ORIGINAL ARTICLES – Laboratory science

Diffusion coefficient through the blood-aqueous barrier using a standard protocol

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Abstract

Aims/Background—Comparison of the diffusion coefficient through the blood-aqueous barrier of healthy volunteers measured in different cities with identical fluorophotometers using a standardised protocol.

Methods—Healthy volunteers aged between 20 and 70 years were studied in seven European cities. The fluorescein concentration in the anterior segment of each eye was measured with a commercial scanning fluorophotometer 30 and 40 minutes after intravenous fluorescein. The decay of non-protein bound fluorescein concentration in blood plasma was determined with the use of three blood samples taken at 7, 15, and 55 minutes after injection. The diffusion coefficient through the blood-aqueous barrier was calculated from the ratio between the fluorescein concentration in the anterior chamber and the time integral of non-protein bound fluorescein concentration in plasma using specially developed software.

Results—The mean values of the diffusion coefficient (SD) ($\times 10^{-4} \text{ min}^{-1}$) were 4.76 (1.51) (n=20, Brussels), 5.48 (2.33) (n=17, Coimbra), 3.47 (2.09) (n=12, Cologne), 6.09 (2.77) (n=21, Frankfurt), 3.85 (1.59) (n=11, Ghent), 4.99 (1.69) (n=23, Leiden), and 4.87 (1.05) (n=20, Madrid). The values between centres were similar (Kruskal-Wallis test $p>0.05$) except for Cologne and Frankfurt ($p=0.013$). No differences were found when repeating measurements (four centres, interval time 1–8 months, Wilcoxon paired test $p>0.39$).

Conclusion—The diffusion coefficients had similar values and standard deviations. The concerted action demonstrated the usefulness of a standardised protocol.

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intraocular lens implantation^{1–8} and to study the effects of topical drugs.^{9–11} The measurements were also performed in diabetic patients,^{10–16} in uveitis patients,^{17,18} and in patients with retinal vein occlusion¹⁹ or retinal detachment.²⁰

Various methods and protocols for the quantification of the permeability of the BAB were used which could result in different outcomes. The easier method was to use the concentration of fluorescein in the anterior chamber (in ng/ml), measured at a fixed time (30, 45, or 60 minutes) after intravenous injection of a fixed amount of fluorescein (7 or 14 mg/kg body weight).^{5,7,10,12,17,18,20} An extra spread (more than 30%) in the values obtained can be expected since the amount of permeated fluorescein strongly depends on plasma fluorescein clearance, binding to plasma proteins, and to some extent on glucuronidation of fluorescein.^{21–24} Another method is to compare the fluorescein concentration in the anterior chamber of a treated eye with that before treatment or with that of the untreated fellow eye.^{2,9,11} In the first case changes in the clearance of plasma fluorescein can still play a role and in the latter case a consensual reaction cannot be excluded.² Still another way is to correct the fluorescein concentration in the anterior chamber by dividing its value by the fluorescein concentration in blood plasma.³ This can only be done at a fixed time after injection since plasma fluorescein decays with time while anterior chamber fluorescein increases. Errors can still occur because the shape of the plasma fluorescein decay curves are not necessarily identical. Other investigators use the mean fluorescence value at one or three fixed locations in the anterior chamber and divide the value by the non-protein bound fluorescein time integral^{4,6,19} or define an apparent transfer coefficient after oral fluorescein.¹

The diffusion coefficient for fluorescein (k_d in min^{-1} or h^{-1}) can be obtained by dividing the peak fluorescein concentration in the anterior chamber at a certain time by the corresponding time integral of non-protein bound fluorescein (NPBF) in plasma. The value of k_d was proved not to depend on plasma fluorescein, time after fluorescein injection, or amount of fluorescein injected and has been used by

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Quantitative measurements of the blood-aqueous barrier (BAB) permeability or of the diffusion coefficient through the BAB (k_d) in humans have been performed previously with fluorophotometry after cataract surgery and

several investigators.^{13 23–28} Glucuronidation was shown to play only a minor role provided measurements took place within 1 hour after intravenous injection.²³ The permeability of the BAB (in nm/s) can also be calculated²³ but this value is questionable since it depends strongly on the fluorescein permeating area of the iris which is difficult to calculate because of its wrinkled state.

