Movement of Fluorescein Monoglucuronide in the Rabbit Cornea

Diffusion in the Stroma and Endothelial Permeability

Katsuichi Shiraya and Shigetoshi Nagafaki

The movement of fluorescein monoglucuronide, a fluorescent metabolite of fluorescein, was studied in the rabbit cornea in vitro and in vivo. A stromal strip was exposed to fluorescein monoglucuronide, and the diffusion rate and the distribution in the stroma were measured every hr for 24 hr. The diffusion coefficient was $0.94 \pm 0.11 \times 10^{-6}$ cm$^2$/sec, and the saline/stroma distribution ratio was in a range of 0.67 to 0.69. The concentration of fluorescein monoglucuronide in the anterior chamber and the cornea was measured every hr for 8 hr following intravenous administration. The endothelial permeability was $4.7 \pm 1.0 \times 10^{-4}$ cm/min, and the aqueous/cornea distribution ratio was $0.56 \pm 0.05$. It appears that the corneal endothelial permeability in the living eye determined hitherto from systemic administration of fluorescein is most likely the permeability to fluorescein monoglucuronide. Invest Ophthalmol Vis Sci 27:24-28, 1986

Fluorescein is commonly used as a quantitative tracer for measuring the endothelial permeability of the living cornea. The previous methods of measuring the permeability to fluorescein may be classified into two groups based on the techniques of administration: topical administration to the cornea or systemic (intravenous or oral). Several inconsistencies, however, appear in the published results between these two groups. For example, the fluorescence distribution ratio between the anterior chamber and the cornea measured from systemic administration of fluorescein$^1$-3 appears to be lower than the equilibrium distribution ratio in the excised cornea.$^4$ Also, the methods using systemically administered fluorescein tend to yield higher values for the endothelial permeability than the methods using topically administered fluorescein both in the human$^5,6$ and the rabbit cornea.$^7$

When fluorescein is given systemically, it is rapidly metabolized to fluorescein monoglucuronide.$^8,9$ This metabolite is $\frac{1}{2}$ to $\frac{1}{3}$ as fluorescent as fluorescein, depending on the excitation wavelength,$^9,11$ and it becomes the dominant fluorophore in the blood plasma shortly after systemic administration of fluorescein.$^{10,11}$ Therefore, fluorescence intensity should not be taken as a measurement of fluorescein concentration when the intensity is measured many hours after the dye has been administered systemically.

Since long-term measurements are required for the permeability of the corneal endothelium to be determined from the time-course of fluorescence intensities of the anterior chamber and the cornea, we wonder whether the reported permeability that was measured from systemic administration of fluorescein is the permeability of fluorescein itself. Thus, we studied the movement of fluorescein monoglucuronide in the rabbit cornea, employing the same techniques as utilized previously for fluorescein. In this paper, the diffusion coefficient of fluorescein monoglucuronide in the corneal stroma, its distribution coefficient between stroma and aqueous humor, and the permeability of the corneal endothelium to fluorescein monoglucuronide are reported.

Materials and Methods

Chemically synthesized fluorescein monoglucuronide, $C_{26}H_{20}O_{11} \cdot 1.72H_2O$ with a molecular weight of 539.42, was used in all experiments. Methods of synthesis and purification were described elsewhere.$^{12}$ The emission peak of this compound at pH 7.4 was 512 nm when excited at 492 nm. The excitation spectrum
at pH 7.4 showed three peaks at 452, 470, and 483 nm when the emission was measured at 512 nm.

A solution of fluorescein monoglucuronide at a concentration of 20 mM was prepared in isotonic phosphate buffered saline, a 9:1 mixture of 0.15 M NaCl and 0.15 M phosphate buffer, and sodium carbonate was added at a concentration of 20 mM; the pH was adjusted to 7.4. Serial dilutions of fluorescein monoglucuronide down to 1 μM were made with the same buffered saline, pH 7.4. Fluorescence intensity was measured with a slit-lamp fluorophotometer, a modification of Topcon photo-slit lamp (Topcon SL-5D, Tokyo Optical Co., Ltd., Tokyo). The photometer was set at 45 deg to the illuminator beam; a slit was 0.13 mm wide in the focal plane, and the window for measuring fluorescence was 0.29 mm long and 0.205 mm wide in air.

