Inward Permeability of the Blood–Retinal Barrier by Fluorophotometry

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A method for assessing the inward permeability of the blood–retinal barrier by fluorophotometry is presented. The permeability value is calculated with the fluorophotometer computer from fluorophotometric scan values in vitreous and non-protein-bound fluorescein concentration values in plasma. No diffusion coefficient of fluorescein in vitreous was required in the calculations. Corrections were performed for lens transmission, corneal transmission, and the spatial resolution of the apparatus. The method was applied to 58 healthy volunteers, aged 13–72 yr. An insignificant average increase of permeability values was found from 4.8 nm/s at 10 yr, up to 6.1 nm/s at 70 yr (P = 0.14; standard deviation: 1.8 nm/s). Permeability values showed an average increase of 10% between 30 min and 60 min after injection ($P < 0.001$). Invest Ophthalmol Vis Sci 28:665–671, 1987

In vitreous fluorophotometry fluorescence is measured in vitreous along the optical axis of the eye. Most fluorophotometric studies concern the function of blood–ocular barriers by determination of the concentration of fluorescein in the eye originating from choroidal and retinal vessels after intravenous (IV) administration. This leakage is attributed to the permeability of blood–ocular barriers for non-protein-bound fluorescein.

Various methods for calculating inward permeability of blood–retinal barrier (BRB) have been described.[1-3] The large range in their values (0.7 nm/s ± 0.4 SD, to 3 nm/s ± 0.8 SD) may be attributed to an inaccurate assessment of the course of non-protein-bound fluorescein concentration in plasma, an absence of correction for lens and/or cornea transmission, and an incorrect determination of fluorescein concentration near retina as a result of the spatial resolution of the fluorophotometer.

This study presents a new, practical method with corrections for the sources of error mentioned above, which yields inward permeability values expressed in meters per second. Permeability values of healthy volunteers are presented.

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Materials and Methods

Material

This study was carried out in 32 male and 26 female volunteers, aged 13–72 years, mean 40.5 y ± 16.2 SD. The age distribution histogram is presented in Figure 1. None of the volunteers was on medical drug therapy, or had a history of ophthalmic or systemic disease. No evidence of opacities in ocular media or fundus disease was found during slit-lamp examination and ophthalmoscopy. Informed consent was obtained after the procedure had been fully explained.

Methods

Fluorophotometric measurements were performed with the Fluorotron Master (Coherent Radiation Inc. Palo Alto, CA) after dilation of the pupil with tropicamide (1%). Two vitreous and two anterior segment scans were performed in each eye prior to fluorescein IV injection. The anterior segment scans were carried out using a special lens (anterior segment adapter). Vitreous scans were performed about 3 min, 30 min, 55 min and 65 min after injection of 8 mg ± 1.8 SD fluorescein per kg bodyweight.

The model used in this study is an improvement of a model described previously.[4,5] The eye is represented by a sphere, and leakage of non-protein-bound fluorescein from retinal and choroidal vessels into vitreous is represented by homogenous radially-oriented diffusion through the surface of the sphere. The inward permeability of this barrier can be calculated[1] by means of the expression:

$$P_{in} = I_{cone}/I_{plasma} \quad (1)$$

where I_{cone} is the volume integral of fluorescein leaked into a cone-shaped segment of the vitreous body with

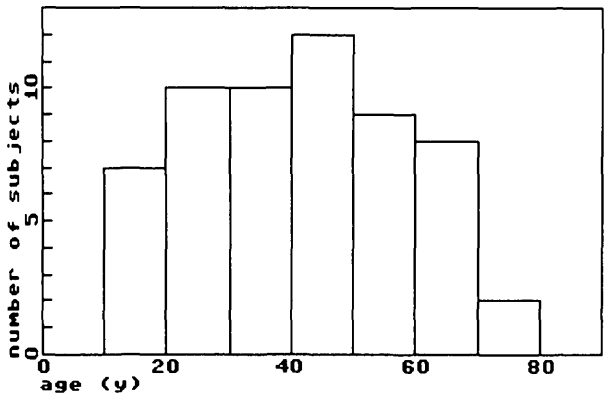

Fig. 1. Age distribution histogram of the volunteers.

a base of 1 square cm, and I_{plasma} is the time integral of non-protein-bound fluorescein concentration in plasma from the time of injection up to that of measurement.

