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turnover and flow rates in humans, fluorophotometry has emerged as the gold standard.\textsuperscript{10–13} Mishima et al\textsuperscript{6} used fluorophotometry to measure rates of fluorescein decay in human eyes,\textsuperscript{14} and the mathematical treatment of the data has been corroborated by several investigators and remains the basis for most fluorophotometric tear flow studies. The purpose of the study reported here was to use fluorophotometry to measure the tear volume, turnover rate, and flow rate in ophthalmologically normal horses.

Materials and Methods

Horses—Twelve mares typically used for teaching purposes at the University of Tennessee College of Veterinary Medicine were used for the study. The mean age was 11.1 years (range, 5 to 15 years), and the mean body weight was 478 kg (range, 426 to 525 kg). Four of the 12 horses were also receiving a nutraceutical \textsuperscript{6} (various omega-3 fatty acids, minerals, and vitamins) to evaluate its effects on the gastric mucosa. All horses were judged to have healthy eyes by means of slit-lamp biomicroscopy and indirect ophthalmoscopy prior to commencement of the study. The STT value in all horses was within reference limits, exceeding 15 mm of wetting in 60 seconds in all tests. All experiments were carried out in compliance with the US Animal Welfare Act and with the approval of the University of Tennessee Institutional Animal Care and Use Committee.

Experimental protocol—Although tear production in horses does not vary diurnally,\textsuperscript{2} all experiments were performed at the same time of day (between 9:00 AM and 11:00 AM) for consistency. The horses received no general sedatives or local nerve blocks that might otherwise confound tear production and measurement. Horses were acclimated to the procedures for 7 days prior to data collection by application of saline (0.9% NaCl) solution eye-drops and tear collection from the inferonasal conjunctival cul-de-sac 2 to 3 times/d in a manner identical to that described for the actual data acquisition.

A 2-µL glass microcapillary tube\textsuperscript{6} with a rubber bulb stopper was used to instill 2 µL of 10% sodium fluorescein\textsuperscript{2} onto the upper bulbar conjunctiva of the left eye without touching the eye. Immediately thereafter (0 minutes), a tear sample was collected from the inferior conjunctival fornix by exerting the inferior eyelid and gently placing a 2-µL microcapillary tube against the conjunctiva for 2 to 3 seconds. The volume of the collected sample was calculated by measuring the length of fluid contained within the microcapillary tube and extrapolating the volume from the volume-to-length ratio of the microcapillary tube. The contents of the microcapillary tube were then expelled into a 1.5-mL Eppendorf tube\textsuperscript{6} containing 1.0 mL of buffered saline solution. The collection procedure was repeated at 2, 4, 6, 10, 15, and 20 minutes. Fluorescein concentrations were measured with a computerized scanning ocular fluorophotometer\textsuperscript{6} fitted with a cuvette holder. Samples were placed in the glass cuvette, and 1.0 mL of buffered saline solution was added to provide an adequate volume for reading.

Calculations of tear parameters—Actual tear film concentrations were calculated from the diluted samples by use of the following formula (formula 1):

\[
C(t) = \frac{C_f(t) \cdot V_d}{V_c}
\]

in which \(C(t)\) is the actual tear film concentration at each sample collection time, \(C_f(t)\) is the concentration of the diluted samples as measured by use of the fluorophotometer, \(V_i\) is the dilution volume (ie, 2.0 mL), and \(V_c\) is the collected volume of each sample.

The tear volume was calculated from the resulting fluorescein concentrations by use of 2 methods: the including and excluding methods. In the including method (also known as the dilution method\textsuperscript{15}), the actual tear film concentration at 0 minutes \((C(t = 0))\) is included in the analysis and the volume is calculated by mathematically determining the change in the original fluorescein concentration immediately following instillation and thorough mixing with the tear film. If \(C\) is the original concentration of the fluorescein solution instilled into the tear film (10% or 10\textsuperscript{6} ng/mL), \(V_i\) is the original volume of instilled fluorescein solution (2.0 µL), and \(C(0)\) is the postinstillation concentration in the tear film at 0 minutes (presuming that thorough mixing and homogenization occur essentially instantaneously upon instillation of fluorescein into the tear film), then the tear film volume \((V)\) is given by a dilution ratio as follows (formula 2):

\[
V = V_i \cdot \left[ \frac{C_i}{C(0)} - 1 \right]
\]

where \(C(0)\) is obtained from the measured concentration at 0 minutes.