All investigations involving animals conformed to the ARVO Resolution on the Use of Animals in Research. The diffusion and the distribution of fluorescein monoglucuronide in the rabbit stroma in vitro were measured at room temperature according to the methods described previously for fluorescein diffusion. A strip of stroma, 5 mm long and 1 mm wide, was prepared from the excised rabbit cornea and mounted in the chamber utilized for the fluorescein diffusion. One cut end of the strip was exposed to 1-μM fluorescein monoglucuronide solution; and the other end was exposed to the buffered saline. The fluorescence intensity was measured at the center of the strip every hr for 24 hr, and the diffusion coefficient was calculated from the change in the intensity at that point using Fick’s diffusion equation.

The fluorescence of fluorescein monoglucuronide in the stromal strip was measured at concentrations of 1, 10, and 100 μM. Both ends of the strip were exposed to the solution, and the stromal fluorescence was measured every 0.625 mm along the strip at 24 hr. In similar experiments, the fluorescence of fluorescein in the stroma was measured at concentrations of 1, 10, and 100 μM.

The endothelial permeability to fluorescein monoglucuronide and the fluorescence distribution ratio between the anterior chamber and the cornea were determined in vivo after intravenous administration of fluorescein monoglucuronide. A Dutch rabbit weighing 2.0 to 2.5 kg was placed in a wooden box, and 20 mM fluorescein monoglucuronide solution was injected intravenously at a dose of 20 μmoles/kg body weight. The slit beam was aligned to pass along the optic axis by tilting the rabbit head and by centering the beam in the pupil. The fluorescence intensity was measured at the center of the optical cross section of the cornea and the anterior chamber. The measurements were repeated every hr for 8 hr, and after subtraction of the background fluorescence, the intensity was converted to the equivalent concentration of fluorescein monoglucuronide. The corneal endothelial permeability coefficient, k_{ac}, and the fluorescence distribution ratio between the anterior chamber and the cornea, f_{ac}, were determined according to the method described previously.

**Results**

The diffusion coefficient of fluorescein monoglucuronide in the corneal stroma was determined in five rabbits. Figure 1 illustrates the time-course of the mean fluorescence intensity at the center of the strip. The intensity increased gradually in the early period, and thereafter approached a steady-state level at 24 hr. The diffusion coefficient calculated from the rise in the intensity was in the range of 0.84 to 1.12 × 10^{-6} cm²/sec; the mean value was 0.94 ± 0.11 (±S.D.) × 10^{-6} cm²/sec.

The fluorescence in the stroma of fluorescein and fluorescein monoglucuronide measured at 24 hr is illustrated in Figure 2. The stromal fluorescence of these fluorophores was considerably higher than that in the buffered saline. The stromal fluorescence of fluorescein was uniformly distributed along the strip, whereas the stromal fluorescence of fluorescein monoglucuronide was high at the center of the strip and lower toward the ends of the strip. The stroma/saline fluorescence ratios at the center of the strip with 1, 10, and 100 μM fluorescein monoglucuronide solutions were 1.49

![Graph showing fluorescence intensity over time](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933126/ on 09/04/2018)
Measurement points along the strip

Fig. 2. Relative fluorescence intensity in the strip at 24 hr. Both ends exposed 1, 10, 100 μM fluorescein (●) or fluorescein monoglucuronide (○) and intensity measured 24 hr later.

± 0.13, 1.53 ± 0.23, and 1.45 ± 0.07, respectively. The ratios of 1, 10, and 100 μM fluorescein at the center of the strip were 1.27 ± 0.07, 1.20 ± 0.08 and 1.11 ± 0.06, respectively. The stroma/saline fluorescence ratios of these fluorophores at this point were not significantly different among the three concentrations except for the difference between 1 and 100 μM fluorescein (P = 0.004). The difference in the ratio between these two fluorophores was statistically significant at each of the concentrations (P < 0.001).

The endothelial permeability to fluorescein monoglucuronide and the fluorescence distribution ratio between the anterior chamber and the cornea were determined in nine rabbits. Figure 3 illustrates the equivalent concentration of fluorescein monoglucuronide in the anterior chamber and the cornea after intravenous dosing of 20 μmoles/kg. The anterior chamber concentration was 3.3 ± 1.4 (±S.E.) μM 1 hr after administration, indicating that fluorescein monoglucuron ide easily enters the anterior chamber, and decreased subsequently. The corneal concentration exceeded the aqueous at 2 hr and thereafter decreased at a slower rate. The time-course of these concentrations was analyzed according to the equation of Ota et al, and the permeability coefficient, kac, and the distribution ratio, rac, were calculated for each of the rabbits (Table 1). The kac averaged 0.78 ± 0.17 (±S.D.) hour⁻¹, and the rac averaged 0.56 ± 0.046. The permeability calculated from the kac and the mean thickness of the rabbit stroma, 0.36 mm, averaged 4.7 ± 1.0 × 10⁻⁴ cm/min.

