

## EXTENDED REPORT

## Minimally invasive, direct, real time measurement of drug concentration in the anterior eye

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**Aims:** To evaluate a corneal contact lens which effectively turns the anterior chamber of the eye into a cuvette, enabling the concentration of a drug to be measured using absorption spectroscopy.

**Methods:** A hand held contact lens incorporating optical fibres connected to a spectrograph enabled a beam of light to be directed in, across, and out of the anterior chamber. The device was used to follow the time course of drug concentration in the anterior chamber of rabbit (sedated) and humans, using topical brimonidine or fluorescein (with or without local anaesthesia). Absorbance measurements were taken for a 5–25 second period, repeated every 30 minutes. Drug concentrations were compared using absorbance peak height.

**Results:** Corneal absorption starts to rise rapidly at wavelengths shorter than 315 nm. The light path within the anterior chamber is 6.9 mm (rabbit) and 5.8 mm (human), the absorbance measured also includes a corneal component. Application of fluorescein (three drops of 2% solution) in rabbit allowed detection, 60 minutes later, of a large absorbance peak at 490 nm. In the human eye, the device could not measure fluorescein (applied as in rabbit), but clearly detected brimonidine for 3 hours following topical application of 0.6 mg. Modification of the device to measure fluorescence resulted in the detection of 5.3 nM fluorescein in the ex vivo rabbit eye, an increase in sensitivity of two orders of magnitude over the absorption measurements.

**Conclusion:** This device has the potential to allow repeated measurements of drug concentrations in the anterior eye provided the drug has suitable absorption or fluorescence characteristics.

Drug absorption following topical application to the eye is a complex process. The vast majority of the drug applied fails to enter the eye and that proportion that does so is likely to accumulate in the cornea and sclera or be absorbed systemically via blood vessels in the conjunctiva and nasal passages. Robinson<sup>1</sup> has calculated absorption rates ( $k_{\text{abs}}$ ) in the range of  $1-5 \times 10^{-4} \text{ min}^{-1}$  with elimination rates ( $k_{\text{loss}}$ ) of  $2-5 \times 10^{-1} \text{ min}^{-1}$ . To improve drug absorption through the cornea, from where it may access lens, iris, and ciliary body, it is necessary to decrease  $k_{\text{loss}}$  and improve  $k_{\text{abs}}$ .

The ability to measure the ocular concentration of a drug remains a fundamental requirement of any attempt to develop an ophthalmic medication. This has often been done in the past by paracentesis, but this technique cannot be repeated at intervals and gives no estimate of the corneal drug content, which is often very substantial. More information can be gained by the harvesting of animal eyes at various times following topical application of the drug.<sup>2</sup> However, this is very wasteful and laborious.

The original idea for using a scleral lens, which would enable light to be transmitted laterally through the anterior chamber, was proposed by Rabinovitch *et al.*<sup>3-4</sup> Their object was to determine glucose concentration in the aqueous humour, but they did not report progress beyond the concept/design stage. McLaren and Brubaker developed the concept of a direct spectrophotometric scan from the aqueous humour.<sup>5-6</sup>

Our group designed a Perspex contact lens and demonstrated propagation of monochromatic light across the anterior chamber of an ovine eye.<sup>7-8</sup> Subsequently, a lens constructed from synthetic fused silica was used to direct monochromatic light across the anterior chamber of a rabbit eye, *in situ*.<sup>9-10</sup> We recently reported detection of brimonidine by absorption spectroscopy in the living rabbit.<sup>11</sup> In this paper

we report the semiquantitative detection of fluorescein in the rabbit anterior eye and of brimonidine in the rabbit and human eye.

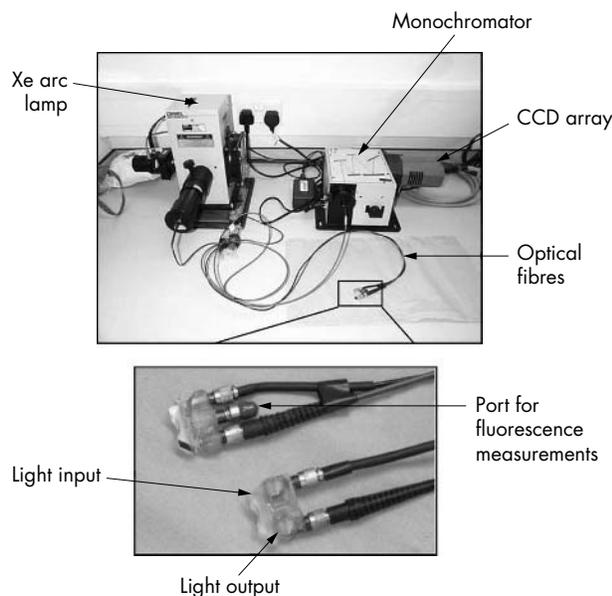
## MATERIALS AND METHODS

The design of the instrument aimed to turn the anterior chamber into a spectrophotometer cuvette, using a corneal contact lens that enabled light to pass through the anterior chamber, perpendicular to the visual axis. This lens lay at the front of the hand held sensor head (fig 1), which carried two optical fibres, one coming from the light source and the other conveying light from the "cuvette" to a spectrograph (for measuring light absorption by the test compound). Also shown in figure 1 is an alternative device where the central port carried fluorescence light emitted from the anterior chamber at right angles to the excitation beam (for measuring fluorescence from the test compound). A diagram of the light path is shown in figure 2A.

