A Novel Method to Evaluate Residence Time in Humans Using a Nonpenetrating Fluorescent Tracer

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Purpose. The purpose of these investigations was to develop an improved method for measuring precorneal residence time (RT) and to demonstrate its efficacy with novel formulations.

Methods. A biomicroscope was adapted for use as a clinical fluorometer. Using a nonpenetrating fluorescent probe (FITC-dextran, 70,000–73,000 molecular weight [MW]), RT was estimated as the time to return to baseline (gross RT) and from parameters derived from least-squares regression fits to the decay data (area under the curve [AUC], elimination rate, and time for 50% of the signal to be eliminated [T_{50}]). One rabbit and two human studies were conducted. The studies were randomized, double-masked, and controlled. Repeatability in humans was examined in 15 subjects (six determinations per subject, $n = 90$ total).

Results. The FITC-dextran tracer did not penetrate into corneal tissue. The rabbit gross RTs were 14.5, 15.0, and 16.0 minutes for three low-viscosity solutions ($\eta = 2.7$–7.7 mPa/sec) and 22.5 minutes for a more viscous solution ($\eta = 357$ mPa/sec). For a high-viscosity ($\eta \cong 30{,}000$ mPa/sec) gel in humans, the method demonstrated approximately a twofold increase in gross RT and AUC compared with buffered saline. Repeatability of the method appeared acceptable, with intersubject variability the most significant factor affecting precision.

Conclusions The new method is safe and convenient and offers comprehensive RT data. Furthermore, it appears to differentiate among formulations. However, as with other tear-influenced parameters, there is significant variability. Thus, sufficient sample sizes are necessary for meaningful comparative investigations. (*Invest Ophthalmol Vis Sci.* 2002;43:1032–1039)

It is often desirable to prolong the precorneal residence time (RT) of ophthalmic formulations for several reasons. Improved RT may lead to a reduction in dosage frequency because of improved bioavailability and a decrease in active ingredient concentration with the concomitant reduced potential for side effects.

An essential adjunct to developing topical ocular formulations that achieve prolonged RT is the ability to obtain reliable measurement of their retention characteristics. An indicator could be covalently bound to the active ingredient(s) comprising the formulation. However, covalent binding can be both challenging and expensive and is therefore not practical for the efficient screening of potential clinical entities. A frequently used alternative has been to admix low-molecular-weight (molecular weight [MW] <500) fluorescent tracers or gamma emitters into the ophthalmic formulation and to follow their elimination from the precorneal area with appropriate instrumentation.

One limitation of the low-MW tracer method is that the tracers may be eliminated at a rate different from the larger active vehicle ingredients because of their disparate size. Another difficulty is that low-MW tracers penetrate the ocular tissues concurrently with the normal reflex and basal tearing elimination processes, and this can lead to a significant increase in the background signal and cause misleading interpretation of the data.

If the penetration characteristics of the tracer as a pseudo drug are desired, small-molecule tracers have some utility. However, MacDonald and Maurice[1] have demonstrated for fluorescein that conjunctival and corneal penetration artificially elevates elimination rate by approximately 25%, compared with a high-MW, nonpenetrating fluorescent tracer. In addition to the penetration concern, the gamma scintigraphy method has been criticized for poor sensitivity and spatial resolution,[2] in addition to requiring a radioisotope pharmacy.

Because the small-molecule tracer methods appear to have limitations, we set out to determine whether an alternate method, using a high-MW fluorescent indicator, could be developed to measure vehicle precorneal RT.[3,4] This report outlines simple, inexpensive modifications to a clinical photographic biomicroscope and the use of an FITC-dextran (MW ~70,000) tracer to measure precorneal RT. Normative data are presented for both rabbits and humans, and these data suggest that the new technique is convenient and can differentiate the efficacy of topical ophthalmic formulations.

Materials and Methods

Instrumentation

A photographic biomicroscope (model FS-2; Nikon Corp., Tokyo, Japan) was used as the base instrument for the fluorometer system. A custom filter (490RDF10, λ_{max} 488 nm, full width at half maximum [FWHM] 10 nm; Omega Optics, Brattleboro, VT) was used for fluorescein excitation. The emitted light was transmitted through the standard instrument optics onto a side-on photomultiplier tube (PMT) mounted on the camera's port (PMT housing, [catalog no.] 70680; PMT, 77349; adapter, 71260; Oriel Corp., Stratford, CT). The emitted light passed through a black metal mask of 10.0-mm diameter (to reduce stray light; mask located just in front of the PMT) and an emission filter (530RDF40, λ_{max} 530 nm, FWHM 30 nm; Omega Optics). The biomicroscope beam measured 1.5 mm wide by 6.0 mm high at the tear film plane. Observations were made at approximately ×30 magnification, which resulted in a 3.8-mm^2 tear film sampling area.

PMT bias voltage was set at 775 mV, and the PMT output was directed to a readout (7070; Oriel Corp.). The final signal was fed to a standard strip chart recorder (Houston Instruments, Austin, TX). A schematic of the assembled system is shown in Figure 1. Amplifier linearity was checked periodically and the amplifier was found to be linear.

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Presented in part at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida, May, 1994.

Supported in part by the Australian Government through the Cooperative Research Centres Scheme.

Submitted for publication February 5, 2001; revised October 2, 2001; accepted October 23, 2001.