Discussion

The diffusion coefficient of fluorescein along the rabbit stroma has been reported to be 1.1 × 10⁻⁶ cm²/sec in vivo at the living corneal temperature, and 1.21 × 10⁻⁶ cm²/sec in vitro at room temperature. The diffusion coefficient of fluorescein monoglucuronide, which we determined employing the method described for the fluorescein diffusion in vitro, was approximately 20% lower than that of fluorescein. In the two experiments, the same method was used in the intensity measurements and the data analysis. In addition, the dissociation constants of these two fluorophores, pH of 6.4 with fluorescein and 5.0 with fluorescein monoglucuronide as determined by Grotte et al, indicate that both compounds are almost completely ionized at physiologic pH. Therefore, the slower diffusion of fluorescein monoglucuronide in the stroma was most likely due to its large molecular weight.

The stromal fluorescence of fluorescein at 24 hr was 20% higher than that in the saline solution, and the fluorescence was distributed uniformly along the strip. This is consistent with the previous findings. By contrast, the stromal fluorescence of fluorescein monoglucuronide was not uniform along the strip. The central part was approximately 50% more fluorescent than...
ends of the strip. The stroma/saline fluorescence ratio of fluorescein monoglucuronide at the center of the strip was significantly higher than that of fluorescein.

We have no good explanation for the observed non-uniform fluorescence distribution of fluorescein monoglucuronide in our 5-mm strip. In our previous paper, we discussed several possibilities which could cause the higher stromal fluorescence, including binding by the stroma and optical phenomena such as scattering and reflection in the stroma. The optical phenomena seem incompatible with the present findings using fluorescein monoglucuronide since the stromal fluorescence was uniform when exposed to the fluorescein solution. Another possible explanation is that these fluorophores were bound to plasma proteins in the stroma, such as albumin, which was known to be present in the corneal stroma, and the concentration of binding sites in the strip became non-uniform by 24 hr because some of the proteins would have been lost at the ends of the strip.

We recently studied the binding of fluorescein monoglucuronide to human albumin and observed that it was bound more loosely than fluorescein, and that its fluorescence was not quenched by the presence of albumin whereas fluorescein was significantly quenched. It is likely that the stromal fluorescence which we measured with fluorescein and fluorescein monoglucuronide includes whatever effect the stromal albumin may have on the measurements, and differences in the quenching between these fluorophores resulted in their different stroma/saline fluorescence distribution ratios. The extent of such effects which the stroma might have on the fluorescence measurement, however, is not readily determined from the present data because the excitation spectra are somewhat different between these two fluorophores and the emission spectrum of bound fluorescein is shifted toward longer wavelengths. Thus, the stroma/saline fluorescence ratios of these fluorophores will be wavelength dependent, and different values would be obtained if the intensity was measured with different filters.

The endothelial permeability to fluorescein monoglucuronide in the rabbit cornea was greater than the reported permeability to fluorescein in the same animals; 2.6 × 10⁻⁴ cm/min using topical administration by Minkowski et al. and 3.1 × 10⁻⁴ cm/min using intravenous injection by Ota et al. McLaren et al have recently studied the aqueous humor concentration of fluorescein and fluorescein monoglucuronide after systemic administration of fluorescein, and reported that fluorescein monoglucuronide becomes the dominant fluorophore after 3 hr. From their data along with our present results on the endothelial permeability, it is most likely that the fluorescein monoglucuronide contributes substantially to the corneal fluorescence when fluorescein has been given systemically. We believe it may be necessary for investigators who are using systemically administered fluorescein to measure the endothelial permeability to determine the relative fluorescence of fluorescein monoglucuronide to fluorescein in the corneal stroma.

**Key words:** fluorescein monoglucuronide, rabbit cornea, diffusion coefficient, distribution ratio, endothelial permeability

### References

3. Sawa M, Araie M, and Tanishima T: A fluorophotometric study