Absorption of topically applied drugs via the conjunctival and nasal mucosae into the bloodstream to elicit side effects has been recognized as a possibility in ocular drug therapy for more than 25 years. Examples of these drugs include anticholinesterase agents, epinephrine, phenylephrine, and steroids. Depending on the physiochemical properties of the drug, varying extents of systemic drug absorption can occur. These extents range from 3% for inulin to approximately 80% for timolol. Nevertheless, the possibility of systemic drug absorption was not considered in various attempts to optimize ocular drug therapy until beta-blockers were widely used in patients with glaucoma. Several approaches have been investigated relative to reducing systemic drug absorption, and the results have been positive. These approaches include nasal and ocular occlusion with or without eyelid closure, use of microdrops, use of vehicles with extended residence time in the conjunctival sac, coadministration of vasoconstrictors such as phenylephrine and epinephrine, use of soft drugs that are rapidly inactivated once they are absorbed into the bloodstream, and use of prodrugs that are either inherently poorly absorbed into the bloodstream or are well absorbed across the cornea, thus permitting the use of a lower dose. Several of the approaches described above, notably those of vehicles and prodrugs, have also been investigated for improvement in ocular drug absorption.

This study was conducted to evaluate the alternative strategy of varying the time of drop instillation to minimize the systemic absorption of topically applied timolol, a nonselective β-adrenergic blocking drug widely used in the treatment of glaucoma. This strategy was based on the expectation that drug absorption into the eye and the bloodstream is governed by a circadian rhythm, as is the case for intraocular pressure and for pharmacokinetic processes elsewhere in the body. For convenience, four dosing times were chosen: 6 AM, 12 PM, 6 PM, and 12 AM. The pigmented rabbit was the experimental animal. All experiments conformed with the ARVO Resolution on the Use of Animals in Research.

Materials and Methods

Male, Dutch-belted, pigmented rabbits, weighing 2.0–2.5 kg, were purchased from American Rabbity (Los Angeles, CA). The animals were housed in standard laboratory rabbit cages, with food and water ad libitum, in a light-controlled room at a temperature of 22 ± 1°C and humidity of 55 ± 10%. They were entrained to a lighting schedule of alternating 12-hr periods of light and dark (12L:12D) for at least 1 week before use. Lights were turned on at 6 AM and off at 6 PM. Experiments during the dark period were conducted under lighting conditions provided by Bright...
Lab Jr. Safelight bulbs (Delta 1, Dallas, TX), which are used primarily in photographic dark rooms and which emit a narrow visible spectrum in the far red range. Timolol maleate and propranolol HCl were purchased from Sigma Chemical Company (St. Louis, MO). Dosing solutions that contained 0.65% timolol maleate, equivalent to 0.5% timolol, were prepared in 10 mM Tris buffer and were adjusted to pH 7.4 with 10 N sodium hydroxide and rendered isotonic by adding sodium chloride. The commercial preparation was not used to avoid the possible confounding variables of acidic pH and benzalkonium chloride in the commercial formulation on the circadian rhythms of ocular and systemic absorption of timolol.

Assay

Timolol was quantitated with the use of reversed phase high-performance liquid chromatography (HPLC) on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm, 5-μm particle size) fitted with a Brownlee Labs Newguard precolumn (1.5 cm) (West Coast Scientific, Hayward, CA), as previously described. The HPLC system consisted of an SCL-6A system controller, two LC-6A pumps, an SIL-6A autoinjector, an SPD-6A spectrophotometric detector, and a CR-3A integrator (Shimadzu Instruments, Baltimore, MD). The mobile phase was a mixture that consisted of 4 parts of 10% acetonitrile in methanol and 6 parts of 0.2% triethylamine HCl in 5% acetonitrile at pH 3. The flow rate was 1 ml min⁻¹. Propranolol HCl (10 μg ml⁻¹) was used as the internal standard. Timolol was monitored at 294 nm. The retention time was 5 min for timolol and 12 min for propranolol. The sensitivity of the assay was more than 5 nmol with respect to timolol in a 2-ml plasma sample. The intra- and inter-run variations were 5% and 7.5%, respectively.

At the time of assay, an aqueous humor sample was mixed with an equal volume of acetonitrile that contained 0.01 N HCl and 10 mg ml⁻¹ propranolol HCl. After centrifugation, 80–120 μl of the supernatant was injected into the HPLC. Excised tissues were rinsed with 1.17% KCl solution and were blotted dry, were transferred to preweighed microcentrifuge tubes that contained 200 μl of 0.6% HCIO₄, and were stored at -70°C. Four to eight eyes were used per time point per dosing period. Both eyes of a given rabbit were used.