Therefore, a protocol for the fluorophotometric determination of k_d in a standardised way was drawn up in detail in cooperation with experts in several countries of the European Community (Belgium, Germany, Portugal, Spain, and the Netherlands).²⁹ Oral administration of fluorescein was not used since this would mainly result in protein bound or glucuronidated fluorescein in blood. The evaluation of the BAB function would become less sensitive because protein bound fluorescein molecules (MW > 50 000) pass the BAB less easily than NPBF (MW = 376) while glucuronidated NPBF molecules (MW = 508) have a 30× lower fluorescence quantum efficiency.^{23 30} Furthermore, the evaluation would become inaccurate as a result of the different excitation and emission spectra of bound fluorescein and the interindividual and time dependent variation in fluorescein binding.^{21 23} The NPBF concentration in blood plasma was determined with the use of three blood samples obtained from an intravenous catheter. Fingertip samples were not appropriate because the determination of NPBF in a sample requires at least 2–3 ml of blood.

All k_d values were determined according to this standardised protocol in healthy subjects at different centres in order to verify the feasibility and usefulness of the procedures.

Materials and methods

CALCULATION OF THE DIFFUSION COEFFICIENT k_d

After intravenous injection, fluorescein reaches the anterior chamber mainly via the iris vessels and leaves via the aqueous humour flow and cornea.

The increase of fluorescein concentration in the anterior chamber can be written as²⁴:

$$\frac{dC_a}{dt} = k_d \cdot (C_p - C_a) + k_f \cdot (C_h - C_a) + k_{a,ca} \cdot (C_c/r_{ca} - C_a) \quad (\text{kg m}^{-3} \text{ s}^{-1}) \quad (1)$$

where C_a , C_p , C_h , and C_c are the non-protein bound fluorescein concentrations (NPBF in kg m^{-3}) in the anterior chamber, blood plasma, pupillary aqueous, and cornea respectively; k_d is the diffusion coefficient of NPBF from plasma (in the iris vessels) into the anterior chamber (in s^{-1}); k_f is the loss coefficient due to aqueous outflow (in s^{-1}); $k_{a,ca}$ is the aqueous to cornea diffusion coefficient referred to the anterior chamber volume (in s^{-1}); and r_{ca} is the correction factor for steady state concentration difference between cornea and anterior chamber, as a result of bound fluorescein in the cornea ($r_{ca} = 1.6$).³¹

After integration of both sides of equation (1) from the average time of the first passage of fluorescein through the iris vessels (t_d) up to the time of measurement (t_m) one obtains for the diffusion coefficient k_d :

$$k_d(t_m) = K \frac{C_a(t_m)}{I_p(t_m)} \quad (\text{s}^{-1}) \quad (2)$$

where K depends on k_f , $k_{a,ca}$, r_{ca} , $C_p(t)$, $C_a(t)$, $C_h(t)$, $C_c(t)$, t_d , and t_m and its value is 1.0 (SD 0.1) provided $10 < t_m < 70$ minutes²³; $C_a(t_m)$ is the fluorescein concentration in the anterior chamber at time t_m after injection; and $I_p(t_m)$ is the time integral of the NPBF in plasma.

The decay of NPBF in blood plasma can be approximated by a power of time function²¹:

$$C_p(t) = \alpha \cdot t^{-\beta} \quad (\text{kg m}^{-3}) \quad (3)$$

where α and β are constants depending on subject and amount of fluorescein injected. Integrating $C_p(t)$ from the time of first appearance in the eye (t_d) up to the time of measurement (t_m) yields the time integral $I_p(t_m)$:

$$I_p(t_m) = \int_{t_d}^{t_m} C_p(\tau) d\tau = \frac{\alpha}{(1-\beta)} \cdot (t_m^{1-\beta} - t_d^{1-\beta}) \quad (\text{kg s m}^{-3}) \quad (4)$$

The diffusion coefficient k_d is calculated using equation (2) wherein the value of $C_p(t_m)$ corresponds to the peak value of fluorescein concentration in the anterior chamber and the value of the time integral is calculated using equation (4). K is assumed to be 1.0 and $t_d = 54$ seconds.²⁹ The values of α and β are obtained by a power regression procedure to the NPBF concentration values in blood plasma samples taken at 7, 15, and 55 minutes after injection.

SUBJECTS

Healthy controls

The purpose was to recruit 20 healthy controls in each centre, four recruited from each of the following age categories: 20–29, 30–39, 40–49, 50–59, and 60–69 years. The criteria were: no medication at present except oral contraceptives, no contact lens wear, visual acuity of 10/10, normal aspect of all corneal layers on slit-lamp examination, no corneal lesions.