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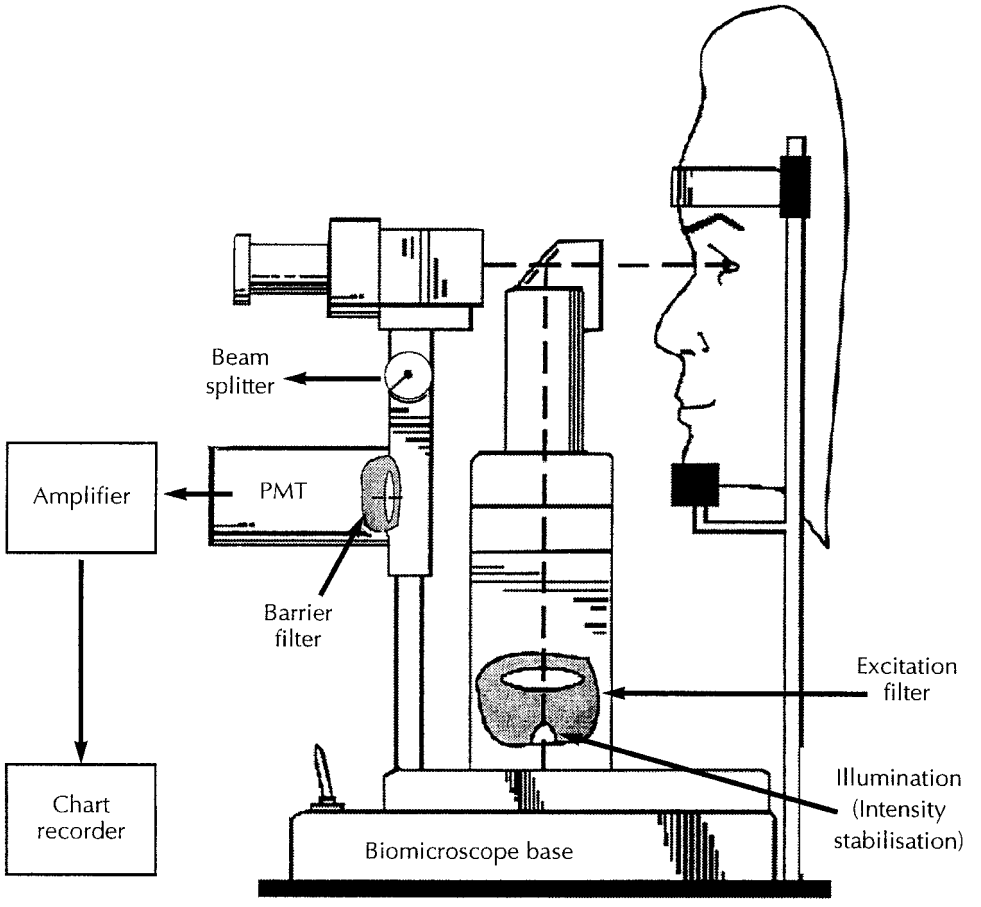

FIGURE 1. Schematic diagram of the biomicroscope fluorometer. The base instrument incorporated a beam splitter for rapid switching from viewing to recording through a side-on photomultiplier. The illumination was stabilized with a voltage-feedback system.

Tracer

We selected FITC-dextran as the tracer, because its spectral characteristics are similar to sodium fluorescein, and it is readily available in various molecular weights. Additional favorable attributes of FITC-dextran are stability, nonaffinity with protein, and proven clinical safety.[5–7]

An important consideration for the tracer MW concerns the size necessary to prevent tissue penetration. To the best of our knowledge, no data are available in the literature concerning dextran's penetration into the human ocular surface. However, Ambati et al.[8] have recently reported in vitro penetration data through rabbit sclera for FITC-dextrans. Scleral penetration was found to be dependent on MW and was reduced as MW increased, with a plateau at approximately 70,000.[8]

A key assumption of the current RT technique is that the tracer is eliminated from the precorneal zone at the same rate as the pharmacologically active compound (e.g., a viscosifying agent, in the case of palliative artificial tears) because both polymers are approximately the same molecular size. Although mindful of this concern, we selected a single tracer of reasonable size (≥70,000 MW) to use in all investigations. The 70,000 MW tracer (Molecular Probes, Eugene, OR) would be unlikely to penetrate the ocular surface, yet would be of a molecular size similar to most of the viscosifying agents studied in these preliminary investigations.

The FITC-dextran was used at a concentration of 0.10% (wt/vol), because it gave a signal that was two to three log units above baseline and did not affect vehicle viscosity.

Data Collection

Measurement sessions were scheduled for each subject (rabbit and human), with washout periods of at least 24 hours, but with no constraint as to time of day. Data were collected during typical working hours, from 8 AM to 5 PM. Biomicroscopy without the use of vital stains was performed before data collection to ensure a normal anterior segment.

For all determinations a sample volume of 25 μL was instilled into the lower fornix with a positive-displacement pipette. The drop size was standardized to 25 μL to simulate a typical ophthalmic drop volume.[9] Drop size is known to influence elimination rate,[10] although the goal of these investigations was to determine washout under typical clinical conditions. Care was taken to place the drop into a pocket at the base of the lower fornix. One eye had a test formulation instilled, followed within 2 to 3 minutes by a test of the control formulation in the contralateral eye to allow elimination measurements to be made at almost the same time. Preliminary investigations indicated that this nearly concurrent approach was efficient and did not affect the initial eye, in that no change in tracer decay (i.e., no increase in elimination rate) was noted when the contralateral drop was instilled.

The volunteers were asked to roll their eyes and blink, to gently distribute the formulation after instillation. In rabbits, the lid was manually blinked three times and then left alone for the remainder of the measurement session. Both volunteers and rabbits were allowed to blink at will during the data collection period.

Fluorometry data were collected as follows. The PMT, at approximately a 300-mV input voltage, and the biomicroscope were warmed up for at least 30 minutes before measuring began. Input voltage at the time of data collection was between 750 and 850 mV. Biomicroscope settings (e.g., beam height, width, lamp intensity, and illumination angle) were kept consistent throughout the study.