Systemic Absorption of Topically Applied Timolol

The dosing procedure was as described above in the ocular absorption experiments. Fifteen minutes before the solution was instilled, the rabbits were cannulated in a central ear artery with polyethylene tubing (PE-50, Intramedic) and were heparinized with 1000 U of Na heparin (Western Medical Supply, Arcadia, CA). Thereafter, 25 μl of a dosing solution was instilled into each eye of a given rabbit. At 0, 3, 6, 10, 15, 30, 45, 60, 90, and 120 min after dosing, 4-ml blood samples were collected into heparinized tubes and stored in the refrigerator until extraction of the drug the next day. The volume of blood that was aspirated was replenished with an equal volume of lactated Ringer’s solution, USP (Abbott Laboratories, Chicago, IL). Preliminary experiments showed that the plasma-timolol concentration was below the detec-
Fig. 1. Concentration-time profiles of timolol in the conjunctiva (plot A), anterior sclera (plot B), corneal epithelium (plot C), corneal stroma (plot D), aqueous humor (plot E), iris-ciliary body (plot F), lens (plot G), and plasma (plot H) of the pigmented rabbit following the topical instillation of 25 μl of 0.65% timolol maleate solutions at 0600, 1200, 1800, and 2400 hr. Error bars represent standard error for n = 4–8 at each dosing time. One-way ANOVA revealed statistically significant difference at P < 0.05 for the majority of time points among the four dosing times. The exceptions were 15 min in the conjunctiva; 15 and 60 min in the sclera; 5, 60, and 150 min in the corneal epithelium; 5 min in the corneal stroma; 5 min in the aqueous humor; 15 min in the iris-ciliary body; and 90, 180, and 240 min in the lens.
tion limit after 120 min; hence, blood was collected
until then. The blood was centrifuged at 1500×g for
10 min to yield 2 ml of plasma, and then was frozen
and stored at −20°C until assayed. At least four rab-
bits were used per dosing time, and rabbits were not
reused.

Time Course of Plasma–Timolol Concentration After
Intravenous Administration at Various Dosing Times

Fifty microliters of a 0.65% timolol maleate solu-
tion was injected into the marginal ear vein of pig-
mented rabbits at 6 AM, 12 PM, 6 PM, or 12 AM.
The sampling schedule and sampling processing pro-
cedures were the same as in the topical solution instil-
lation experiment described above. At least four rab-
bits were used per dosing time, and rabbits were not
reused.

Pharmacokinetic Data Analysis

The data were plotted as timolol concentration vs
time, and were subjected to noncompartmental phar-
macokinetic analysis.26 The following pharmacoki-
netic parameters were obtained: peak time (tmax),
peak timolol concentration (Cmax), area under the
concentration–time curve (AUC), and mean resi-
dence time (MRT). The plasma data from the intrave-
nous administration experiment were fitted to a two-
compartment model26 to yield the pharmacokinetic
parameters k12, k21, k10, and Vdss, where k12 is the rate
constant for drug transfer from the central to the pe-
ripheral compartment, k21 is the rate constant for the
opposite direction, k10 is the rate constant for drug
elimination from the central compartment, and Vdss
is the volume of distribution at steady state.

Statistical Data Analysis

Statistical significance of difference was first tested
with one-way analysis of variance (ANOVA). Where
there was a statistical difference, multiple compar-
sions were conducted among the various dosing times
with the use of the Fisher’s Protected Least Significant
Difference (PLSD) test. In the systemic absorption ex-
periments, statistical analysis was conducted on
Cmax and AUC. In the ocular absorption experi-
ments, statistical analysis was conducted on timolol
concentrations in each ocular tissue at each time
point.

Results

Regardless of dosing time, timolol concentrations
reached a peak within 5 min in the conjunctiva,
sclera, corneal epithelium, and corneal stroma; 15–30
min in the aqueous humor; 90–150 min in the iris–ci-
liary body; and 30–60 min in the lens. This is shown
in Figs. 1A–G and summarized in Table 1, which also
shows the pharmacokinetic parameters Cmax, AUC,
and MRT. The extent of timolol absorption into the
anterior segment tissues, expressed as AUC, was de-
pendent on the time of dosing. For the majority of
time points, one-way ANOVA showed a statistical
difference at P < 0.05 in timolol concentrations in a
given tissue among the four dosing times. In all ante-
rior segment tissues except the lens, timolol concen-
trations were highest from 12 PM dosing, followed by
either 6 PM or 12 AM dosing, and then by 6 AM
dosing (Fig. 2). Moreover, an approximately two-fold
difference was seen in ocular tissue concentrations be-
tween 6 AM and 12 PM dosing for the majority of
time points (P < 0.05 by Fisher’s PLSD test). As
shown in Figure 3, the AUC in the aqueous humor
 correlated well with that in the corneal epithelium
and iris–ciliary body. This finding suggests that
changes in timolol concentrations in the iris–ciliary
body as a function of dosing time were due to changes
in timolol concentrations in the aqueous humor,
which were due to changes in timolol concentrations
in the corneal epithelium.