In the excluding method (also known as the zero time method\textsuperscript{15}), \(C(t = 0)\) is excluded from the analysis and the mathematical treatment is the same as that described for the dilution method, except that \(C(0)\) is extrapolated from the regression equation of the ln[FI] versus time plot from 2 to 20 minutes.

To confirm the tear volume calculations, tear volume was estimated on the basis of geometric considerations. Tears were assumed to be homogeneously distributed, and tear film thickness was assumed to be equal on the corneal surface and under the eyelids.\textsuperscript{16} The volume contributed by the marginal tear strips was ignored. A schematic model was used in which the globe of the eye was taken to be a sphere with a radius \((r)\) of 20 mm (in approximate accordance with measurements reported elsewhere\textsuperscript{17}) and in which the conjunctival cul-de-sac extended to the equator of the sphere such that the surface of the globe covered by tears would approximate a hemisphere. The volume of the hemisphere without tears was calculated as follows:

\[
V = \frac{1}{2} \left( \frac{4}{3} \pi r^3 \right)
\]

and the volume of the hemisphere with tears was calculated as follows:

\[
V = \frac{1}{2} \left( \frac{4}{3} \pi (r + d)^3 \right)
\]

in which \(d\) is the thickness of the tear film. The tear film volume was subsequently calculated by subtracting the
The regression lines were calculated for a range of plausible volumes in horses, which were then compared with the volume determined by use of the excluding method.

The tear turnover rate was defined by the percentage decrease in fluorescein concentration from an initial time (0 minutes) and was calculated as follows (formula 3):

\[ T(t) = 100 \cdot \frac{C(t) - C(t + 1)}{C(t)} \]

in which \( T(t) \) is the turnover rate, \( C(t) \) is the concentration at 0 minutes, and \( C(t + 1) \) is the concentration 1 minute later. Assuming a semilogarithmic decay pattern, the concentration at any time \( t \) is represented by use of the following formula (formula 4):

\[ C(t) = C(0) \cdot e^{-kt} \]

where \( k \) is the decay constant, which is the slope of the regression line of the semilogarithmic plot of the concentration versus time. The regression lines were established with the method of least squares by use of commercially available software. By substituting \( C(t) \) into \( T \), the turnover rate could then be simplified as follows (formula 5):

\[ T = 100 \cdot (1 - e^{-k}) \]

Tear flow rate \( (T) \) was determined by multiplying the decay constant \( k \) by the tear volume \( V \) as follows (formula 6):

\[ T = k \cdot V \]

Comparison between right and left eyes and consistency of parameters over time—In addition to the preceding calculations, tear parameters were measured in the right eye as well as the left in 3 of the horses by use of the excluding method to compare right and left eye values. In an additional 3 horses, the left eye parameters were remeasured 8 to 9 days after the original measurements were made to assess the consistency of parameters over time.

Statistical analysis—Tear volumes, turnover rates, and flow rates calculated by use of the including and excluding methods were compared between horses receiving the nutraceutical and those not receiving the nutraceutical by use of a Student test. Values are reported as mean ± SD. In all statistical analyses, a value of \( P < 0.05 \) was considered significant. Correlation coefficients and coefficients of determination were calculated for \( \ln[\text{Fl}] \)-versus-time plots for both methods of calculation.

Results

Tear volume, turnover rate, and flow rate did not differ significantly (\( P > 0.05 \); power > 0.80) between horses receiving and those not receiving the nutraceutical. Therefore, data from all horses were combined for the remainder of the analysis.