Endogenous corneal fluorescence was measured for each eye four to six times as a baseline signal, for later subtraction from all fluorescence intensity values. After instillation of the drop, the fluorescence

Table 1. Topical Formulations for Rabbit Investigation

Formulation*	Major Components† (MW)‡	Surface Tension (mN/m)	Viscosity§ (mPa/sec)
CMC 0.5%	Carboxymethylcellulose 0.5% (90,000)	68.7	3.5
HPMC 0.3%	Hydroxypropylmethylcellulose 0.3% (80,000), dextran 70 (70,000), 10 ppm polyquaternium	44.7	7.7
PVA 1%	Polyvinylalcohol 1.0% (20,000–30,000) polyethylene glycol 400 1.0% (400,000)	43.2	2.7
CMC 1%	Carboxymethylcellulose 1.0% (700,000)	NA	357.0

* All formulations admixed with 0.1% wt/vol FITC-dextran.
† Concentrations are wt/vol.
‡ Nominal molecular weight (i.e., before sterilization).
§ Viscosity measured at shear rate of 2.2 sec^{-1}, 25°C.

intensity was measured during the middle of the interblink period, which was identified by observing the cessation of upward tear film movement after a blink.

After instillation of the formulation, repeated fluorescent measurements were made for approximately 3 seconds, starting immediately after instillation and followed by measurements at selected time points until two consecutive near-baseline fluorescence intensities were recorded.

The RT end point was reached when the fluorescence intensity was within 1.5 times that of the preinstillation endogenous corneal fluorescence. The 1.5-fold value was used, because it was more than three SDs above the preinstillation mean fluorescence. Thus, the fluorescence intensity end point was well above the measurement noise, although it represented a conservative estimate of RT. RT was defined as the time from formulation instillation to reach the first time point of the two consecutive near-baseline measurements.

Rabbit Studies

New Zealand albino rabbits (n = 10 young adult females) with nictitating membranes removed were used to determine the retention characteristics of four marketed artificial tear formulations (Table 1). No saline control was included, because the study was designed to be a direct comparison of the four preparations. The carboxymethylcellulose (CMC) 0.5% and 1.0% solutions (Cellufresh and Celluvisc, respectively) were commercial products from Allergan, Inc. (Irvine, CA), the hydroxypropylmethylcellulose (HPMC) tears (Tears Naturalle II) were produced by Alcon Laboratories (Fort Worth, TX), and the polyvinylalcohol (PVA) 1.0% solution (Hypotears) was from Iolab (Claremont, CA). All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was a randomized, double-masked, crossover design.

Human Studies

Subsequent to the rabbit feasibility studies, human investigations were undertaken to determine the clinical utility of the method. Two major investigations, in two groups of subjects, were performed. The first was an investigation of a proprietary topical gel formulation, and the second was a repeatability study. In addition, tracer penetration was examined in a subset of subjects. This was accomplished by remeasuring corneal fluorescence, after saline irrigation, at the completion of several formulation data collection sessions.

All subjects were young, healthy volunteers without evidence of ocular surface abnormalities or dry eye. Subjects did not wear contact lenses or take tear-influencing medication (e.g., antihistamines). All participants provided written informed consent after a description of the study procedures. The human study adhered to the tenets of the Declaration of Helsinki.

Gel Formulation Study

The test formulation was a proprietary in situ gelling vehicle (viscosity 55,000 to 80,000 mPa/sec, measured at 2.6 $seconds^{-1}$ and 37°C) comprising cross-linked polyacrylic acid (0.3% wt/vol, Carbopol 950; B. F. Goodrich Co., Akron, OH) and methylcellulose (0.50% Methocel A4M Premium; Dow Chemical Co., Midland, MI). The control was borate-buffered isotonic saline at pH 7.4. The gel study was randomized (for eye and formulation), double-masked, and controlled. Eleven normal subjects were recruited (four women, seven men; mean age, 32.4 years; range, 21–45). Three had blue irides, seven had brown, and one had hazel.

Repeatability Study

The repeatability study was conducted over a period of 3 months, using borate-buffered commercial saline (Lens Plus; Allergan, Inc.) admixed with FITC-dextran. Fifteen normal subjects were included (4 men, 11 women; age range, 22–37 years). The eye for saline instillation was selected at random but was always the same eye. For scheduling reasons, measurements were not controlled for time of day, despite evidence that there are diurnal influences on tear dynamics.[11] Data were collected between 8 AM and 5 PM.

Tracer Penetration

Tracer penetration was determined in six subjects from the saline repeatability study. Preinstillation measurements were obtained (n = 4–5 in each subject). After the saline RT measurement (20–30 minutes), the eye was irrigated with additional buffered saline and the intrinsic corneal fluorescence remeasured (n = 4–8 measurements). The Wilcoxon signed rank test was used to determine whether the difference was statistically significant.

Data Analysis

The three RT parameters of interest in these investigations were gross RT, area under the fluorescence versus time curve (AUC), and the elimination rate constants.

Gross RT was obtained directly from the chart recorder printout, taken as the elapsed time to the first of two consecutive intensities within 1.5 times that of the intrinsic corneal fluorescence. Commercial graphing software (Kaleidagraph, ver. 3.0.1; Synergy Software, Reading, PA) was used to calculate AUC and the curve fits for the elimination rate determinations. The AUC was calculated from the time of instillation to the first of the two consecutive measurements that were within 1.5 times the baseline intrinsic fluorescence.