In the plasma, timolol concentrations reached a
peak within 6–10 min, regardless of dosing time (Fig.
1H and Table 1). Like ocular timolol absorption, sys-
temic timolol absorption, expressed as Cmax and
AUC, was also dependent on the dosing time (P
< 0.008 by ANOVA). On the basis of the AUC shown
in Figure 2H, absorption of timolol into the blood-
stream was most extensive at 6 AM dosing, then at 6
PM, 12 AM, and 12 PM dosing, respectively. An 1.8-
fold difference was seen in the plasma AUC between 6
AM and 12 PM dosing (P < 0.05 by Fisher’s PLSD
test) (Table 1). The important observation was the
negative correlation between ocular and systemic ab-
sorption on the basis of AUCs in the aqueous humor/
iris–ciliary body and the plasma, respectively, as
shown in Figure 4. Specifically, dosing at 12 PM af-
forded the best ocular absorption and the poorest sys-
tematic absorption, a desirable situation from a thera-
peutic point of view.

After intravenous administration, diurnal changes
in the disappearance of timolol in the plasma were
also seen, as shown in Figure 5. The AUC was the
highest at 12 PM dosing, then at 6 AM, 12 AM, and
6 PM dosing (P < 0.05 by ANOVA) (Table 2). The
Fisher’s PLSD test showed a statistical difference in
plasma AUC between 12 PM dosing and the other
three dosing times, but no statistical difference among
these three dosing times at P < 0.05. As shown in
Table 2, time-dependent changes were probably due
to changes in the volume of distribution at steady
state (Vdss) and the distribution rate constants k12 and
k21, rather than to changes in the elimination rate
constant (k10) of timolol from plasma according to
two-compartment pharmacokinetic analysis.
ple, intraperitoneally administered valproic acid in patient's urine flow was highest. As another exam-
phrotic when given at 6 PM, presumably when the day. Thus, cisplatin was 25% less ne-
MRT (min)

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* See text for explanation of abbreviations. Key: Conj, conjunctiva; CE, corneal epithelium; CS, corneal stroma; AH, aqueous humor; ICB, iris-ciliary body.

Discussion

This study shows, for the first time, that ocular and systemic absorption of topically applied ophthalmic drugs may be influenced by the time of drop instillation. In the pigmented rabbit, absorption of topically applied timolol into the eye at 12 PM, as shown by AUC, was about two times greater than at 6 AM, 6 PM, or 12 AM. Interestingly, at 12 PM, when the extent of ocular absorption was best, the extent of absorption of topically applied timolol into the bloodstream was worst. If this trend prevails in humans, the ratio of ocular to systemic absorption of topically applied timolol could be maximized, thereby improving its safety, by judiciously selecting the time of drop instillation.

It may be possible to minimize drug toxicity by optimizing the time of drug administration. The rationale is that receptor density, drug absorption, metabolism, and excretion may vary at different times during the day. Thus, cisplatin was 25% less nephrotoxic when given at 6 PM, presumably when the patient’s urine flow was highest. As another example, intraperitoneally administered valproic acid in ICR male mice was more toxic when injected at 5 PM and least toxic when injected at 9 AM or 1 PM, probably due to rhythmic changes in sensitivity of the central nervous system to the drug. However, this situation concerning timolol is complicated by the fact that, in rabbits, maximum ocular absorption occurs at the time when intraocular pressure is not the most responsive to timolol. This could be true in humans, although humans have diurnal rhythms of intraocular pressure and aqueous flow that are approximately 180° out of phase with these rhythms in rabbits.