I_{cone} can be calculated from a fluorophotometric scan in vitreous concentration measurements, and I_{plasma} from fluorescein concentration measurements in bloodsamples. All calculations were performed by the Fluorotron computer using software developed by the authors.

Correction Procedures

Each fluorophotometric measurement scan was submitted to several corrections.

Autofluorescence and reflection: A pre-injection fluorophotometric scan of the eye, prescan, was subtracted from each post-injection scan, to correct for autofluorescence and reflections in the eye (Fig. 2, curves A and B). Subtraction was performed point by point by the computer after alignment of both scans using peak fluorescence of retina and anterior part of the lens (Gray peak) as reference points. Scan alignment was required to correct for a possible shift in the distances due to variation in focussing the Fluorotron.

Spatial resolution: A high concentration of fluorescein in fundus vessels causes erroneous measurements of fluorescein concentration in the vitreous near retina, due to the limited spatial resolution of the fluorophotometer. The spread function resulting from spatial resolution was postulated to have the same shape in all measurements. Furthermore, the magnitude of this function was supposed to depend linearly on the peak fluorescein concentration measured in fundus vessels. A correction for this effect was achieved by scaling down a scan performed a few minutes after injection (bolusscan), so that its peak fundus value corresponds to that of the measurement scan.[1,6] The scaled down bolus scan was then subtracted from the measurement scan after alignment (Fig. 2, curve C). Leakage of fluorescein into vitreous was supposed to be negligible during the first minutes after injection.

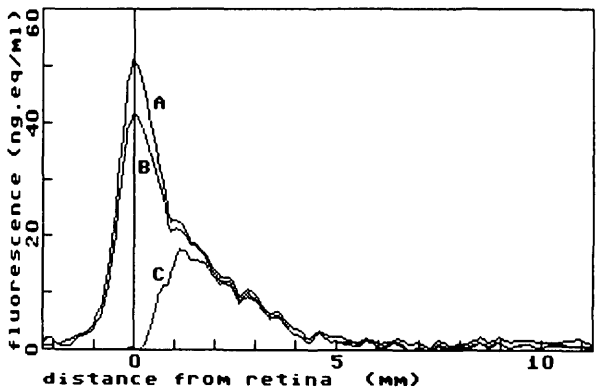

Fig. 2. Fluorescence distribution along part of the optical axis of the left eye of a 39 year old volunteer. A, measurement scan 55 min after fluorescein injection. B, same as A after correction for autofluorescence and reflections. C, same as B after correction for spatial resolution.

Retinal region: The correction procedure for spatial resolution will result in an overcorrection for concentration values measured near retina (Fig. 2, curve C), since fluorescein leaked into vitreous near retina also contributes to peak fundus fluorescence. The following procedure was developed to compensate for this overcorrection.

In several fluorophotometric scans, the spatial distribution of fluorescein along the optical axis was found to decrease according to a single exponential function starting at about 2–3 mm from retina. Therefore, part of the measurement scan near retina, which was supposed not to be affected by the spread function correction, was selected by setting two vertical lines in the displayed computer plot (Fig. 3, lines 1 and 2). Exponential regression through the data points between both lines was performed by computer, and the resulting curve was extrapolated exponentially to retina (Fig. 3, curve D). The extrapolated curve represented the spatial decay of fluorescein concentration in the first 1–2 mm from retina.