The graphing software has the ability to customize curve fits by the general-curve-fit option. The general curve fit involves a user-defined equation and the Levenberg-Marquardt least-squares algorithm. This algorithm is widely used, because it is efficient[12] and robust.[13]

Fluorescence intensities were measured as deflection distances from the hard copy of the chart recorder output. Figure 2 shows a typical chart recorder trace. These distances were converted into arbitrary fluorescence units and standardized to one amplifier setting. Raw elapsed time (from drop instillation) and arbitrary fluorescence units were entered into the graphing software data sets. Approximately four to six fluorescence intensity–time point data pairs were available for the initial rabbit and human data. Approximately 20 to 30 data pairs

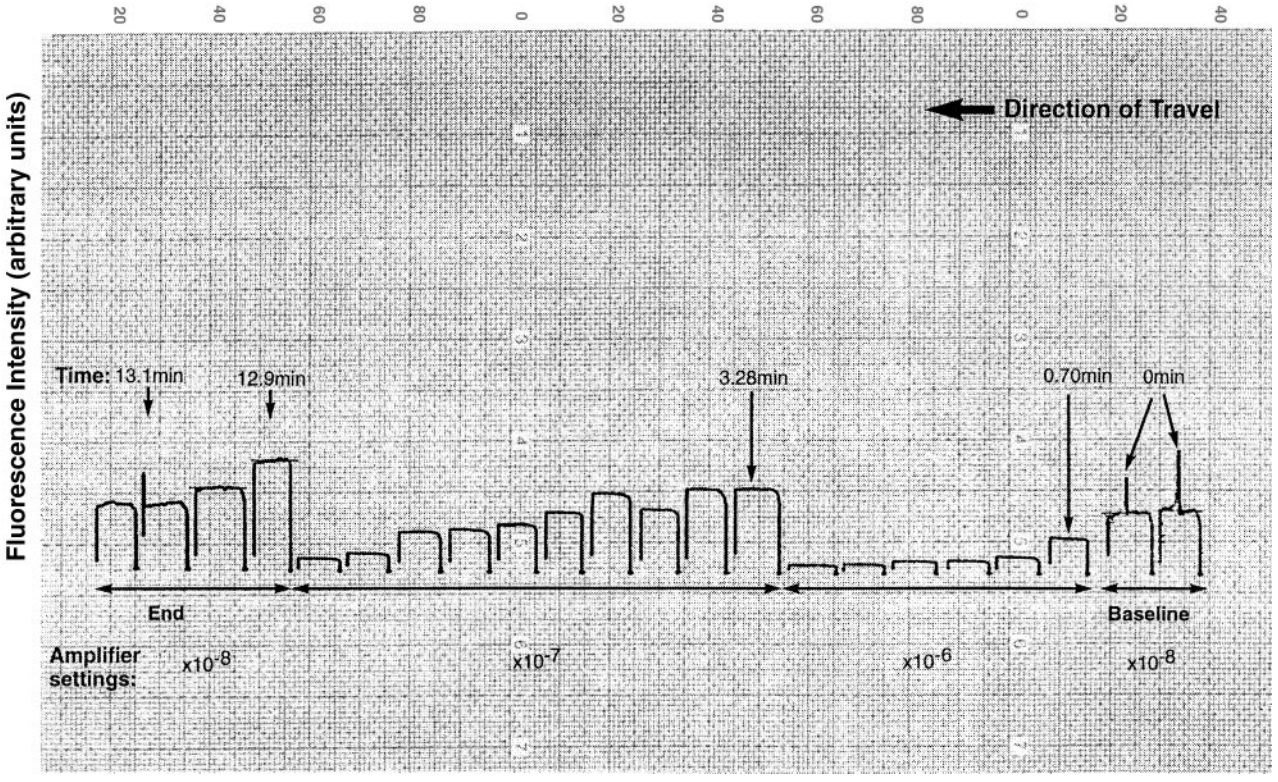

FIGURE 2. Sample chart recorder trace. Square waves represent recording periods of 2 to 4 seconds.

were available for human saline repeatability decay curve fits. This latter approach was used as the technique evolved, because enhanced fitting accuracy was observed with the additional points.

Fluorescence intensity data were normalized to obviate variation due to various sources (e.g., differences in anatomy). The endogenous, or autofluorescence, intensities were subtracted from all postinstillation data before normalization. Because the drop was instilled into the lower fornix, some delay occurred with the more viscous preparations (e.g., the proprietary gel in the human studies) before the peak fluorescence signal was observed.

Preliminary evaluation of several decays suggested that a custom exponential curve fit (either single- or double-exponential) would most accurately describe the data. The following equations were used.

Single exponential (two-parameter fit)

$$Y = Ae^{-t/\tau} \quad (1)$$

In this form, A is expected to be unity, because the data were normalized by dividing by the peak response; t is the elapsed time in minutes, and τ is the life of the decaying species in minutes. The inverse of the lifetime represents the fractional loss per minute, or elimination rate k, as is commonly reported in tear flow studies.

Double exponential (three-parameter fit)

$$Y = fe^{-t/\tau_1} + (1 - f)e^{-t/\tau_2} \quad (2)$$

Lowercase t is again elapsed time in minutes; f is the rapidly decaying fraction; τ_1 is the shorter lifetime related to the rapid elimination rate constant ($k_1 = 1/\tau_1$); $(1 - f)$ is the slowly decaying fraction; and $1/\tau_2$ is the long lifetime related to the slower, more physiological elimination rate constant ($k_2 = 1/\tau_2$).