Trial group

The purpose was to recruit 15 diabetic patients. The criteria were: age category 20–39 years, insulin dependent diabetes mellitus (IDDM), 8 years diabetes duration or more, normal blood pressure (WHO: diastolic pressure < 95 mm Hg), retinopathy according to Wisconsin 2–3–4 grading, no nephropathy.

The studies have been conducted according to the principles established in the Declaration of Helsinki and were approved by local medical ethics committees. All subjects gave their informed consent after explanation of the nature of the procedures.

Figure 1 Example of fluorescence scans along the optical axis of the eye made before intravenous injection of fluorescein (prescan, thin curve) and 40 minutes thereafter (measurement scan, thick curve). The fluorescence values are expressed in ng equivalent fluorescein/ml. The distance along the optical axis is calculated from the number of scan steps per mm. The horizontal broken lines represent the fluorescence values at the anterior chamber. The instrumental vitreous lens was used.

MEASUREMENT PROCEDURE

(1) The fluorophotometer (Fluorotron Master, Coherent Radiation Inc, Palo Alto, CA, USA) was turned on at least 20 minutes before measurements. Stability was checked with a fluorescence standard (Zeiss fluorescence reference F-53, Zeiss, Germany).

(2) Slit-lamp examination of both eyes was performed by an ophthalmologist without the use of fluorescein since this could interfere with the measurements.

(3) Four fluorophotometric (pre-)scans (scan duration about 15 seconds) were performed in each eye for determination of the autofluorescence values of the anterior chamber and the cornea (Fig 1, thin curve, 4 scan steps/mm, 100 ms gate time). The scans were registered on disk.

(4) Disodium fluorescein was injected intravenously into an antecubital vein through an indwelling Teflon catheter (about 14 mg/kg body weight, given as 10% or 20% solution at an injection rate of about 10 ml per 12 seconds). The time of injection was noted. After injection of fluorescein 10 ml of dilute heparin solution was injected through the catheter. Injection was performed with the patient seated. Fundus photography was permitted provided blood sampling or fluorophotometry was not interfered with.

(5) Blood samples were collected from an intravenous catheter into evacuated tubes approximately 7, 15, and 55 minutes after injection. Three blood samples were taken at each sampling time; the first one (at least 15 ml) was discarded and the following two samples (5–10 ml each) were taken in two separated EDTA tubes (Vacutainer, Becton-Dickinson). The time of each blood sampling was noted. The catheter was washed after each

sampling by injection of 2–5 ml of dilute heparin solution.

(6) Two fluorophotometric scans of each eye were performed alternately at about 30 and 40 minutes after fluorescein injection (Fig 1, thick curve). The time of each scan was noted.

(7) Slit-lamp examination of both eyes was performed by the same ophthalmologist, now with the use of fluorescein. Any irregularity was registered. IOP could be determined.

If possible, all measurements were repeated 1 to 8 months after the first series in order to check reproducibility.

PRACTICAL CONSIDERATIONS

(1) The first blood samples must be taken between 6 and 8 minutes after fluorescein injection since otherwise the calculation of the time integral of the NPBF in plasma will not be reliable.

(2) Intravenous injection of fluorescein can also be performed directly in one arm and two samples of 3 ml each can be taken at 7, 15, and 55 minutes after injection by means of a syringe from the contralateral arm vein.

(3) Fluorescein angiography can be performed between the fluorophotometric measurements provided it does not interfere with the timing of the first plasma sample.¹⁵

(4) Simultaneous measurements of the BAB diffusion coefficient and the blood-retinal barrier permeability can be performed provided the short (vitreous) fluorophotometer lens is used and a mydriatic agent is instilled after point (2) of the previous section.¹⁵ The same instrumental lens should be used for eye and plasma scans. Note that a mydriatic agent can alter the blood flow in the iris and grossly change its geometry. However, the functional area of the barrier will remain relatively unchanged, which results in unchanged values of k_d . A pilot study in healthy controls and diabetic patients in one of the centres (Leiden) revealed a slightly decreased value of k_d (at most 20%) when excessive amounts of mydriatic agents were applied.