Including method—With the including method, in which the concentration of tears at 0 minutes was used in calculations (formula 2), a mean tear volume of 360.09 \( \mu \text{L} \) was obtained (Table 1). The decay constant, as determined from the slope of the regression line of \( \ln[\text{Fl}] \) versus time, was 0.1319. The mean tear turnover rate was 12.22%/min, and the mean tear flow rate was 47.77 \( \mu \text{L/min} \). The mean ± SD correlation coefficient for \( \ln[\text{Fl}] \) versus time was 0.93 ± 0.12, and the mean coefficient of determination was 0.88 ± 0.18. Seven of the 12 horses had concentrations immediately following fluorescein instillation (0 minutes) that were lower than the subsequent measurement at 2 minutes.

Excluding method—The regression lines were recalculated for data from the 2-minute to 20-minute measurements, without use of the 0-minute values (Figure 1; Table 1). Tear film concentration at 0 minutes was then equal to the y-intercept of the calculated regression line and did not depend on the assumption of instantaneous homogenization of fluorescein in the tear film immediately after instillation. The decay constant was 0.1437, and mean tear volume was 233.74 \( \mu \text{L} \). The mean turnover rate was 13.21%/min, and the mean tear flow rate was 33.62 \( \mu \text{L/min} \). The mean ± SD correlation coefficient for \( \ln[\text{Fl}] \) versus time was 0.98 ± 0.03, and the mean coefficient of determination was 0.95 ± 0.06.

Tear volume from geometric considerations—When the radius of the globe of the eye was assumed to be 20 mm and values of 3 and 40 \( \mu \text{m} \) were used as the thickness of the tear film, the calculated range of tear

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**Table 1—Mean ± SD values for tear parameters in left eyes of 12 ophthalmologically normal horses calculated by use of including and excluding methods.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Including method</th>
<th>Excluding method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (( \mu \text{L} ))</td>
<td>360.09 ± 111.67</td>
<td>233.74 ± 90.96</td>
</tr>
<tr>
<td>Turnover rate (%/min)</td>
<td>12.22 ± 5.20</td>
<td>13.21 ± 5.22</td>
</tr>
<tr>
<td>Flow rate (( \mu \text{L/min} ))</td>
<td>47.77 ± 24.34</td>
<td>33.62 ± 19.46</td>
</tr>
<tr>
<td>Decay constant for instilled fluorescein</td>
<td>0.1319 ± 0.0930</td>
<td>0.1437 ± 0.0603</td>
</tr>
<tr>
<td>Natural logarithm of initial tear film concentration</td>
<td>13.56 ± 0.27</td>
<td>13.72 ± 0.37</td>
</tr>
</tbody>
</table>

*In the including method, the actual tear film concentration at 0 minutes was included in the analysis and the volume is calculated by mathematically determining the change in the original fluorescein concentration immediately following instillation and thorough mixing with the tear film. In the excluding method, the initial tear film concentration was excluded from the analysis and was then extrapolated from the regression equation of \( \ln[\text{Fl}] \) versus time from 2 to 20 minutes. Values for all parameters differ significantly (\( P < 0.05 \)) between methods.
film volumes in ophthalmologically normal horses was 7.54 to 100.73 µL. Similarly, when a tear film volume of 233.74 L was used to solve for the volume of the globe hemisphere with tears and the resulting value was used to solve for tear film thickness, the data predicted a tear film thickness in ophthalmologically normal horses of approximately 92.6 µm.

Right eye versus left eye and consistency across time—In the 3 horses in which right and left eyes were measured, there were no significant differences in tear volume, turnover rate, or flow rate (Table 2). Similarly, values of parameters did not change across time in the 3 horses in which measurements were made twice (Table 3). However, the small sample sizes for these analyses resulted in low statistical power, so it is possible that true differences could exist but that larger samples would be needed to detect them.