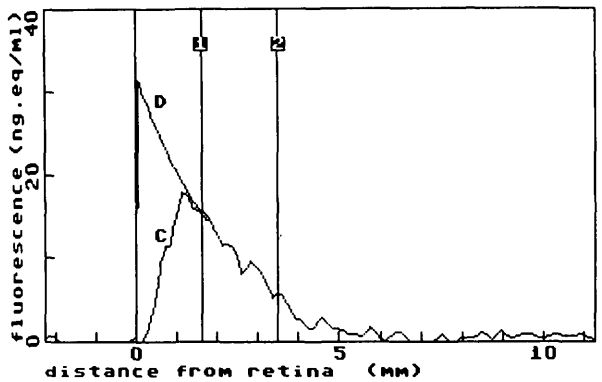

Fig. 3. Same scan as in Fig. 2. C, as in Fig. 2. D, after exponential extrapolation to retina. Line 1 and 2: Selection of a part of curve for calculation of extrapolation to retina. Calculated permeability: 7.7 nm/s.

Lens and cornea transmission: Loss of excitation (λ = 490 nm) and fluorescence light (λ = 510 nm) in lens and cornea was corrected by dividing all fluorophotometric scan values in vitreous by the product of squared lens and cornea transmission values for blue–green light ($\lambda = \pm 500$ nm). The lens transmission of each eye was calculated from the square root of the ratio between posterior and anterior lens autofluorescence, as described previously.[7,8] The autofluorescence measurements were performed with the anterior segment adapter.

Since it was not possible to determine corneal transmission for blue–green light for each eye separately, the corneal transmission was calculated from:

$$T_c(a) = 0.751 - 3.46 \times 10^3 \times a \qquad (2)$$

with a = age in years. This expression was obtained by linear regression to the data points of several investigators.[9,10,*]

Calculation of Volume Integral

The cone integral, I_{cone} in equation 1, is calculated in accordance with Zeimer[1] by numerical computer approximation of the expression:

$$I_{cone}(t_m) = (1/R^2) \int_{r=0}^{R} C(t_m, r) \times r^2 \times dr \qquad (3)$$

where R is the eye radius, t_m is the time of fluorophotometric measurement, r is the distance from the center of the eye, and $C(t_m,r)$ is the fluorescein concentration measured along the optical axis. The center of the eye was situated in the middle between cornea and retina. The position of cornea and retina were identified as peaks in the prescan.

The computer procedure for calculating the volume integral was verified by performing all calculations by hand, using Fluorotron fluorescein concentration graphs. Distances in vitreous, determined by the fluorophotometer, were verified by positioning a diffusely-reflecting surface at different distances in an eye model. Linearity was within 2%. Concentration values in vitreous as measured by the fluorophotometer had been verified previously in rabbits.[11]

The reproducibility of the approximation procedure was assessed by repetitive processing of the same fluorophotometric scan, and was estimated 5%. This only applies to a sufficiently high signal-to-noise ratio near retina. This is achieved for the Fluorotron at fluorescence values in excess of about 2 ngEq/ml.

* Den Haan, G., Best, J. A. van, Oosterhuis, J. A.: in preparation.

Plasma Time Integral

The time integral of the non-protein-bound fluorescein concentration in plasma from the time of injection to the time of fluorophotometry was determined according to a method described previously.[12] In this method, the concentration of non-protein-bound fluorescein as a function of time is approximated by asum of two exponential decay functions:

$$U(t) = U_a \times e^{-t/t_a} + U_b \times e^{-t/t_b} \qquad (4)$$

The time integral was calculated by integrating expression (4).

A fixed value for parameter t_b was used (t_b = 60.68 min). The values of parameters U_a, t_a and U_b were determined for each subject with the use of the non-protein-bound fluorescein concentration in plasma directly after injection, and in two blood samples taken about 5 and 60 min after injection. The concentration of non-protein-bound fluorescein in plasma directly after injection was determined from the total fluorescein concentration by linear extrapolation of the unbound fraction in both samples. The total fluorescein concentration was calculated from the amount of fluorescein injected and the total blood volume. Non-protein-bound fluorescein in plasma was obtained by ultrafiltration of the samples.