DATA PROCESSING (OFF LINE)

(1) Each blood sample was centrifuged (5 minutes at 3000 rpm). The supernatant was ultrafiltrated for 5 minutes at 3000 rpm by means of an ultrafiltration system (Amicon MPS-1 with YMT membranes 40420; Amicon Corp, Danvers, MA, USA) at an angle of 45° and at a temperature of 30°C. The ultrafiltrate was diluted 40× in phosphate buffer at pH 7.4. The fluorescein concentration of the latter dilution was measured in a cuvette in front of the fluorophotometer and the NPBF concentration of the sample was calculated.

(2) A power of time regression procedure was applied to the plasma NPBF values and the corresponding sampling times (determination of α and β in equation (3); Fig 2).

(3) The mean anterior chamber autofluorescence value of each eye was determined from the prescans (Fig 1).

Figure 2 Example of the non-protein bound fluorescein (NPBF) concentration (solid line, left scale) and the time integral of NPBF concentration in blood plasma (broken line, right scale) as a function of the time after fluorescein injection. The squares represent the NPBF concentration values measured in the plasma samples. The curves represent equations (3) and (4) with $\alpha=27.1$ and $\beta=0.580$.

(4) The peak anterior chamber fluorescence was determined from each measurement scan (Fig 1) and the corresponding mean autofluorescence value was subtracted.

(5) The diffusion coefficient k_d was calculated for each measurement scan using equations (2) and (4).

(6) The mean value of k_d measured at 30 and 40 minutes was calculated for each eye since these values did not differ significantly.²³

All data processing was performed using software which was specially designed for the concerted action ('ANT_SEGMENT BAB'). This program can be used off line, corrects for autofluorescence and light loss in the instrumental lens used, performs calculations of decay and time integral of NPBF in blood plasma, and calculates the mean value of k_d .

EVALUATION

The significance of differences in the values of diffusion coefficient and NPBF time integral among the different centres was evaluated with the non-parametric Kruskal-Wallis rank test with correction for tied ranks. A non-parametric multiple comparison test with corrections for tight values and unequal numbers of

data in the groups was applied on each pair of groups.³² Non-parametric tests were required since some of the data were not normally distributed according to the D'Agostino test for normality.³²

Results

SUBJECT DATA OF THE PARTICIPATING CENTRES
The data of the healthy volunteers and diabetic patients of the different centres are given in Table 1. Sex and age data from the Cologne centre could not be obtained.

FLUORESCENCE IN BLOOD PLASMA

NPBF concentration and time integral values

The calculated mean value and SD of the NPBF concentration and time integral at 35 minutes after injection of fluorescein are given for each centre in Table 2. The values were calculated with the use of equations (3) and (4). No significant differences in the time integral values between centres were found ($p \geq 0.15$) except between those of the Cologne centre and every other centre ($p < 0.007$) and between those of the Leiden and Coimbra centres ($p = 0.043$).

Reproducibility

The plasma NPBF time integral values at 35 minutes after injection measured at two different times with a mean interval of 3.06 (SD 2.0) (range 0.7–8.0) months did not differ significantly (double sided Wilcoxon paired test $p = 0.46$, $n = 57$).

Age dependency

A low but significant correlation between age and plasma integral value at 35 minutes after injection was found for all healthy controls together (except Cologne): correlation coefficient = 0.25, $p = 0.0086$. The mean increase in time integral value between 20 and 80 years amounted to 31% and the coefficient of relative variation was 41%.

Diabetic patients

The plasma NPBF time integral values of all diabetic patients ($n = 30$) at 35 minutes after injection did not differ significantly from those of the healthy controls (all controls: $p = 0.063$; all controls except those of Cologne: $p = 0.26$).

INTRAOCULAR PRESSURE

The IOP values of right and left eyes were correlated in each centre as well for healthy volunteers and for diabetic patients ($r > 0.69$, $p < 0.002$ and $r > 0.66$, $p < 0.019$, respectively). The mean IOP values of both eyes were 15.3 (SD 2.7) (range 9.5–22.5) mm Hg and 15.7 (2.6) (range 10.3–20.0) mm Hg for volunteers and patients, respectively.