### Discussion

In the present study, tear volume, turnover rate, and flow rate were determined in ophthalmologically normal horses. Fluorescein administration and tear collection appeared to be well tolerated by all horses, with no obvious reflex lacrimation (Figure 1). This finding is consistent with that of another study in which no significant difference was detected between results of STTs I and II in horses, implying a relative lack of reflex lacrimation in response to placement of a paper tear strip into the inferior conjunctival cul-de-sac. In each horse in the present study, fluorescein concentrations decreased according to first-order kinetics between 2 and 20 minutes after instillation of fluorescein. An axiom in ocular pharmacokinetics is that the highest concentration of a topical compound in the tears must occur immediately after instillation, with concentrations decreasing thereafter because of dilution by newly formed tears. However, our data indicated higher concentrations at 2 minutes after instillation rather than immediately after instillation (0 minutes; ie, C(2) > C(0)) in 7 of 12 horses. Given that it is physiologically impossible for fluorescein concentration to increase following initial instillation into the tear film, the lower concentrations measured at 0 minutes were most likely unrepresentative of the actual concentration attributable to the fluorescein not having had adequate time to distribute homogenously in the tear film. In such instances, the primary assumption leading to volume calculation as determined by use of the including method would be violated, and the artificially lower concentrations at 0 minutes would alter the regression model by reducing the decay constant. The result would be overestimation of tear volume and flow rate and underestimation of tear turnover rate. Thus, the excluding method should provide more accurate estimations of tear volume, turnover rate, and flow rate in horses.

Comparing the including method analysis with the excluding method analysis, there was typically an increase in the correlation strength and coefficients of determination with concomitant reduction in range and SD with the latter, corroborating that the excluding method may be the more robust approach. Similar decreases in range were detected when tear volumes and flow rates were calculated in humans with the excluding method. Researchers in that study also surmised that the excluding method was the more accurate method, hypothesizing that a combination of reflex lacrimation and poor homogenization in the first few minutes following fluorescein instillations created artifacts that were problematic for the dilution method. Our observations were similar.

All horses in the present study had a semilogarithmic decline of fluorescein concentration with a simple exponential decay pattern, indicative of first-order kinetics over the period evaluated (Figure 1). Coefficients of determination for these plots were > 0.9, indicating...
that a single exponential equation was appropriate. Mishima et al\textsuperscript{10} reported similar instances of decay with a single turnover rate in human subjects, but most of their subjects had an initial rapid decay, followed by a slower decay after 4 to 5 minutes. The single turnover rate was evident in subjects who claimed to be irritated with the instillation of fluorescein, producing a high turnover rate. The decreased decay rate was associated with subjects who claimed the least irritation with the technique. It was concluded that the more rapid decay in the first 4 to 5 minutes after fluorescein instillation was caused by reflex lacrimation, and that the slower decay after 5 minutes was the actual physiologic tear turnover. Similar biphasic decay in humans was detected in another study.\textsuperscript{10}

The horses in the present study appeared to tolerate the application of fluorescein well (ie, there were no signs of blepharospasm or epiphora), and care was taken not to directly contact the conjunctiva with the tip of the capillary tube during instillation. However, contact with the inferior conjunctival fornix surface to collect tears was inevitable and this may have resulted in reflex lacrimation, which would increase the turnover rate. If irritation had been caused by fluorescein instillation or tear collection, it appeared to be fairly constant with each horse and did not result in much fluctuation of the tear turnover rate. We could not assess our horses for increased decay rates during the early period after instillation because we did not have enough sampling points prior to 5 minutes with which to establish a regression line. Increasing the number of samples collected within the first few minutes might have revealed the biphasic pattern detected in humans.\textsuperscript{10,11} Interestingly, the physiologic tear turnover rate (ie, the rate when those early points associated with reflex lacrimation were excluded) in humans was 13\% to 18\%/min (depending on subject age), which was close to our value (calculated via the excluding method) of approximately 13\%/min.

Studies of tear volume and flow rate in other species are limited. The mean tear volume, turnover rate, and flow rate in humans have been reported to be 7.0 \textmu L, 13.1\% to 30.0\%/min, and 1.2 \textmu L/min, respectively.\textsuperscript{10,12,13} Rabbits reportedly have a tear volume of 7.5 \textmu L and a flow rate of 7\%.\textsuperscript{19} Cows in 1 study\textsuperscript{18} had a flow rate of 32.6 \textmu L/min, which is comparable to the flow rate of 33.62 \textmu L/min for an animal with a comparably sized eye. The tear volume was not determined in that study; therefore, the turnover rate could not be calculated.