Results

Interocular Spread

The interocular spread in BRB permeability values was assessed by calculating the ratios between mean permeability values of left and right eye measured at 55 and 65 min after fluorescein injection for all 58 subjects. The ratio distribution is presented in a histogram (Fig. 4). Although an average ratio of 1.09 $\pm$ 0.29 SD was found, a mean value for both eyes was used in this study.[13,14,15] The correlation coefficient between permeability values for left and right eye amounted to 0.77 ($P < 0.001$).

Reproducibility

The spread in repetitive measurements was assessed by various measurement sessions in the same male subject taken at intervals of 1 week. The mean permeability values calculated from measurements of both eyes at 55 and 65 min (four values) are presented in Table 1. The maximum deviation from the mean value of all sessions was 24%.

Permeability and Measurement Time

The BRB permeability value of each eye of 58 subjects was determined at about 30, 55 and 65 min after

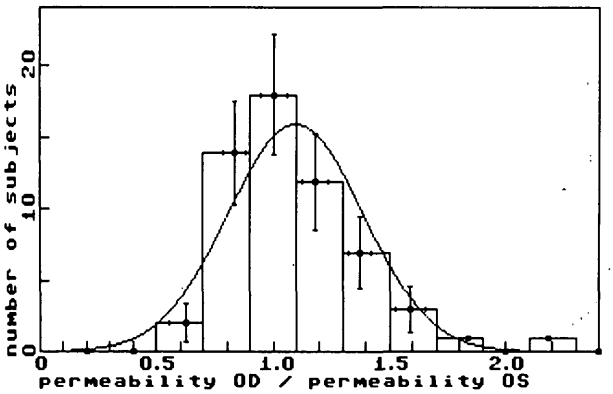

Fig. 4. OD/OS permeability ratio histogram. Vertical bars stand for square root of the number of subjects. Horizontal bars stand for standard deviation. Smooth curve: Gaussian approximation.

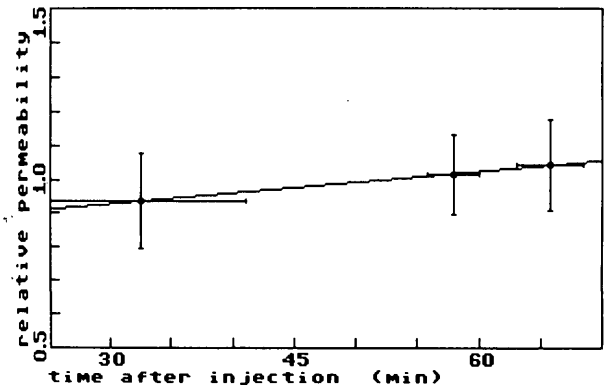

Fig. 5. Relative permeability as a function of time after injection. Each point represents the mean of the 58 subjects (2 eyes per subject). Bars correspond with 1x standard deviation. Solid line: linear regression to the 174 data points.

injection. A significant average increase in permeability value of 10.2% was found between 30 min and 60 min after injection ($P < 0.001$). This is shown in Figure 5.

Permeability and Age

The average value of BRB permeability of both eyes at 55 and 65 min after injection (four measurements) was determined for each subject. The mean value of the 58 subjects was 5.5 nm/s ± 1.8 SD (range 2.6–10.8 nm/s). The values are presented as a function of age in Figure 6. Linear regression analysis yielded an insignificant increase with age from 4.8 nm/s at 10 yr, to 6.1 nm/s at 70 yr (SD 1.8 nm/s; correlation coefficient 0.2; $P = 0.14$). Similar results were obtained by using the average value of both eyes at 30, 55, and 65 min (six measurements).