Table 1 Data of participants

		<i>Age (years)</i>		<i>Diabetes duration (years)</i>	
<i>Centre</i>	<i>Number (M/F)</i>	<i>Mean (SD)</i>	<i>Range</i>	<i>Mean (SD)</i>	<i>Range</i>
Healthy controls:					
Brussels	20 (10/10)	37.7 (15.2)	24–81		
Coimbra	17 (5/12)	41.6 (15.9)	21–62		
Cologne	12 (–)	–			
Frankfurt	21 (14/7)	43.8 (15.4)	23–70		
Ghent	11 (5/6)	36.0 (14.4)	21–65		
Leiden	23 (13/10)	44.7 (14.6)	24–67		
Madrid	20 (10/10)	44.4 (14.4)	22–68		
All	124* (57/55)†	41.9 (15.0)†	21–81†		
Diabetic patients:					
Coimbra	12 (5/7)	40.0 (10.1)	23–58	19.6 (9.8)	8–40
Frankfurt	17 (9/8)	30.6 (5.2)	19–38	13.1 (3.0)	10–18
Ghent	1 (0/1)	28		14	
All	30 (14/16)	34.3 (8.7)	19–58	15.7 (7.2)	8–40

*All centres. †Excluding the Cologne centre.

Table 2 Non-protein bound fluorescein in blood

Centre	Concentration ($\mu\text{g ml}^{-1}$)*		Time integral ($\mu\text{g min ml}^{-1}$)†	
	Mean (SD)	Range	Mean (SD)	Range
Healthy controls:				
Brussels	4.85 (1.07)	3.28–7.41	318 (64)	195–466
Coimbra	3.94 (1.36)	1.50–6.75	242 (63)	98.1–344
Cologne	7.39 (2.01)	3.92–11.4	1930 (1170)	451–4090
Frankfurt	4.50 (1.83)	1.46–9.33	273 (89)	135–493
Ghent	3.74 (0.60)	2.79–4.83	275 (44)	185–354
Leiden	5.06 (1.22)	2.34–7.60	324 (68)	145–414
Madrid	4.06 (1.69)	1.01–7.28	288 (114)	95.6–499
All	4.73 (1.74)	1.01–11.4	449 (605)	95.6–4090
All except Cologne	4.44 (1.45)	1.01–9.33	290 (83)	95.6–499
Diabetic patients:				
Coimbra	3.64 (1.13)	1.98–5.29	243 (44)	186–311
Frankfurt	4.79 (1.81)	2.53–10.7	311 (94)	183–589
Ghent	3.20		190	
All	4.28 (1.63)	1.98–10.7	279 (84)	183–589

*NPBF concentration in blood 35 minutes after injection.

†Time integral of NPBF concentration in blood 35 minutes after injection.

DIFFUSION COEFFICIENT THROUGH THE BLOOD-AQUEOUS BARRIER

Mean values

The diffusion coefficient through the blood-aqueous barrier (k_d) of each healthy control and patient is presented as a function of age in Figure 3. The mean value of both eyes was used because the values of right and left eyes were significantly correlated in each healthy control and patient group ($r > 0.85$, $p < 0.0001$ except Madrid: $r = 0.51$, $p = 0.023$). The mean value, standard deviation, and total range of k_d in each group are given in Table 3.

Reproducibility

The mean values and standard deviations of k_d measured a second time and the corresponding interval times are presented in Table 4 for each

of the four centres that repeated the measurements. The values did not differ significantly from the first ones (double sided Wilcoxon paired test $p > 0.39$).

Differences between centres

The significance of the differences in the values of k_d between centres is presented in Table 5. The values between pairs of centres were not different (Kruskal-Wallis test $p > 0.05$) except between Cologne and Frankfurt ($p = 0.012$). No significant difference was found between the values of all diabetic patients and those of all healthy controls (Mann-Whitney test $p = 0.061$; excluding Cologne: $p = 0.15$).

DIFFUSION COEFFICIENT VERSUS AGE OR SEX

No significant correlation between age and diffusion coefficient was found in each of the centres Ghent, Leiden, and Coimbra ($r \leq 0.4$, $p \geq 0.09$) but a significant correlation was found in Brussels, Frankfurt, and Madrid ($r \geq 0.7$, $p \leq 0.0006$). In all healthy controls together (except those of Cologne because of missing data) a small but significant correlation was found (increase between 20 and 80 years: factor 2.2, $r = 0.54$, $p < 0.0001$, Fig 3).

No significant differences were found between the values of k_d of male and female healthy participants (mean values: 5.15×10^{-4} and $5.04 \times 10^{-4} \text{ min}^{-1}$, respectively; Mann-Whitney test: $p > 0.5$).