Concentrations in the aqueous humor and iris-ciliary body, particularly between 6 AM and 12 PM, may be attributed to corresponding changes in precorneal drug loss, corneal drug penetration, or drug elimination from the target tissue/liquid. One-compartment pharmacokinetic analysis of the data in the elimination phase in aqueous humor (Fig. 1E) shows a rate constant of 0.82 ± 0.18 min⁻¹ at 6 AM, 1.33 ± 0.26 min⁻¹ at 12 PM, 1.11 ± 0.17 min⁻¹ at 6 PM, and 1.51 ± 0.85 min⁻¹ at 12 AM (P < 0.025 by ANOVA). Thus, elimination of timolol from the aqueous humor is about 60% slower at 6 AM than at 12 PM (P
Fig. 2. Area under the concentration–time curves (AUC) of timolol in the conjunctiva (plot A), anterior sclera (plot B), corneal epithelium (plot C), corneal stroma (plot D), aqueous humor (plot E), iris–ciliary body (plot F), lens (plot G), and plasma (plot H) of the pigmented rabbit following the topical instillation of 25 μl of 0.65% timolol maleate solutions at 0600, 1200, 1800, and 2400 hr. Error bars represent standard error for n = 4–8. No error bars were shown for the ocular tissues since pooled data were used.
< 0.01 by Fisher’s PLSD test.) Although the slower rate of timolol clearance from the aqueous humor at 6 AM is consistent with the slower aqueous humor flow rate at that time,23 it is inconsistent with the lower aqueous humor concentration achieved at 6 AM dosing (Fig. 1E), because a higher aqueous humor drug concentration would be expected. Consequently, diurnal variations in precorneal clearance or corneal permeability are plausible explanations.

Work completed in the corresponding author’s laboratory showed that corneal permeability to timolol at 12 PM (1.13 ± 0.06 × 10⁻⁵ cm s⁻¹) was about 30% greater than at 6 AM (0.87 ± 0.09 × 10⁻⁵ cm s⁻¹) (unpublished data). Clearly, this magnitude of increase alone cannot account for the two-fold difference in extent of timolol absorption into the eye. It thus appears that time-dependent changes in precorneal drug loss, a major component of which is solution drainage,35 play a more important role. Although no information exists on how the solution drainage or how the tear flow rate varies during a 24-hr period, there is evidence that, in human subjects, tear enzymes,36,37 proteins,38 Ca²⁺,38 pH,39 and osmotic pressure,40 show diurnal variations. Some of these factors, such as pH,41 osmolality,42 and protein concentration,35 alter ocular drug bioavailability.

Because solution drainage determines the rate at which the administered dose reaches the nasal cavity,44 from which more than 70% of an instilled dose of timolol is absorbed,8 it is not surprising that time-dependent changes in systemic absorption bear an opposite trend to those seen in ocular absorption (Fig. 4). Like corneal permeability, nasal permeability to timolol may vary with time, being lower at 12 PM than at 6 AM. This possibility is suggested indirectly by the time-dependent changes in plasma–timolol pharmacokinetics after intravenous administration (Fig. 5 and Table 2). Plasma–timolol concentrations were highest at 12 PM than at other dosing times. This finding is directly opposite to the trend afforded by topical solution instillation (Fig. 1H). The higher timolol concentrations achieved after intravenous administration at 12 PM were probably due to slower drug clearance from systemic circulation, as with other beta-blockers, including atenolol, sotalol, metoprolol, and propranolol.45 It is unlikely that diurnal changes in plasma protein binding46 due to diurnal changes in plasma protein concentrations47 are a contributing factor, because timolol is poorly protein bound (7% at 100 ng ml⁻¹).48
Gregory and co-workers\textsuperscript{22-24} and others\textsuperscript{19-21} have provided conclusive evidence that the IOP of albino rabbits follows a circadian rhythm that is in phase with the circadian rhythm of aqueous flow.\textsuperscript{23} IOP is high during the dark phase and low during the light phase.\textsuperscript{20,22-24} Experiments are being conducted in the corresponding author's laboratory to determine whether the ocular pharmacokinetics of topically applied timolol also follow a circadian rhythm. This would be shown by dosing time-dependent changes in ocular absorption that vary in phase with the light:dark cycle and must persist in constant dark.

In conclusion, the ratio of ocular:systemic absorption of topically applied timolol in the pigmented rabbit could be improved by varying the time of drop instillation. Although a similar time dependency of ocular and systemic pharmacokinetics may exist in humans, the time at which the above ratio is maximized would probably be different. Studies are in progress to elucidate the mechanisms that cause the variations in ocular and systemic pharmacokinetics of topically applied timolol with the time of drop instillation and to determine whether such variations are circadian.

Key words: chronopharmacokinetics, circadian rhythm, ocular timolol bioavailability, systemic timolol bioavailability, therapeutic index

Acknowledgments

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References