The light source for absorption spectroscopy was a xenon arc lamp and for fluorescence measurements was an argon ion laser ( $\lambda = 488 \text{ nm}$ ). The spectrograph comprised a monochromator (model MS127) and an open electrode charge coupled device (CCD) array (both supplied by Oriol Ltd, Leatherhead, UK).<sup>10-11</sup>

The spectral region available for analysis is limited by the transmission properties of the anterior eye. The cornea and aqueous humour of the human eye, being relatively transparent between 300 and 1400 nm, transmit light in the ultraviolet (UV), visible (VIS), and the near infrared (NIR) spectral regions.<sup>12</sup> For this study we have chosen to analyse the UV/VIS spectral region as many of the drugs of pharmaceutical interest have broad, easily resolvable peaks in

**Abbreviations:** CCD, charge coupled device; ddw, deionised and distilled water; NIR, near infrared; UV, ultraviolet; VIS, visible



**Figure 1** Ocular spectrograph: the two alternative sensor heads (incorporating the contact lens) attached to the rest of the instrumentation via fibreoptic cables.

this region. The detection range of the spectrograph was chosen for this purpose. Alternative equipment choices would enable the analysis of the portion of the NIR spectrum transmitted through the eye, although a more complex absorption peak structure would be expected.

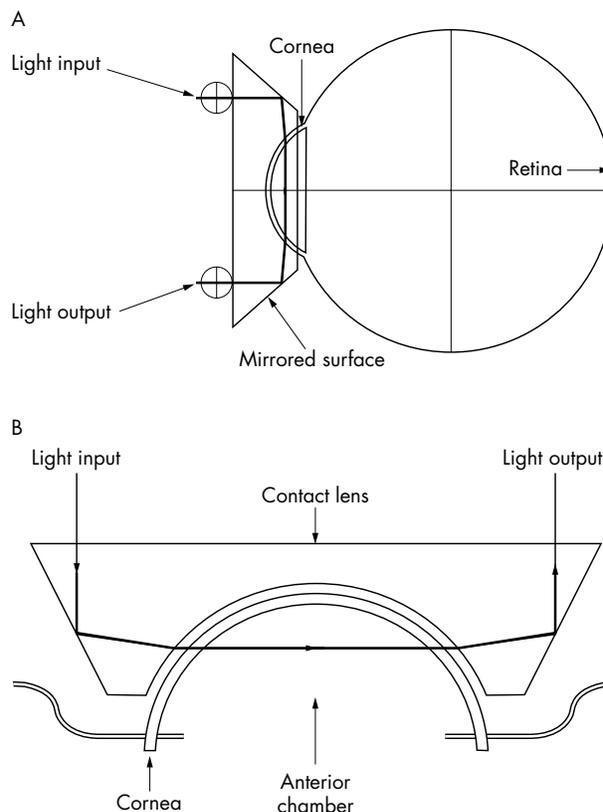
All in vivo and ex vivo absorption spectra were generated in a room with subdued lighting, using the following procedure. A reference spectrum was obtained by filling the concave lens surface of an upturned sensor head with deionised and distilled water (ddw); the light path then passed through the ddw and a reference spectrum ( $I(\lambda)_{\text{reference}}$ ) was recorded. The lens was dried with disposable lens wipes, then coated liberally with carbomer 980, 2% (Viscotears; Novartis Ophthalmics, Camberley, UK) in order to cushion the cornea, before being placed on the surface of the cornea. The light now followed the path shown in figure 2A and a signal spectrum ( $I(\lambda)_{\text{signal}}$ ) was recorded. The absorption spectra were then calculated:

$$A(\lambda) = \log_{10} \left( \frac{I(\lambda)_{\text{reference}}}{I(\lambda)_{\text{signal}}} \right) \quad (1)$$

After each measurement the concave surface of the lens was cleaned three times with ddw, once with ethanol (70% v/v in water), and dried.

In vivo testing was performed on both humans and rabbits (New Zealand White). The rabbit experimental protocol was approved by the UK Home Office. Each animal was tested no more than four times and had a minimum of 2 weeks' rest period after each test. The rabbits were sedated with fentanyl, 1 mg/kg, and fluanisone, 3 mg/kg (Hypnorm; Janssen-Cilag Ltd, High Wycombe, UK), and the cornea locally anaesthetised with topical lidocaine hydrochloride (2%).