Evaluation of Different Methods and Corrections

Concentration at 3 mm from the retina: Concentration values of fluorescein at 3 mm from the retina were used by some authors[16,17] to evaluate blood–retinal barrier function from fluorophotometric scans. This procedure was applied on the data from the 58 subjects for comparison with results of our method. To make both methods comparable, 3-mm concentrations in scans made 55 and 65 min after injection were determined in both eyes, and the four values were averaged. The 3-mm concentration values as a function of permeability are presented in Figure 7 after correction for lens and cornea transmission and plasma time integral.

The straight line in the figure was obtained by linear regression to the data points, and yielded a correlation coefficient of 0.81 ($P < 0.001$).

Mean value and SD are presented in Table 2, with and without various correction procedures.

Average concentration in posterior vitreous: The average concentration values in posterior vitreous as a function of permeability are presented in Figure 8 after correction for lens and cornea transmission and plasma time integral. The straight line in the figure was obtained by linear regression and yielded a correlation coefficient of 0.92 ($P < 0.001$).

The average fluorescein concentration in posterior vitreous was calculated from the fluorescein concen-

Table 1. Reproducibility of blood–retinal barrier permeability determination

*Session**	*Permeability (nm/s) mean ± SD† (SD in %)*	*Range (nm/s)*
1	4.1 ± 0.7 (17)	3.3–4.9
2	3.7 ± 0.5 (13.5)	3.3–4.4
3	4.0 ± 0.3 (7.5)	3.7–4.3

* Time between sessions was 1 wk.

† Mean of four measurements (2× OD and 2× OS) of the same 40-year-old subject.

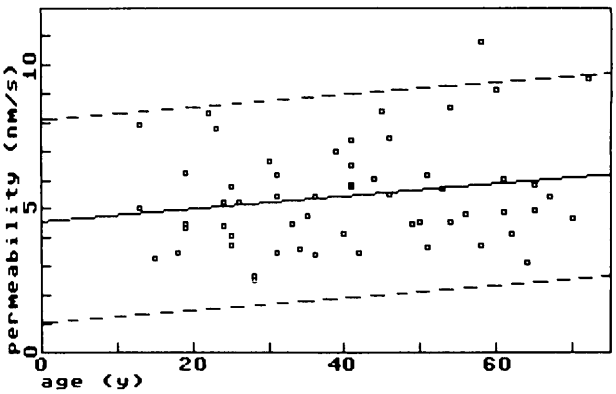

Fig. 6. Permeability of blood-retinal barrier as a function of age. Each data point corresponds to the average value of OD and OS measured at about 55 min and 65 min after injection (4 measurements). The solid line was obtained by linear regression and the broken lines represent 95% probability limits.

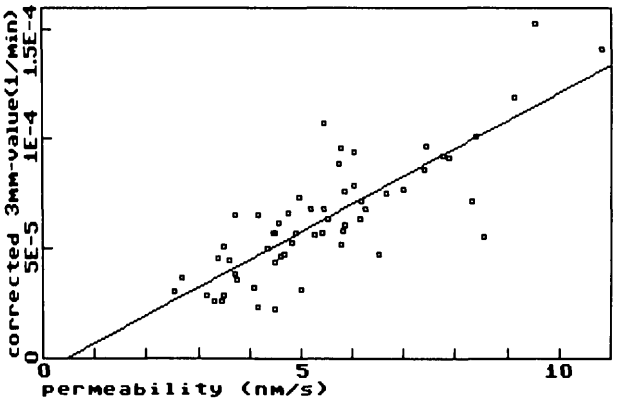

Fig. 7. Comparison between fluorescein concentration values at 3 mm from retina and permeability values calculated according to this study. The concentration values at 3 mm from retina were corrected for plasma time integral and lens and cornea transmission. Each data point represents mean value of OD and OS measured at 55 min and 65 min after injection. Solid line: linear regression to the data points.

