Nanoparticles as carrier for improve therapeutic efficacy of pioglitazone in ocular inflammatory disorders: development and validation of a high throughput HPLC–MS/MS method for its quantitation in ocular tissues.

Esther Miralles-Cardiel[1,2*], Marcelle Silva-Abreu[3,4], Ana Cristina Calpena-Capmany[3,4*], Isidre Casals-Ribes[1].

[1]CCiTUB (Scientific and Technological Centers), University of Barcelona, 08028 Barcelona, Spain; emiralles@ub.edu; isidre@ccit.ub.edu.
[2]Department of Analytical Chemistry, Faculty of Chemistry, University of Barcelona, 08028 Barcelona, Spain.
[3]Department of Pharmacy, Pharmaceutical Technology and Physical Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; silvadesabreu@ub.edu.
[4]Institute of Nanoscience and nanotechnology (IN2UB). Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; anacalpena@ub.edu.
*Correspondence: emiralles@ub.edu; Tel.: +34 93 403 4652/anacalpena@ub.edu; Tel.: +34 93 402 4578.

INTRODUCTION

In ocular therapies one of the challenges is the effective penetration of the drugs through the eye's tissue barriers to reach targets and to sustain it. Normally, when ophthalmic formulations are used, less than 5% of the drug permeates the cornea which means that it is necessary to instill frequently. One of the most successful approaches to overcome this inconvenient is the use of colloidal suspensions of nanoparticles (NPs) as delivery systems [1,2]. Polylactic-co-glycolic acid (PLGA) is one of the most studied synthetic polymers due to its biocompatibility and biodegradability. The advantage of the PEGylated polymer PLGA-PEG is that the hydrophobicity of the polymer decreases, thus increasing the stability and solubility in aqueous media and avoiding aggregation. Moreover PEG (polyethylene glycol) is biocompatible.

In recent years some studies of our research group have been focused on pioglitazone (PGZ) nanoparticles of PLGA-PEG. These nanosystems have been optimized and characterized, and the ocular anti-inflammatory activity as well as the tolerance have been proven [3].

PGZ is a hypoglycemic therapeutic drug used in the treatment of type 2 diabetes. According to the Biopharmaceutical Classification System (BCS) falls into Class II, i.e., slightly soluble and highly permeable. This drug is an agonist of the peroxisome proliferator-activated receptor (PPARγ) which has reported functions as anti-inflammatory [4]. Some studies proved that PGZ has effects on inflammatory ocular processes [3,5,6], skin [7,8], heart [9,10] or Azlheimer [11,12].

PURPOSE

The objective of this research was to develop and validate an HPLC-MS/MS method following the guidelines of the European Medicines Agency-2019 and U.S. Food and Drug Administration-2018 for bioanalytical methods validation [13,14] to focus the analysis on the application of PGZ-NPs in the eye via an *in vivo* model for its use in inflammatory processes.

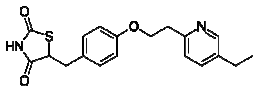

Figure 1. Pioglitazone structure.

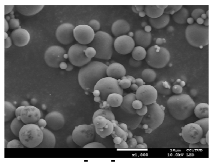

Figure 2. SEM image of PGZ-NPs.

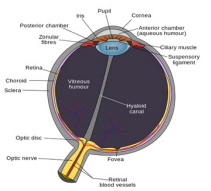

Figure 3. Eye anatomy (from *differencebetween.com*).

METHODS

Chromatographyc conditions:

Agilent 1260 liquid chromatograph
Kinetex C18 column (2.6 µm, 50x2.1 mm) (Phenomenex)
Gradient: formic acid 0,1 % in water (A) and formic acid 0,1 % in acetonitrile (B)
(t(min), %B), (0, 10), (4, 74), (4.5, 90), (6, 90), (6.1, 10), (10, 10)
Column temperature 35 °C
Flow rate 0.6 ml/min
Injection volume 1 µl
Triple quadrupole mass spectrometer 4000 QTRAP (AB Sciex Instruments)
Multiple reaction monitoring (MRM) mode. PGZ m/z transition pairs: quantitation (precursor ion/product ion) 357.2/134.1 (most sensitive), confirmation 357.2/119.1.