The variables of interest from the decay analysis were the overall rate constant (k) in the case of a single exponential fit and the rate constant (k_2) in the case of a biexponential fit. The retention of the single- or double-exponential fit was made on the basis of the best χ^2 and R statistics from the graphing software output. The software determined the inflection point for the double-exponential fit and was not subject to influence by the analyst. The more physiological decay k_2 was used as the parameter of interest, rather than the initial, rapid-decay constant k_1, because it was thought to be a more reliable indicator of RT behavior. Once the best-fit equation was generated, it was used with a commercial spreadsheet to calculate the T_{50}, or time for 50% of the dose to be eliminated.

Statistical Analysis

Overall and pair-wise comparisons of the rabbit data for gross RT and AUC were made using Friedman's test and the Wilcoxon signed rank test, respectively (StatXact 3; Cytel Software, Cambridge, MA). Probability adjustments were made for the pair-wise comparisons. The Wilcoxon signed rank test was also used for the human penetration and gel study data, again providing exact probabilities. The repeatability data were tested for between-day variability, by using an ANOVA model with effect terms for subject and day.[14] Sample size estimates for future investigations were based on the SD of the differences, day 6 minus day 1 (the greatest difference of the six repeatability days) for the gross RT measure, and were calculated on computer (nQuery Advisor statistical software; Statistical Solutions, Ltd., Boston, MA).

RESULTS

General

Postmeasurement biomicroscopy demonstrated very little corneal epithelial disruption from any of the test or control formulations in either the rabbits or humans. Fewer than six punctate abrasions were observed in two human subjects on one occasion each. There were no long-term sequelae from either the formulations or the procedures in any animal or human subject.

TABLE 2. Dye Penetration in Humans

Subject	Preinstillation Fluorescence*	Postinstillation Fluorescence*	Difference
1	8.30 ± 0.7	7.75 ± 0.4	0.55
2	9.87 ± 0.2	7.92 ± 0.9	1.95
3	6.97 ± 0.4	6.94 ± 0.3	0.03
4	6.90 ± 0.2	6.44 ± 0.8	0.46
5	9.12 ± 0.3	9.75 ± 0.5	0.63
6	8.44 ± 0.6	8.17 ± 0.3	0.27

Data are mean fluorescence ± SD.
* Arbitrary fluorescence units.

The data from the penetration study are given in Table 2. The Wilcoxon signed rank test with exact probability was used to test for preinstillation versus postinstillation differences in the six tested subjects. There was insufficient evidence to demonstrate a significant difference ($P = 0.3125$, two-tailed test).

Rabbit Studies

All four artificial tear formulations demonstrated similar exponential decay when administered to the rabbits, although the measurement time points were too sparse to allow quantitative curve fitting. The data for the HPMC 0.3% and CMC 1.0% formulations are shown in Figure 3. Only nine sets of rabbit data are depicted for each solution, because one run of the 0.3% HPMC and 1% CMC formulations was not evaluable from the original 10 rabbits. Gross RT and AUC are summarized in Table 3.

The gross RTs ranged to a maximum of 22.5 minutes (CMC 1.0%) from a low of 14.5 minutes (PVA 1.0%). All three low-viscosity formulations had similar gross RTs, whereas the higher viscosity CMC 1.0% solution had a slightly longer RT at 22.5 minutes. The result of Friedman's overall test was not significant across the four formulations ($P = 0.2300$), although the CMC 1.0% gross RT was significantly longer than that for the HPMC 0.3% solution (two-tailed $P = 0.008$). That some of these differences did not reach statistical significance was somewhat surprising, given the similar viscosities of three of the formulations compared with the much greater 1.0% CMC material viscosity.

TABLE 3. Summary of Rabbit Data

Formulation	Gross RT (min)	AUC*
CMC 0.5%	16.0 ± 9.7	6.247 ± 5.17
PVA 1.0%	14.5 ± 6.4	5.525 ± 3.12
HPMC 0.3%	15.0 ± 7.1	4.773 ± 1.53
CMC 1.0%	22.5 ± 9.5	6.572 ± 2.89

Data are the mean ± SD.
* In arbitrary units.

The AUCs did not demonstrate a viscosity effect similar to that for the gross RT (i.e., CMC 1.0% being greater than the low-viscosity formulations). All formulations demonstrated nearly equivalent AUCs. There was no statistically significant difference among the formulations for AUC overall ($P = 0.932$) or relative to the pair-wise comparisons. Rabbit data variability, as demonstrated by the coefficient of variation for gross RT and AUC, was substantial (42%–61% of the mean for gross RT, 32%–83% of the mean for AUC). This variability probably explains why the results were generally not statistically significant.

Human Data

Representative data for subject 131 from the gel formulation investigation are shown in Figure 4 and for the sample ($n = 10$ subjects) in Figure 5. Figure 4 demonstrates the obvious difference in AUC for the control and test formulations and the complete return to baseline. With one control and three individual gel formulation decays (i.e., only one gel formulation was used in all subjects), there was a delay of several minutes in attaining the maximum fluorescence signal. In all other cases the maximum signal was achieved by the 2-minute time point, which was the initial data collection period. This suggests minimal delay in transit time for the tracer to reach the center of the precorneal area, a surprising finding for the relatively viscous gel formulation.