No significant correlation between k_d and age was found in the individual diabetes groups ($r < 0.2$, $p \geq 0.47$) nor in all diabetes patients together ($r = 0.15$, $p = 0.43$).

DIFFUSION COEFFICIENT VERSUS DIABETES DURATION

No significant correlation between k_d and diabetes duration was found in the individual centres ($r \leq 0.21$, $p \geq 0.49$) nor in all patients together ($r = 0.13$, $p = 0.49$).

Discussion

GENERAL CONCLUSIONS

The method presented is straightforward, highly evolved, and worked out in detail. It consists of an injection of fluorescein, the taking of three blood samples, and six fluorophotometric measurements per eye. After handling of the blood samples, the value of k_d can be obtained in a few minutes by non-specialised personnel with the sophisticated but easy to use software.

The diffusion coefficient (k_d) through the blood-aqueous barrier was measured in each of the seven centres without problems in about 1 hour and 15 minutes. The results were found to be reliable and reproducible provided the protocol was strictly followed.

The utility of the method is obvious. It can be used when changes of the BAB function are suspected and a reliable quantitative outcome

Figure 3 Diffusion coefficient through the blood-aqueous barrier as a function of age. The solid line was obtained using a linear regression procedure to the data points of the healthy controls in all centres except Cologne. The dotted lines represent the 95% probability intervals for the regression line and the broken lines the 95% probability intervals for the data points. Healthy controls: Brussels (solid triangles), Coimbra (solid squares), Frankfurt (open diamonds), Ghent (open triangles), Leiden (inverted triangles), Madrid (open squares). Diabetic patients: Coimbra (x), Frankfurt (+), and Ghent (*).

is required, such as in studies concerning the recovery time of the BAB function after medication or surgery or the effect of drugs and drug dosage on the barrier function.¹⁻²⁰

The Concerted Action study demonstrated the feasibility of international multicentre studies even if protocols and procedures are complex. Such studies are beneficial for future studies on ocular pathophysiology, especially in case of diseases occurring sporadically and for analysis of the effects of geographical differences on disease and treatment.

SPECIFIC REMARKS

The values of k_d found in the seven centres had similar values and standard deviation (Table 3). No significant differences between the values of the various centres were found ($p > 0.05$, Table 5) except between Cologne and Frankfurt ($p = 0.012$). These results clearly demonstrate the reliability of the absolute values of k_d obtained.

The reproducibility of the measurement of k_d was assessed in four centres by repeating measurements after 1 to 8 months. No significant differences between the values of the first and second measurements were found in each of the centres (Wilcoxon paired test $p > 0.390$). These

findings suggest that the methodologies described can be used for long term studies of k_d .

The mean value and standard deviation of k_d ($\times 10^{-4} \text{ min}^{-1}$) for healthy volunteers in this study (4.94 (2.04), $n = 24$) correspond with values found in previous studies (9.7 (4.6), $n = 24^{25}$; 6.8 (3.7), $n = 20^{33}$; 6.1 (2.7), $n = 10^{24}$; 4.7 (1.5), $n = 23^{23}$; 3.7 (0.7), $n = 15^{27}$).

Care should be taken when using fluorophotometry for evaluating the BAB after an ocular lens extraction followed by implantation of an iris claw lens. The aqueous flow will be hindered by this lens and the fluorescein originating from the iris vessels will be forced to follow other ways out. This can result in a lower fluorescein concentration in the anterior chamber and a higher one in the posterior chamber or anterior vitreous,¹⁸ in comparison with an eye without such an implant lens. The concentration values at both sides of the implant lens should be taken into consideration for evaluation of the BAB permeability. The method presented takes only the anterior chamber into consideration, assuming that the fluorescein concentration at the posterior side of the lens is negligible.