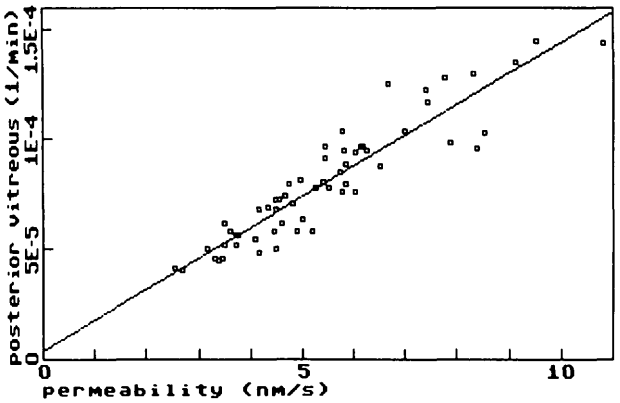

Fig. 8. Comparison between average fluorescein concentration values in posterior vitreous and permeability values calculated according to this study. Applied corrections as in Fig. 7.

tration curve corrected for spread function by means of exponential extrapolation; posterior vitreous was defined as the region along the optical axis from retina up to one-third the distance between retina and posterior lens capsule.

Mean value and SD are presented in Table 2, with and without the correction procedures.

Permeability: Mean permeability values and SD with and without correction for cornea and lens transmission are presented in Table 2.

Plasma fluorescein: Mean non-protein-bound fluorescein concentration in plasma, and the time integral of this concentration value at 60 min after injection, are also presented in Table 2 with the corresponding SD. Linear regression to the data points representing the plasma time integral as a function of age yielded a positive correlation coefficient of 0.54.

Discussion

The method described in this study is based on several assumptions stated by Palestine and Brubaker[4] and by Zeimer.[5] Compared with previous methods,[1-3] various improvements were introduced, such as the correction for age-dependent corneal transmission, the determination of non-protein-bound fluorescein decay in plasma, and the approximation of fluorescein concentration levels near retina. The method does not necessitate separate determination of diffusion coefficients of fluorescein in vitreous, which excludes an important source of error.

One volunteer was excluded from this study because of vitreous liquefaction, diagnosed by contact glass inspection. Initial measurement yielded high mean permeability values (14.7 nm/s ± 1.7 SD). A second measurement session was performed 1 yr later to assess the reproducibility of these values, resulting in a mean value of 13.5 nm/s ± 3.9 SD. Increased fluorophotometric values in liquefied vitreous were reported be-

Table 2. Evaluation of different methods and corrections

*Method**		*Corrections applied†*	*58 Subjects* mean ± SD	*(SD in %)*
3-mm values	(ng/ml)	A, IN	4.3 ± 1.7	(39)
	(min^{-1})	A, PI	$2.1 \times 10^{-5} \pm 8.6 \times 10^{-6}$	(42)
	(min^{-1})	A, PI, C, L	$6.3 \pm 10^{-5} \pm 2.7 \times 10^{-5}$	(44)
Posterior vitreous	(ng/ml)	A, IN	5.6 ± 2.0	(36)
	(min^{-1})	A, PI	$2.7 \times 10^{-5} \pm 1.1 \times 10^{-5}$	(41)
	(min^{-1})	A, PI, C, L	$8.0 \times 10^{-5} \pm 2.7 \times 10^{-5}$	(34)
Permeability	(nm/s)	A, PI	1.8 ± 0.7	(37)
	(nm/s)	A, PI, C, L	5.5 ± 1.8	(33)
concentration in plasma	(μg/ml)	IN	1.7 ± 0.52	(30)
plasma time integral	(μg/min/ml)	IN	215 ± 47	(22)

* Average of values determined in OD and OS at 55 and 65 min after injection.
† A = Autofluorescence.
IN = Normalized to 7 mg fluorescein injected per kg bodyweight.
PI = Time integral of non-protein bound fluorescein in plasma.
C = Cornea transmission.
L = Lens transmission.