Summary data for the gel formulation study are listed in Table 4. Both the gross RT and the AUC for the gel formulation were approximately two times greater than for the buffered

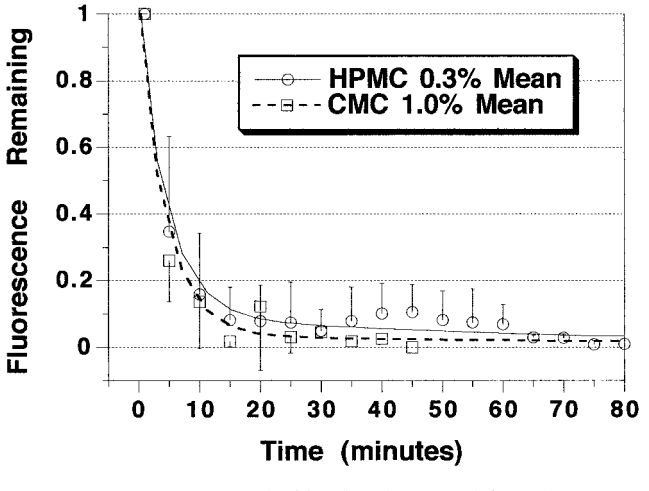

FIGURE 3. Demonstration of rabbit data for two of the palliative tear preparations. Data are the mean ± SD ($n = 9$ sets of useable rabbit decays; one 0.3% HPMC and one CMC 1% rabbit decay was not evaluable). End points were return to baseline, which explains the time difference between the two formulations. Note that there was only slight difference in behavior, despite significant differences in viscosity.

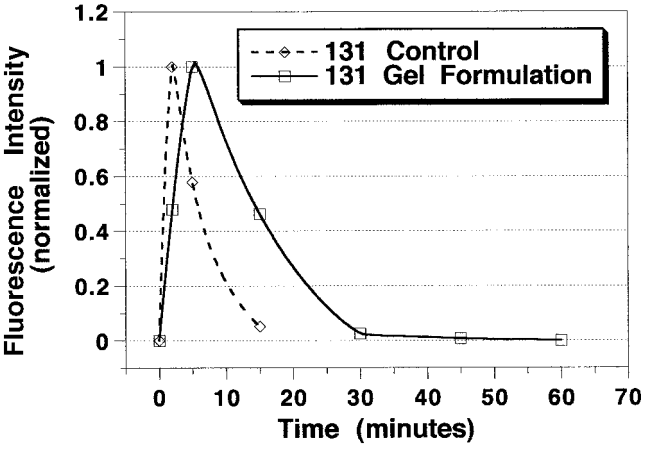

FIGURE 4. Control and gel formulation RT behavior for subject 131. Starting from the peak value, single exponential curve fits best described the decay data: $k = 19.5\%$ per minute and 9.1% per minute for control and gel, respectively (χ^2 and R were 0.001, 0.999 and 0.010, 0.993 for control and test fits, respectively). The AUCs for the control and gel formulations were 6.519 and 13.909, respectively.

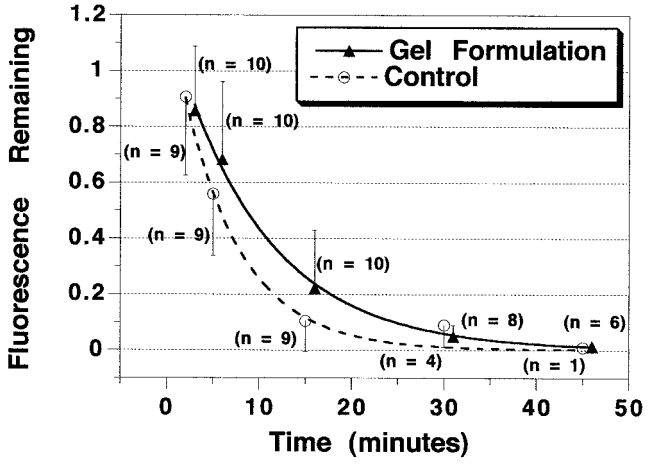

FIGURE 5. Mean normalized fluorescence intensity values at each time point (gel data offset for clarity; n = number of subjects with data available). Only nine control decays were evaluable at the beginning of the experiment. Various numbers of subjects are shown, because times for individual subjects to return to baseline varied. Single exponential curve fits best described the decay behavior: k = 15.7% per minute and 10.0% per minute for control and gel, respectively ($\chi 2$ and R were 0.007, 0.995; and 0.002, 0.998 for control and gel fits, respectively).

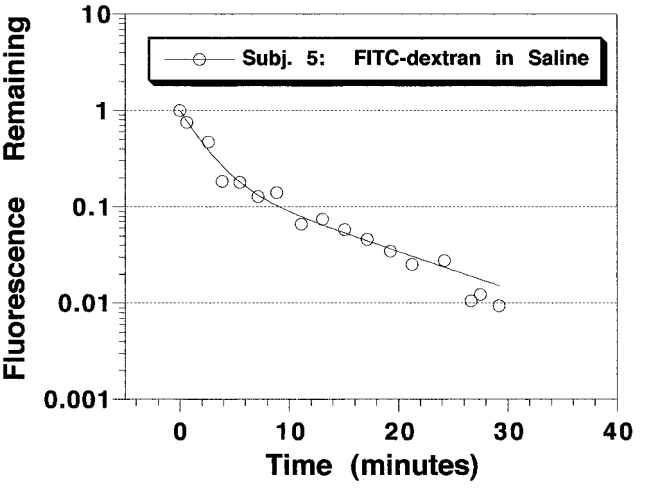

FIGURE 6. Fluorescence decay in one subject in the repeatability study. Gross RT = 31.2 minutes, AUC = 5.116. Biexponential curve fit ($\chi 2$ = 0.016, R = 0.988): k_1 (initial decay) = 47.9% per minute, k_2 (physiological decay) = 8.6% per minute.

saline control, and the difference was statistically significant (Wilcoxon signed rank test, two-tailed $P = 0.002$ for both gross RT and AUC).