COMPARISON WITH THE LASER FLARE CELL METER

The BAB function can be evaluated also with the laser flare cell meter (LFCM). The LFCM assesses the relative protein content in the anterior chamber by measurement of the intensity of scattered laser light.¹⁹ The measurement is rapid, simple, and non-invasive and is therefore of value for daily clinical practice, while the fluorescein injection and blood sample takings make fluorophotometry less applicable.²⁸ The value of relative protein content is expressed in photon counts per millisecond and depends on the detection geometry of the instrument. Furthermore, this value depends on size and shape of the protein molecules in the anterior chamber. These features contrast with those of the diffusion coefficient k_d as measured by fluorophotometry which provides a physical value for the leakage of small fluorescein molecules (MW=376) through the BAB. This makes fluorophotometry more sensitive in detecting early changes in BAB function.^{34 35} Note that the k_d value is independent of aqueous flow²³ while the LFCM measures a steady state value of protein contents which depends on aqueous flow.²⁶

PROBLEMS ENCOUNTERED

The interval time between the first plasma sample and the fluorescein injection was critical. A time less than about 5 minutes resulted in an inaccurate time of sampling while the fluorescein concentration in plasma was changing rapidly and a time greater than about 9 minutes resulted in an inaccurate first part of the plasma time integral. In both cases the values of k_d were unreliable. The data of five volunteers were rejected as a result of incorrect time of the first plasma sample.

Table 3 Diffusion coefficient through the blood-aqueous barrier

Centre	Number of participants	Mean (SD)* ($\times 10^{-4} \text{ min}^{-1}$)	Range ($\times 10^{-4} \text{ min}^{-1}$)
Healthy controls:			
Brussels	20	4.77 (1.51)	2.88-7.99
Coimbra	17	5.48 (2.33)	2.62-10.88
Cologne	12	3.47 (2.09)	1.13-8.49
Frankfurt	21	6.09 (2.77)	2.33-11.36
Ghent	11	3.85 (1.59)	2.19-7.90
Leiden	23	4.99 (1.69)	2.71-9.32
Madrid	20	4.87 (1.05)	2.90-6.75
All centres	124	4.94 (2.04)	1.13-11.36
All centres except Cologne	112	5.10 (1.98)	2.19-11.36
Diabetic patients:			
Coimbra	12	5.65 (1.55)	3.24-8.86
Frankfurt	17	5.35 (1.89)	3.05-9.87
Ghent	1	4.60	
All centres	30	5.45 (1.71)	3.05-9.87

*Mean value (SD) of k_d (both eyes averaged).

Table 4 Reproducibility of the diffusion coefficient through the blood-aqueous barrier

Centre	Time interval* (months)	No of participants	Mean (SD)† ($\times 10^{-4} \text{ min}^{-1}$)	Range ($\times 10^{-4} \text{ min}^{-1}$)	p Value‡
Healthy controls:					
Brussels	2.1 (1.7)	17	4.70 (0.94)	3.24-6.49	>0.5
Coimbra	3.4 (1.5)	15	5.78 (2.31)	3.73-13.1	0.39
Frankfurt	4.7 (2.1)	15	6.04 (3.10)	2.45-13.78	>0.5
Ghent	1.3 (0.4)	8	3.68 (2.36)	1.70-8.96	>0.5
All centres	3.1 (2.0)	55	5.21 (2.36)	1.70-13.78	>0.5
Diabetic patients:					
Coimbra	0.8 (0.2)	12	5.37 (1.24)	2.94-7.74	>0.5

*Mean time (SD) between first and second measurements.

†Mean value (SD) of k_d measured the second time (both eyes averaged).

‡Double sided p value obtained with the Wilcoxon paired test for significance of the difference between the first and second measurements.

Table 5 Significance of differences in blood-aqueous barrier diffusion coefficient between centres*

	Coimbra	Cologne	Frankfurt	Ghent	Leiden	Madrid
Brussels	>0.5	0.37	>0.5	>0.5	>0.5	>0.5
Coimbra		0.09	>0.5	>0.5	>0.5	>0.5
Cologne			0.012	>0.5	0.12	0.082
Frankfurt				0.16	>0.5	>0.5
Ghent					>0.5	>0.5
Leiden						>0.5

*Probability for equal values of the diffusion coefficient through the blood-aqueous barrier (double sided Kruskal-Wallis test).

One centre (Cologne) demonstrated plasma time integral values largely deviating from the values of the other centres (factor 6.7; $p < 0.007$). On the other hand the values of the diffusion coefficient through the BAB found at this centre did not differ significantly from the other centres except Frankfurt (Table 5). This contradiction may be explained by an incorrect calibration of the fluorophotometer in Cologne resulting in apparent excessive fluorescein concentration values in the plasma samples and in the anterior chamber; these errors cancel out in the calculation of the diffusion coefficient.

(One major problem encountered with the Concerted Action study was the time consuming communication between the hospitals in the different countries, mainly caused by language problems.)

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