Biological material
Ocular specimens from pigs.
Validation: non treated eyes.
In vivo bioavailability study: topical administration of 0,05 ml of a PGZ-NPs suspension 1 mg/ml PGZ (4h)

Extraction
Cornea, sclera, lens: 125 mg
Aqueous, vitreous humours: 250 mg
Extraction with 2 ml methanol-30 min
Centrifugation, filtration for HPLC

Spiked levels
Cornea, sclera, lens: 160, 320 , 1600 µg/kg
Aqueous/vitreous humours: 80, 160 , 800 µg/kg

Validation parameters
selectivity
specificity
matrix effect
calibration curve
LOD, LOQ
accuracy
precision
recovery
carry-over
dilution integrity (50-fold dilution)
stability

ACKNOWLEDGE: to HPLC/MS Technicians Alberto Adeva-Antón and Olga Jáuregui-Pallarés of Separative Techniques Unit of the Scientific and Technological Centres of the University of Barcelona (CCiTUB) for help with the HPLC/MS management. The authors would like to thank Lídia Gómez-Segura and Álvaro Gimeno-Sandig of the Bellvitge Hospital of Barcelona for her assistance in the management of the animals used in the experiments.

RESULTS

Table 1. Absolute recovery (values in mean percentages) of PGZ. Intra-day results (n=3). (*Mean±SD)

Nominal concentration in extract	Recovery (%)				
ng/ml	Lens	Cornea	Sclera	Aqueous humour	Vitreous humour
10	89,0 ± 1,9* CV(%)= 2,1	96,9 ± 0,5 CV(%)= 0,5	97,7 ± 3,3 CV(%)= 3,4	92,2 ± 0,4 CV(%)= 0,4	89,5 ± 1,6 CV(%)= 1,7
20	85,0 ± 1,4 CV(%)= 1,7	100,9 ± 2,2 CV(%)= 2,2	98,5 ± 2,7 CV(%)= 2,7	91,6 ± 1,2 CV(%)= 1,3	89,4 ± 0,4 CV(%)= 0,4
100	85,2 ± 0,5 CV(%)= 0,6	93,9 ± 3,2 CV(%)= 3,4	88,4 ± 2,7 CV(%)= 3,1	86,4 ± 1,1 CV(%)= 1,3	86,7 ± 1,7 CV(%)= 1,9
2500	109,6±3,8 CV(%)= 3,5	85,0±0,7 CV(%)= 0,8	88,0 ± 0,4 CV(%)= 0,4	97,2 ±1,8 CV(%)= 1,9	99,6 ± 0,4 CV(%)= 0,4

Table 2. Absolute recovery (values in mean percentages) of PGZ. Inter-day results (n=9). (*Mean±SD)

Nominal concentration in extract	Recovery (%)				
ng/ml	Lens	Cornea	Sclera	Aqueous humour	Vitreous humour
10	93,1 ± 4,9* CV(%)= 5,2	97,8 ± 1,5 CV(%)= 1,5	89,7±7,9 CV(%)= 8,8	92,3 ± 8,4 CV(%)= 9,1	98,3 ± 6,7 CV(%)= 6,8
20	91,8 ± 5,3 CV(%)= 5,8	97,4 ± 3,1 CV(%)= 3,2	89,1 ± 8,2 CV(%)= 9,2	92,5 ± 5,7 CV(%)= 6,2	98,5 ± 6,9 CV(%)= 7,0
100	87,8 ± 2,2 CV(%)= 2,4	93,0 ± 6,9 CV(%)= 7,4	85,0 ± 3,1 CV(%)=3,6	87,7 ± 4,8 CV(%)= 5,4	92,3 ± 4,3 CV(%)= 4,7

Table 3. Intra-day accuracy and precision data (n=3) for tissues spiked with PGZ-NPs. (*Mean±SD)

Nominal concentration in extract	Recovery (%)				
ng/ml	Lens	Cornea	Sclera	Aqueous humour	Vitreous humour
8,4	87,2± 2,3* CV(%)=2,6	88,3±0,5 CV(%)=0,6	92,0±3,4 CV(%)=3,7	87,4±3,7 CV(%)=4,2	98,4±6,0 CV(%)=6,1
16,8	107,7±3,2 CV(%)=3,0	92,1±1,0 CV(%)=1,1	94,9±2,3 CV(%)=2,4	85,2±1,1 CV(%)=1,3	98,1±1,9 CV(%)=2,0
84,2	102,8±2,2 CV(%)=2,2	90,2±1,6 CV(%)=1,8	88,5±1,1 CV(%)=1,3	85,3±0,6 CV(%)=0,7	95,2±2,4 CV(%)=2,5
2094	109,6±3,8 CV(%)=3,5	85,0±0,7 CV(%)=0,8	88,0±0,4 CV(%)=0,4	99,9±0,4 CV(%)=0,4	110,8±2,1 CV(%)=1,9

Table 4. Analysis of samples: pioglitazone found in tissues after PGZ-NPs ocular administration (n=2).