Single-exponential curve fits best described both the control and gel decay behavior and yielded calculated elimination rate constants of 24.1% ± 10.1% per minute and 16.6% ± 11.2% per minute, respectively. Taken together, the three measures suggest that the gel formulation retarded the elimination of the fluorescence indicator by a clinically meaningful amount compared with the control.

Repeatability

The rabbit and human gel formulation investigations provided acceptable pilot data, but from those results two points were made clear: that substantial individual variability occurs in elimination behavior, as evidenced by the significant coefficient of variation (e.g., 35% and 61% for the control and test gross RT, respectively, Table 4), and that enhanced accuracy of the decay curve fits would be likely with additional data collection time points.

With those considerations in mind, a power calculation was undertaken before the conduct of the human repeatability study, to determine an appropriate sample size. Sample size calculation was based on a type I error $\alpha = 0.05$, type II error $\beta = 0.20$ (i.e., 80% statistical power), a two-tailed test, and a clinically significant difference in RT of 15 minutes. A SD of 10.4 minutes from the gel formulation study (control gross RT) was used in the calculation. The sample size was adjusted to correct for a repeated-measures reduction in statistical significance. On this basis, the calculated sample size was 12 subjects. Four extra subjects (to add a complete reserve block for the randomized block design) were recruited in case volunteers withdrew.

A typical elimination repeatability (i.e., using buffered saline) study in humans is shown in Figure 6. Whereas the gross RT and the AUC were straightforward to obtain, the curve fitting improved with the additional data points. For the entire sample (i.e., 90 individual curve fits) the curve fit correlation coefficients were 0.90 or more. One significant result was the finding of an elimination rate of 13.9% to 19.5% per minute in the repeatability study (Table 5), in line with data in the literature for the normal tear elimination rate.[1] However, the saline elimination rate in humans was much greater (~24% per minute; Table 4). The larger sample size in the repeatability experiment (n = 15 subjects vs 8 subjects for the gel experiment) may explain the discrepancy. Summary data for the gross RT and the AUC are presented in Table 5 and are depicted graphically in Figure 7.

DISCUSSION

The purpose of these investigations was to develop a new fluorophotometric method to improve measurement of ocular surface RT. The use of a photographic biomicroscope modified with appropriate filters and a modular photographic detection apparatus, combined with a nonpenetrating tracer (FITC-dex-

TABLE 4. Summary Data, Gel Formulation Investigation

Instilled Drop	Gross RT (min)	AUC*	Decay Constant† (%/min^{-1})
Control	16.9 ± 10.4 (10)	5.46 ± 3.4 (10)	24.12 ± 10.1 (8)
Gel	36.0 ± 12.7 (10)	10.02 ± 3.6 (10)	16.56 ± 11.2 (9)

Data are the mean ± SD, with number of subjects in parentheses.

* In arbitrary units.

† Calculated from the single-exponential curve fit of individual subject data. Although the χ^2 goodness-of-fit values were all less than 0.056 (the worst individual curve fit statistic) and the R values were greater than 0.95 for all fits, the decay constants should be interpreted with caution, because of the few (n = 3–5) data points available for curve fitting.

TABLE 5. Summary Data, Buffered Saline Repeatability

Measure	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	P†	R‡
Gross RT (min)	24.1 ± 7.4	23.0 ± 7.5	25.6 ± 11.3	24.1 ± 11.4	24.7 ± 8.5	21.1 ± 9.3	0.391	0.62
AUC	4.6 ± 1.5	4.2 ± 1.4	3.9 ± 1.9	3.4 ± 1.6	3.5 ± 1.5	2.8 ± 1.0	<0.001	0.42
Elimination rate (%/min)	13.9 ± 11	19.5 ± 10	16.7 ± 12	17.2 ± 10	17.0 ± 10	18.3 ± 10	0.647	0.28
T_{50} (min)	1.53 ± 0.9	1.65 ± 1.1	1.31 ± 1.3	1.13 ± 1.0	1.37 ± 1.1	0.82 ± 0.6	0.178	0.23

* $n = 15$ except for day 1, T_{50} data where $n = 14$ subjects with evaluable data.

† Test for between-day variability from an ANOVA model with effect terms for subject and day.

‡ Estimate of reliability assuming equal day effects, based on results of the ANOVA model.[14] Values close to 0 indicate poor reliability and values close to 1 indicate excellent reliability. Even though there is an apparent day effect in the analysis of AUC, the reliability is moderate. The reliability for T_{50} and elimination rate looks relatively poorer, probably because these were derived rather than actual measures.

tran, ~70,000 MW), appeared to provide useful and desirable RT data. The technique is simple, convenient for subjects, and differentiates among topical formulations. There are several advantages and limitations.

Compared with gamma scintigraphy, the use of a fluorometric approach may allow wider clinical application due to more common equipment availability and the economy and ease of admixing a fluorescent tracer into the formulation. For example, the fluorescent approach obviates the need for a radioisotope pharmacy, and fluorescein derivatives have a longer lifetime and greater ease of disposal than does technetium-99, a commonly used gamma emitter. Moreover, the larger tracer molecule prevents penetration and allows a direct measure of RT (time to return to baseline, or gross RT) in addition to the commonly reported measures of AUC and elimination rate.

A further advantage is that the fluorescent tracer can be selected to match the size of the viscosifying agent under consideration, providing that the minimum MW to avoid penetration is achieved (approximately 70,000 for FITC-dextran).[8] The tracer–viscolyzer MW-matching presumably provides a more accurate estimate of the elimination of the investigated component than would a small tracer, such as sodium fluorescein or technetium-99. However, this key assumption remains to be evaluated, possibly by covalently binding a suitable tracer to the viscolyzer.

