European Journal of Pharmaceutical Sciences 123 (2018) 106–110

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

# Esterase activity in porcine and albino rabbit ocular tissues

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ARTICLE INFO

*Keywords:*
Esterases
Ocular metabolism
Ocular ADME
Ocular prodrugs
Ocular drug bioavailability

ABSTRACT

Corneal esterases are utilized in the activation of topically applied ester prodrugs. Esterases may also be involved in the metabolism of drugs in posterior eye tissues, but their physiological activity is unknown. Furthermore, extrapolation of the esterase activity from protein level to the tissues is missing.

The aims of the current study were to determine esterase activities in porcine and albino rabbit ocular tissues, calculate the activities for whole tissues and compare esterase activity between the species. We conducted a hydrolysis study with ocular tissue homogenates using an esterase probe substrate 4-nitrophenyl acetate. The hydrolysis rates were first normalized to protein content and then scaled to whole tissues.

The hydrolytic rate normalized to protein content was high in the cornea and iris-ciliary body and low in the lens and aqueous humor, and in general, the rabbit tissues had higher hydrolytic rates than the porcine ones. Esterase activity scaled to whole tissue was high in cornea and iris-ciliary body and low in aqueous humor and retinal pigment epithelium in both species.

The current study revealed differences in esterase activities among the ocular tissues and the species. This basic knowledge on ocular esterases provides background information particularly for posterior segment drug development.

## 1. Introduction

Ocular ADME processes, such as permeability, active transport (Vellonen et al., 2017), melanin binding (Pelkonen et al., 2017; Rimpelä et al., 2017) and metabolism, are important aspects for ocular drug delivery (Argikar et al., 2017). Drug-metabolizing enzymes in the eye are not yet well characterized, even though ocular metabolism is known to affect pharmacokinetics of topical drugs (Argikar et al., 2017). For example, metabolic capacity of the cornea activates topically applied prodrugs, releasing parent compounds that are active in the inner eye tissues.

Several drug metabolizing enzymes, such as esterases, peptidases, alcohol and aldehyde reductases and cytochrome P450s have been detected in the eye (Duvvuri et al., 2004). Interestingly, whilst cytochrome P450s are the most important drug metabolizing enzymes in the liver, in the eye their expression levels are low and their functional role in drug metabolism and pharmacokinetics of the eye is unclear (Nakano et al., 2014). Esterases, a class of phase I hydrolytic enzymes cleaving ester bonds, in contrast have been shown to affect ocular pharmacokinetics of topical drugs (Anderson et al., 1980; Chang and Lee, 1982; Chang et al., 1987; Dias et al., 2002; Hellberg et al., 2003; Lee et al., 1982b; Lee et al., 1982a; Lee, 1983; Redell et al., 1983; Sjöquist et al., 1998). This hydrolytic reaction is employed in the design of ester prodrugs that have higher lipophilicity and permeation through biological membranes than their parent drug. For example the hydrolysis of latanoprost (Sjöquist et al., 1998), tafluprost (Fukano and Kawazu, 2009) and dipivefrin (Anderson et al., 1980; Mandell et al., 1978) to their active parent drugs by corneal esterases is utilized in glaucoma treatment.

The interest in ester prodrug approach has stimulated studies on esterase activity in ocular tissues (Lee, 1983). These studies have focused on anterior tissues, such as cornea, iris-ciliary body and aqueous humor. A single study with ganciclovir ester prodrugs (Dias et al., 2002) showed prodrug hydrolysis in posterior tissues, such as choroid, but the report did not clarify whether the prodrugs were cleaved by esterases. Esterase activity in the posterior segment is important since it may affect the drug delivery to the posterior eye.

In the present study, we investigated hydrolysis of enzyme substrate 4-nitrophenyl acetate (NPA) in porcine and albino rabbit ocular tissues. The study aims were to 1) determine the esterase activities in anterior

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https://doi.org/10.1016/j.ejps.2018.07.034
Received 27 March 2018; Received in revised form 29 June 2018; Accepted 16 July 2018
Available online 17 July 2018
0928-0987/ 

and posterior ocular tissues (conjunctiva, cornea, aqueous humor, iris-ciliary body, lens, vitreous, neural retina, retinal pigment epithelium (RPE), choroid and sclera) dissected from pig and albino rabbit; 2) establish hydrolytic activities for whole ocular tissues; 3) compare esterase activities in the rabbit and porcine eye. To our knowledge, this is the first comprehensive report on esterase activities in the ocular tissues.

## 2. Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Espoo, Finland).

### 2.1. Tissue Collection

#### 2.1.1. Porcine Eye Tissues

Enucleated porcine eyes were received from a local slaughterhouse and transported to the laboratory on ice in phosphate-buffered saline (PBS) within 5 h after the slaughter. The dissection of the eye was started by collecting a piece of bulbar conjunctiva. Then, the extraocular tissue was removed. Aqueous humor was collected by aspirating through limbus with a needle and a syringe. The eyeball was cut open from limbus and lens, iris-ciliary body and cornea were collected from the anterior part. Vitreous was collected from the posterior eye cup and traces of pigment and neural retina were removed. The neural retina was collected. One milliliter of PBS was pipetted to the eyecup and retinal pigment epithelium was suspended into the buffer by scratching with a small brush. RPE suspension was collected and, thereafter, the procedure was repeated twice. The total RPE suspension was centrifuged at 6000*g* for 5 min at +4 °C and the supernatant was discarded to obtain a RPE pellet. Choroid was collected from the remaining posterior eyecup, the outer surface of sclera was cleaned from any remaining extraocular tissues, optic nerve was cut off and the sclera was collected. All tissues were weighted, stored at −80 °C and thawed on ice before the experiment. Tissues were collected in triplicate, except for conjunctiva $n = 6$ and choroid $n = 4$.