The protocol used in humans was approved by a University of Glasgow ethics committee and adhered to the tenets of the Declaration of Helsinki. Both of the two human male subjects were free of eye pathology. One (32 years of age) used lidocaine eye drops when required, while the other (59 years;



**Figure 2** (A) Light path through the anterior eye. When the contact lens is placed in contact with the eye the light path traverses a total distance of approximately 8.5 mm through the cornea and anterior chamber. The light input and output points are optical fibres (not shown). (B) Schematic of ex vivo eye; the aqueous humour was replaced with a range of concentrations of fluorescein in Krebs solution.

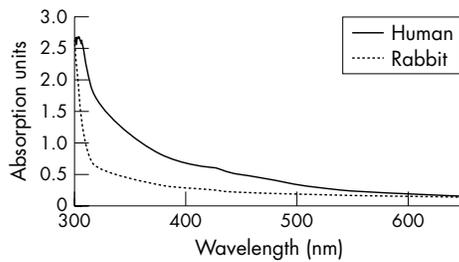
a long term contact lens wearer) required no local anaesthesia.

After the above preparation, the sensor head with Viscotears cushion was gently applied to the cornea and a baseline absorption spectrum  $A(\lambda)_{\text{baseline}}$  was recorded. Finding the optimal position for the lens took 5–25 seconds and the recording itself took 0.5 second (each spectrum was captured in 0.025 seconds and 20 spectra were summed) for absorption measurements. The test drug was then topically applied and further recordings, termed measurement spectra ( $A(\lambda)_{\text{measurement}}$ ) were taken at intervals of 30 minutes. Subtraction of the baseline absorption spectrum from the measurement spectrum yielded the difference spectrum  $A(\lambda)_{\text{difference}}$

$$A(\lambda)_{\text{difference}} = A(\lambda)_{\text{measurement}} - A(\lambda)_{\text{baseline}} \quad (2)$$

Despite repeated application of the device, no discomfort was experienced by the subjects. The potential for damage to the corneal epithelium was considerably less than by tonometry, since the area of contact for our device was much larger than a tonometer head and it was not applied with force required to alter the shape of the cornea, as is inevitable in using a tonometer. Fluorescence signals were relatively weaker and were collected over 1 second or 10 seconds.

Ex vivo measurements were performed on fresh rabbit carcasses. These were obtained approximately 30 minutes



**Figure 3** Typical absorption spectra for the anterior chamber of human and rabbit.

after death. Cannulas (23 gauge needles) were inserted through the cornea to the anterior chamber, to allow the aqueous humour to be easily flushed out and replaced with test solution (fig 2B). A water manometer connected at the inflow cannula maintained an intraocular pressure of 11 mm Hg in order to preserve the curvature of the cornea. Infusion of 3 ml of fluorescein (in Krebs solution) was sufficient to give a constant spectroscopic signal. A series of fluorescein solutions of various concentrations was then tested. Absorption and emission spectra were both recorded from the same eye.

#### Ocular absorption of fluorescein or brimonidine

Fluorescein solution (2%; Minims; Chauvin Ltd, Romford, UK) was applied to human and rabbit eyes. Three drops (approximately 25  $\mu$ l per drop) were topically applied at 20 minute intervals. A baseline spectrum was taken before fluorescein application. Brimonidine solution (30  $\mu$ l of tartrate salt, 2% w/v in isotonic saline) was topically applied to the test eye. To avoid false positives as a result of either drug being retained in the tear film, the test eye was briefly irrigated with isotonic saline 5 minutes after the application of the last drop. A series of measurement spectra were then recorded, starting 25 minutes after application of the last drop. Difference spectra showing only the changes caused by application of the drug were then calculated as above.

#### Path length

An estimate was made of the path length of the light beam in the rabbit eye. Comparing the absorption maxima generated for the same range of fluorescein solutions in three separate configurations, with each having a different path length produced three sets of data. *Configuration 1*: In the ex vivo rabbit eye, the aqueous humour was replaced by a series of increasing concentrations of fluorescein (0, 1, 3, 10, 30  $\mu$ M). An absorption spectrum was taken at each concentration. *Configuration 2*: The sensor head was held with its concave side upwards and the hollow was filled with the same five solutions and absorption spectra taken for each. *Configuration 3*: absorption spectra were measured for the five solutions using a spectrometer and cuvettes with a 10 mm light path. For each of the three sets of data the spectrum obtained for the Krebs solution alone was subtracted from each of the other four spectra to give a series of spectra with absorption

peaks at 490 nm. Plots of these peak heights versus fluorescein concentrations produced three straight lines each with a different gradient. Using the Beer-Lambert law and the fact that the path length is known for the third set of measurements enables the path length to be determined for the first two. The Beer Lambert law is:

$$A = \epsilon c l \quad (3)$$

where A = absorption,  $\epsilon$  = molar absorption coefficient, c = concentration and l = path length.