	Pioglitazone in extract (ng/ml)		Pioglitazone in tissue as is (mg/kg)		
	Average	SD	Average	SD	CV (%)
Sclera	735,5	16,0	11,81	0,26	2,18
Cornea	153,4	2,4	4,84	0,07	1,55
Aqueous humour	89,8	1,9	0,74	0,02	2,08
Vitreous humour	185,0	5,4	1,59	0,05	2,92

Method Validation Results

-Selective
-Specific
-No matrix effect
-Calibration curve:
  5-100 ng/ml
  Linearity: r^2>0.99
  Back-calculated 91-108%
-LOD: 0,4-0,8 µg/kg
-LOQ:
  160 µg/kg cornea, sclera, lens 80 µg/kg aqueous and vitreous humours
-Accuracy: -15% to +10%
-Precision:
  intra-day CV<5%
  inter-day CV<10%
-Recovery: 85-110%
-Carry-over: <4% of LOQ.
-Dilution integrity (50-fold dilution):
  agrees accuracy
  agrees precision
-Stability:
  8h room temperature
  24h refrigerated
  3 months -20°C

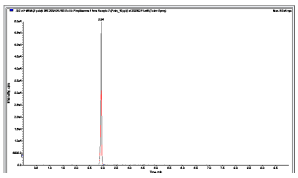

Figure 4. MRM chromatogram of PGZ in swine cornea treated with PGZ-NPs.

CONCLUSIONS

An accurate, sensitive, selective, reproducible and high throughput HPLC-MS/MS method was developed and fully validated for the quantitative determination of PGZ over a wide concentration range in different eye tissues. This method has the advantage of a simple sample preparation thus reducing assay time. The sensitivity and selectivity achieved for the detection of PGZ with respect of HPLC-UV makes it suitable for analysing very low levels of concentration in complex biological matrices. Moreover, HPLC-MS/MS allows the unambiguous identification of PGZ. Published data show that HPLC/MS has been used for PGZ quantitation in liquid biological samples (urine, plasma, serum) [15] but not in biological tissues. The sensitivity was slightly improved compared to data found in the literature [15].

This new biodistribution experiment supports the results of our previous studies [3], and the validated interval of the method covers the concentration range that could be present in eyes after a treatment. Sclera is the tissue which most PGZ accumulates after 4h of the instillation, followed by cornea. Aqueous humour presents low concentrations inside the quantitation range of the method.

REFERENCES

1.Bachu, R.D.; *et al. Pharmaceutics*, **2018**, 10, 28.
2.Ghafoorianfar, S.; *et al. Journal of Drug Delivery Science and Technology*, **2020**, 57, 101765.
3. Silva-Abreu, M.; *et al. Pharm. Res.*, **2018**, 35: 11.
4.Yamamoto, A.; et al. *PPAR Research*. Volume **2011**, Article ID 840194, 8 pages.
5.Okunuki, Y.; *et al. Experimental Eye Research*, **2013**, 116, 291-297.
6.Uchiyama, M.; *et al. Mol Vis.*, **2013**;19:2135–50.
7.Silva-Abreu, M.; *et al. Int. J. Mol. Sci.*, **2017**, 18, 2548.
8.Kanemaru, M.; *et al. Journal of Dermatological Science*, **2019**, 93, 41–49.
9.Matoba, T.; *et al. Journal of Cardiology*, **2017**, 70, 206–211.
10.Tokutome, M *et al. Cardiovascular Research*, **2019**, 115, 419–431.
11.Jojo, G.M.; *et al. Drug development and industrial pharmacy*, **2019**, 45 (7), 1061–1072.
12.Silva-Abreu, M.; *et al. European Journal of Pharmaceutical Sciences*, **2019**, 129, 173–180.
13.European Medicines Agency. EMA/CHMP/ICH/172948/2019. ICH Guideline M10 on Bioanalytical Method Validation.
14.U.S. Food and Drug Administration. Bioanalytical method validation, guidance for industry (2018).
15. Satheeshkumar, N.; *et al. J. Pharm. Anal.* **2014**; 4(5):295–302.