#### 2.1.2. Rabbit Eye Tissues

Eyes of adult female albino New Zealand White rabbits, with body mass of 3.5–5.0 kg, were frozen after enucleation and stored at −80 °C. On day of the experiment, the eyes were thawed at room temperature until unfrozen and dissected in the same manner as the porcine eyes. The isolated tissues were weighted and stored on ice. For aqueous humor, choroid, conjunctiva and RPE $n = 3$; for cornea, neural retina, sclera and vitreous $n = 4$; for iris-ciliary body $n = 5$ and for lens $n = 6$.

#### 2.1.3. Rat Liver

An adult male albino Wistar Han rat was euthanized with carbon dioxide and perfused intra-cardially with 0.9% sodium chloride solution. Liver ($n = 1$) was collected, rinsed with PBS, homogenized (see 2.2) and stored as cell homogenate at −80 °C until the experiment day.

### 2.2. Tissue Homogenization and Dilution

#### 2.2.1. Porcine and Rabbit Eye Tissue Homogenates

On the day of the experiment, all ocular tissues except the aqueous humor were first homogenized with Dounce homogenizer in PBS (200 µl/tissue for rabbit choroid, RPE and vitreous; 400 µl/tissue all the rest; tissue/buffer ratio 0.1–7.6 g/ml). Then, they were sonicated with an ultrasonic processor and microtips (Vibra-Cell VCX750 and four-element probe; Sonics & Materials Inc., Newton, CT) on ice 4 times for 45 s with 15 s pause in between. The tissue homogenates were centrifuged at 12000*g* for 2 min at +4 °C and the supernatants were collected for further use.

#### 2.2.2. Rat Liver Homogenate

Rat liver homogenate was prepared after liver isolation by adding 4 ml of PBS to 1 g of liver and homogenizing on ice with a dispersing device (Ultra-Turrax T8; IKA Works, Inc., Wilmington, NC) in 10 s bursts 3 times, and stored at −80 °C. On the day of the experiment, liver homogenate was thawed on ice and centrifuged at 10000 g for 20 min at +4 °C. Supernatant was then collected.

#### 2.2.3. Dilution of Tissue Homogenate Supernatants for Incubations

Protein concentrations of the collected supernatants were quantified with Bradford assay (Bradford, 1976) and bovine serum albumin standards. The tissue homogenates were then diluted with PBS to protein concentrations of 0.1, 0.3 and 0.5 µg/µl. This dilution was done to decrease sample background absorbance and standardize the protein binding of the hydrolysis substrate NPA, and the product 4-nitrophenol (NP) in the sample.

### 2.3. Hydrolysis of 4-Nitrophenyl Acetate

#### 2.3.1. Hydrolysis Study with Tissue Homogenates

On the day of experiment, 20 mM stock solutions of NPA and NP in ethanol were prepared and NPA stock was further diluted to 200 µM with PBS. Tissue homogenate dilutions of 80 µl were pipetted to clear 96-well plates as triplicates and pre-incubated at +37 °C for 10 min. Equal volume of 200 µM NPA was added to samples with a multichannel pipet and the sample absorbance was immediately measured at 405 nm every 90 s for 45 min in +37 °C. In sample wells, the initial NPA concentration was 100 µM and protein concentrations were 0.05, 0.15 and 0.25 µg/µl. Ethanol concentration in the incubation was < 0.5%.

#### 2.3.2. 4-Nitrophenol Standards

NP stock solution at 20 mM was diluted with PBS to 200 µM, from which 5, 20, 40, 60, 80, 100 and 120 µM NP standards were prepared with PBS. NP standards (160 µl/well) were included to all plates.

#### 2.3.3. Chemical Hydrolysis of 4-Nitrophenyl Acetate

Chemical hydrolysis of NPA was studied in PBS and 5 M sodium hydroxide similar to Section 2.2. NPA hydrolysis in PBS was negligible. Furthermore, in the presence of sodium hydroxide, NPA yielded equal molarity of NP, indicating that NPA is fully converted to NP. To further clarify the non-enzymatic hydrolysis of NPA, ocular tissue homogenates ($n = 2$) were prepared as in Section 2.2, heated in +80 °C for 30 min to denature the enzymes and cooled before incubation. The homogenates (80 µl), were incubated with 80 µl of 200 µM NPA as described above. The experiment showed that NPA hydrolysis was negligible in all tissues except for the porcine vitreous and the rabbit aqueous humor (Supplementary Fig. 1). Therefore, we excluded the results for these tissues.

### 2.4. Calculation of Esterase Activity

#### 2.4.1. Normalization to Protein Concentrations

NPA hydrolysis rate was calculated on the slope of linear part of NP concentration-time curve, that starts at 0 and typically ends from 1.5 to 20 min depending on the tissue and the homogenate protein concentration.

#### 2.4.2. Scaling to Whole Tissues

From NPA hydrolysis rates in tissue homogenates, we calculated the reaction rates for the whole ocular tissues (Fig. 1). NPA hydrolysis rates from incubations at 0.15 µg/µl protein concentration (homogenate protein concentration 0.3 µg/µl) were used in the calculation of esterase activity for whole tissue. First, tissue protein yield was calculated from total tissue mass and tissue protein content. Tissue mass in incubation was calculated from tissue protein yield and protein concentration in the incubation. Then, NPA hydrolysis rate was divided by the tissue mass in incubation to get reaction rate per gram of tissue, which was