg/mL) were plotted against time (Figure 7). PG was first detected after 60 minutes in
244 the diffusion experiments using corneas as the membrane whereas in experiments with sclera PG was
245 detected after 15 minutes. Statistical differences ($p<0.05$) were detected between corneal and scleral
246 concentrations for all sample times after PG was detected (Figure 7).

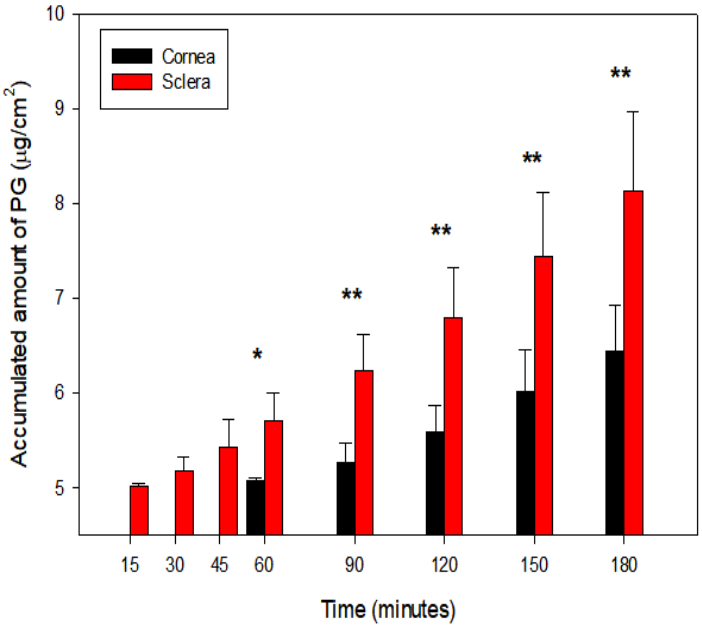

Figure 6. Amounts of PG accumulated in receptor chamber (µg/cm^2) *vs.* time (minutes) obtained in the ocular *ex vivo* experiments developed with cornea and sclera. Error bars show standard deviation of the observed values (n = 6). Significant differences were found between amounts of PG diffused through cornea and sclera at different times, *($p<0.05$) and **($p<0.01$).

After 3 hours of experiment, PG retained in the cornea and sclera were 40.87 ± 9.84 µg/cm^2 and 56.11 ± 16.67 µg/cm^2 (mean ± SD; n = 6) respectively. Statistical differences were found between the amount of PG retained in cornea and sclera (p = 0.009).

The accumulated amount of PG in receptor compartments was 6.57 ± 0.37 µg/cm^2 (n = 6) for trans-corneal and 8.13 ± 0.85 µg/cm^2 (n = 6) for trans-scleral diffusion. Thus, trans-scleral diffusion of PG was statistically higher than trans-corneal diffusion (p= 0.002). PG has two predicted pKa values, one in acid media (pKa = 18.92) and one in basic media (pKa = -4.8) so the protonation state of the drug is dependent on the pH of the solution; at physiological pH the molecule would not be charged. PG is a lipophilic and small molecule (log P_{oct} = 3.9; MW = 314.5 g/mol) [27], therefore, as it is expected, PG has a reasonable trans-ocular permeation [28]. The accumulated results showed greater amounts of PG permeating through sclera than through cornea. T he may be due to different histological characteristics of the two tissues. The cornea is a complex tissue with a minimum of five different layers [29] with an outer epithelial layer (stratified squamous non keratinized) Bowman and Descemet membrane, which would interfere differently with hydrophilic and lipophilic molecules [29]. Diffusion to the internal part of the eye through the cornea may be rather difficult. On the other hand, the sclera is made up of collagen fibers arranged to create a dense connective tissue which may make trans-scleral diffusion of PG easy [30].

After 3 hours of experiment, the amount of PG retained in corneal and scleral tissue was quantified and found to be 40.87 ± 9.84 µg/cm^2 and 56.11 ± 16.67 µg/cm^2 (mean ± SD; n = 6) respectively. Statistical differences were found between the amount of PG retained in cornea and sclera (p = 0.009).

The results obtained show that PG is quantifiable and passes through cornea and sclera accumulating moderately in these structures, probably due to its lipophilicity. Furthermore, a modification of this HPLC-UV analytical method has been used for the quantification of PG (without cyclodextrins) in encapsulated micelles of Soluplus and Pluronic F68 [31]. Although the target concentration of PG in the retina needed to produce a significant therapeutic effect is unknown, the results obtained in this study provide interesting data for the development of eye formulations of PG as a possible treatment for RP. Future studies will complement these results with new formulations with greater capacity to control release and with a longer ocular residence time than the aqueous or micellar drops previously studied.

4. Conclusions

We report the validation of a simple chromatographic method for the rapid and precise determination of PG after ocular *ex vivo* diffusion studies. The specificity, limits of detection and quantification, accuracy, precision and robustness of the HPLC method here reported allow quantitative determination of PG contained in samples obtained from *ex vivo* ocular permeation experiments adequately. The stability results obtained, showed that PG can be stored refrigerated (4 ºC) or frozen (-80 ºC) for at least 7 days without its concentration dropping to less than 10%. Trans-corneal and trans-scleral diffusion of PG has been characterized under passive diffusion conditions. Therefore, we can confirm that the described method is adequate for the quantification of PG *ex vivo*. The present work will lay the foundations for future research studies of new ocular formulations for the release of PG after its topical application to the eye.

5. Funding

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