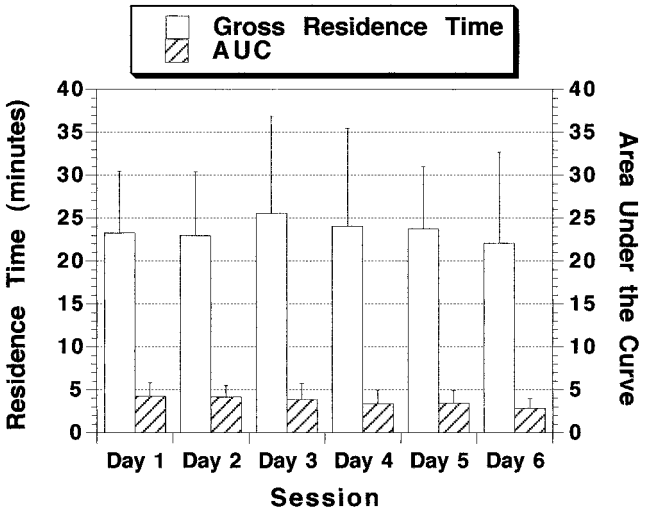

FIGURE 7. Summary repeatability data for saline in humans: gross RT and AUC. Sample size was 15 subjects on all 6 days (mean ± SD). AUC is in arbitrary units.

Normative Data

It is difficult to make direct comparisons to reports appearing in the literature, because of the differences in technique (e.g., topical versus lower fornix drop instillation), different approaches to data analysis (e.g., calculation of elimination rate in seemingly arbitrary segments of 0–195 and >195 seconds[15]), and the interference of absorbed signal. Because small-molecule decays have been characterized by regression curve fits as in the present investigation, the elimination rate and T_{50} measures can be used for comparison.

Snibson et al.[16] have presented T_{50} values for buffered saline in normal humans of approximately 1 minute. This compares favorably with our range of 0.82 to 1.65 minutes from the repeatability study (Table 5). However, it is interesting that the elimination rates were similar when the tracers were significantly different in size.

A noteworthy aspect of the present data is the average gross RT of the saline controls used in the human investigations (Tables 4, 5). The RT of the control solution was approximately 17 minutes in the gel study and varied from 21 to 25 minutes in the repeatability study. This seems to be a remarkably long RT for a nonviscous solution. Although there are no studies in the literature in which similar measures were used (i.e., gross RT with a nonpenetrating tracer) to compare with the present study, there may be several partial explanations for the duration of gross RT.

First, all drops were instilled into the lower fornix, which may have served to delay the total elimination through a reservoir effect. Although signal was detected in the central corneal area essentially instantaneously, there could have been small amounts of tracer released from the inferior fornix over time until the tracer was totally eliminated. MacDonald and Maurice,[17] using a small (0.2-μL drop size) tracer instilled into the lower fornix, found a mean time to appearance of approximately 4 minutes, suggesting that there is, in fact, some lag. Given that we used a much larger drop (25 μL), a reservoir effect would not be surprising.

Another contributing factor is probably that the tracer did not penetrate. MacDonald and Maurice[1] have shown in the same subjects that tracer penetration overestimates the elimination rate by as much as 25%. Thus, due to the fluorescence detection sensitivity and the confinement of the tracer to the precorneal tear film, a longer RT would not be unexpected.

Repeatability and Study Design

Although all four measures demonstrated reasonable repeatability (Table 5), there is significant variability in the RT estimates (e.g., coefficients of variation ranging from 25% to 60%). This has major significance for effective study design.

To estimate the sample size required for a comparative investigation, the gross RT and AUC normative results were evaluated. The presumed investigation is a crossover study to compare two ophthalmic formulations using a two-tailed test, a significance level set at 0.05, and a type II error set at 0.20 (i.e., 80% statistical power). Of the two variables, the gross RT data demonstrated the greatest day-to-day variability (day 1 compared with day 6) and was used for the sample size estimate.

The day 6 minus day 1 SD of the differences was 9.108 (n = 15 subjects). In conjunction with this value and the parameters just described, various sample sizes were estimated depending on the desired clinically meaningful differences in gross RT (in minutes), as follows. For a 5-minute difference, 28 subjects are needed; similarly, for a 6-minute difference, 20 subjects; 7-minute, 16; 8-minute, 12; 9-minute, 10; 10-minute, 9; 15-minute, 6; and 20-minute 4.

Future Directions

Although this novel technique appears promising, several fundamental aspects remain to be examined. First, the "similar elimination based on the similar MW" assumption of the tracer compared with the viscosifying agent(s) under consideration should be tested. It is probable that chemical structural and behavioral differences are present in this tracer compared with the viscosifying agents that affect RT. One approach might involve comparison of the admixed FITC-dextran RT data with those obtained from observation of a labeled "active" agent.

Another aspect that should be examined is the comparison of this new method with other commercial fluorometers. As well, the choice of saline as a control should be examined. It may be that a solution containing a viscolyzer comparable in size to the tracer, yet with minimal viscosity, may give more realistic control data.

Furthermore, a multitude of investigations have been performed with ophthalmic formulations, using small molecule methods, and a direct comparison in the same group of subjects would be helpful. To our knowledge, such a direct comparison has not been reported.

Conclusions

We have described a new technique for RT assessment that appears to be simple, has wide clinical applicability, and offers semiquantitative RT measurements. The technique apparently can discriminate among low- and high-viscosity formulations in rabbits and humans. Furthermore, we have provided extensive normative data for future reference and have established that the results are repeatable. Overall, this new method may be useful and complementary to the several established methods.

Acknowledgments

The authors thank Ronald C. Chatelier, PhD, for advice on regression curve fitting and review of the manuscript.

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