Table 3. Permeability of blood–retinal barrier

		Inward permeability	
Reference	*No. of subjects*	*Correction applied**	*Value ± SD (nm/s)*
1	6	A, PI	0.72 ± 0.44
20	15	A, PI	1.2 ± ?
21	11	?	1.21 ± 0.1
22	13	A, PF	3 ± 0.83
3	6	A, PF	1.1 ± 0.4
2	14	A, PI	1.9 ± 0.94
†	58	A, PI	1.8 ± 0.7
†	58	A, L, C, PI	5.5 ± 1.8

* A = Autofluorescence.
L = Lenstransmission.
C = Cornea transmission.
PI = Time integral of non-protein-bound fluorescein in plasma.
PF = Fixed value for non-protein-bound fluorescein in plasma.
† Current study.

fore.[18,19] These increased values were explained by assuming currents in vitreous, which is incompatible with the diffusion model used in this report.

An average permeability value of 5.5 nm/s ± 1.8 SD (range 2.6–10.8 nm/s) was found in healthy subjects. Fluorophotometric values in humans reported by other investigators are shown in Table 3. These values are lower than those in this study, which may in part be attributed to differences in correction and calculation procedures. The mean correction factor for corneal transmission amounts to 2.8 ± 0.5 SD, and should not be neglected.

It should be emphasized that corneal transmission applied here concerns axially-transmitted light at 490–510 nm wavelength, and is based on two published reports only. Therefore the precise amount of correction may be questionable.

The increase of permeability value with age was hardly significant (correlation coefficient 0.2). An increase of the plasma time integral with age was also found (correlation coefficient 0.5). However, no correlation could be found between permeability value and plasma time integral (correlation coefficient 0.014; $P = 0.92$).

Permeability values were compared with fluorescein concentration values at 3 mm from retina, and with average fluorescein concentration values in posterior vitreous in the same 58 subjects. After correction for autofluorescence, the normalized 3-mm values (to 7 mg fluorescein injected per kg bodyweight), the normalized average posterior vitreous concentration values and the permeability values showed an almost equal SD (Table 2; SD = 39%, 36% and 37%, respectively). After correction for plasma time, integral and lens and cornea transmission the SD of the 3-mm values showed no decrease, and the SD of the posterior vitreous and permeability values showed some decrease (SD = 44%, 34% and 33%, respectively).

In this study, an increase of 10% in permeability values between 30 min and 60 min after injection was found statistically significant ($P < 0.001$). Such an increase was reported before,[23,24] and might be caused by the time-dependent increase of the amount of glucuronized fluorescein. Percentage of fluorescence monoglucuronide 1 hr after administration is reported to be 83% ± 3%.[23] This metabolite (M = 508) has properties different from that of free fluorescein, such as decreased fluorescence, reported 2.9%[24] or 4.2%,[25] an increased affinity for water, and altered pharmacokinetics. However, a ratio between values of vitreal cone integral and plasma time integral of fluorescein concentration is used in BRB permeability calculations. If the BRB permeability for glucuronized and free fluorescein would have had the same value, then both integral values would have been equally affected by the lower fluorescence efficiency of glucuronized fluorescein. No time dependency after injection would then be found. The 10% increase between 30 min and 60 min in this study might be attributed to a lower permeability for glucuronized fluorescein than for free fluorescein. However, this explanation is speculative, since the problem of fluorescein monoglucuronide plagues all such studies, and its presence has not been adequately addressed in any study published to date.

Since disease does not always progress at the same rate in both eyes, clinically it was necessary to monitor each eye separately. The reproducibility of permeability values was better than 20%, as was the case for measurements within 1 hr after injection, and for measurement sessions separated by weeks. The method described has been applied without complications on more than 200 volunteers and patients, provided pupillary aperture was sufficient.

Key words: fluorophotometry, permeability, blood–retinal barrier, fluorescein

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