The tear volume predicted by the excluding method (233.74 \textmu L) was in approximate agreement with the higher end of the estimate calculated on the basis of geometric considerations of the globe in horses (100.73 \textmu L). Because the geometric estimation is based on both the radius of the approximated sphere of the globe and the tear film thickness, it is likely that the geometric estimation would agree even more closely with our kinetically calculated value if the tear film thickness in horses was known to be thicker than the human tear film thickness estimates used in our calculations. It is reasonable to assume that the tear film is thicker in horses than in humans in that the horse ocular surface area is much greater, and a thicker film would be needed to maintain stability stretched over that greater surface area. Our geometric estimates predicted a tear film thickness of 92.6 \textmu m, which is twice as thick as even the thickest estimate in humans, but corroboration of this prediction is required.

Knowing the tear volume and tear turnover rate is clinically important because these variables affect the concentration versus time profiles of topically applied drugs. Consider, for example, topical administration of tobramycin for the treatment of a corneal ulcer infected by \textit{Pseudomonas aeruginosa}. The concentration in the tear film immediately upon instillation can be calculated with the following equation:

\[
C(t) = \frac{C_i \cdot V_i}{V + V_i}
\]

in which \(C_i\) is the concentration of the tobramycin eye-drops, \(V\) is the instillation volume of the tobramycin eye-drops, and \(V\) is the volume of tears. Assuming an instillation volume of 0.200 mL (as is commonly suggested for application via a subpalpebral catheter\textsuperscript{20}), assuming a tear volume of 234 \textmu L (0.234 mL) as calculated with the excluding method, and given that the concentration of commercially available tobramycin is 0.3\% (or 3 mg/mL), the concentration immediately following instillation can be calculated as follows:

\[
C_0 = \frac{3.0 \cdot 0.200}{0.234 + 0.200} = 1.382 \text{ mg/mL}
\]

In other words, the concentration in the tear film is diluted by more than half immediately upon instillation into the tears. In addition, using the tear turnover rate of 13.21\%/min (as calculated via the excluding method for horses in the present study), the concentration \(C(t)\) at any time after initial administration that results from dilution with newly formed tears can be calculated as follows:

\[
C(t) = C(t - 1) - 0.1321(C(t - 1))
\]

Assuming the MIC\textsubscript{90} for tobramycin against \textit{P aeruginosa} is 2 \textmu g/mL,\textsuperscript{21} by 50 minutes following instillation, the tobramycin would be diluted to a concentration (1.16 \textmu g/mL) lower than the MIC\textsubscript{90}; therefore, a dosing interval of every 30 to 45 minutes might be selected to ensure maintenance of a concentration that exceeds the MIC\textsubscript{90}. This is a simplification in that it does not allow for factors other than lacrimal dilution that can influence tear film concentrations (ie, tissue binding, protein binding, and tear film metabolism), but it does serve as a good first approximation. In addition, infectious keratitis in horses is a disorder of the corneal epithelium and stroma, not the tear film, so concentrations in corneal tissue would provide more valuable information but are much more difficult to obtain.

Obvious caveats to the foregoing discussion include the fact that the horses in our study were limited to females of a narrow age range, which was necessitated because those were the only horses available. Some authors claim that tear flow decreases
with advancing age in humans, whereas 1 study revealed no relationship between tear flow and age. In another study, older dogs were found to have reduced tear production, compared with younger dogs; however, another study in horses revealed no such correlation. In humans, tear flow rates appear to be little affected by sex across all age ranges, and sex differences in tear flow rates in horses have not been found.

More importantly, results of the study reported here provided kinetic data for tear parameters in ophthalmologically normal horses, but the horses with ocular disease that might benefit from dosing regimens derived from these data will have altered kinetics. It is intuitive that the lacrimation associated with corneal disease would result in higher tear volumes, turnover rates, and flow rates, and those parameters will vary widely from case to case. In fact, tear film turnover rates can increase up to 300% in humans after mild touching of the cilia of the lower lid with a small piece of filter paper, even after application of topical anesthetic. Therefore, the present data should be used only as a first approximation until additional studies similar to the one reported here establish kinetic parameters in horses with corneal disease.

References