## RESULTS

### Absorption spectroscopy: corneal UV cut off for rabbit and human

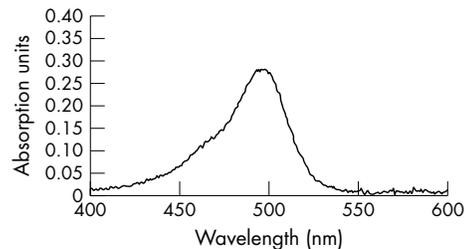
Typical baseline absorption spectra for human and rabbit eyes are shown in figure 3. Both curves show an effective cut off in transmission below 300 nm.

### Path length

In the ex vivo rabbit eye (configuration 1) we assumed that the cornea did not take up any fluorescein in the brief period (<90 seconds) between infusion and recording the spectrum. This record gave a path length of 6.9 mm within the anterior chamber. Configuration 2 gave the combined path length through the tear film, cornea, and anterior chamber of 8.6 mm. Using the geometry shown in figure 2 and the reported rabbit peripheral corneal thickness of 0.4 mm,<sup>13,14</sup> we calculated the "tear film" (largely Viscotears) thickness to be 0.2 mm (see table 1). Assuming the total path length was identical in both rabbit and humans and a peripheral corneal thickness of 0.67 mm,<sup>15</sup> equivalent values were calculated for humans (table 1).

### Absorption spectroscopy: fluorescein

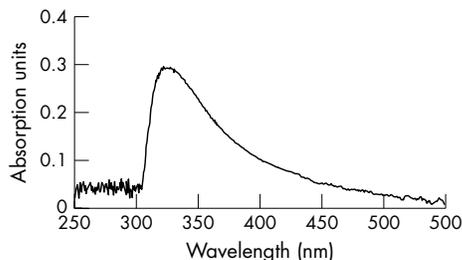
Difference spectra following application of fluorescein (2%) in rabbit eyes all showed an absorption peak at 490 nm (fig 4), indicating that this compound enters the cornea in this species. The path of the light beam was clearly visible as a green track, both at the points where it crossed the cornea and as it traversed the anterior chamber. In human eyes after the application of the fluorescein (2%), all measurement spectra were similar to baseline spectra, indicating little penetration of fluorescein across the cornea.



**Figure 4** Absorption change after the application of fluorescein to the in vivo rabbit eye.

**Table 1** Calculated path lengths (mm) in the rabbit and human eye

	Path length (mm)			
	Total	Aqueous humour	Cornea	Tear film (including Viscotears)
Rabbit	8.6	6.9	1.5	0.2
Human	8.6	5.8	2.4	0.4



**Figure 5** Absorption change 25 minutes after the topical application of brimonidine to an in vivo human eye.

### Absorption spectroscopy: brimonidine in human and rabbit eyes

Following the application of brimonidine (0.6 mg) in volunteers, the difference spectrum in each eye showed a peak at 320 nm. One of these spectra is shown in figure 5. Using equation (3) and the total path length (table 1), the concentration of brimonidine in the combined cornea and anterior chamber was calculated (fig 6).

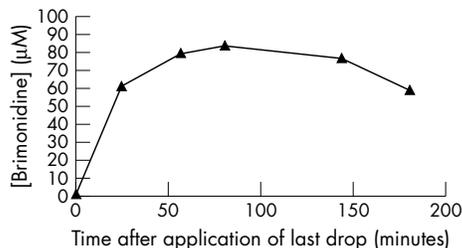
One (0.6 mg), two (1.2 mg), or three (1.8 mg) drops of brimonidine, spaced at 20 minute intervals, were applied to rabbit eyes and concentrations were calculated from absorption spectra. Figure 7 shows the time course of brimonidine concentration in two eyes after the application of 1.2 mg brimonidine. The dose dependence of concentration is shown in figure 8.

### Fluorescence spectroscopy: ex vivo rabbit

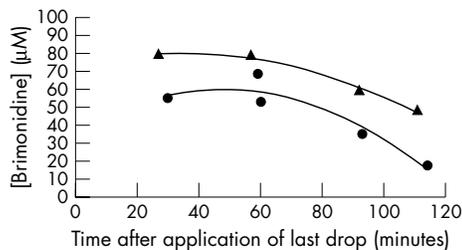
Using the sensor head with fluorescence detection, spectra were recorded over various times. Using a 1 second collection time it was possible to obtain clearly resolvable fluorescence spectra at a fluorescein concentration of 53 nM. Using a 10 second collection time spectra were obtained for concentrations as low as 5.3 nM; one of these is shown in figure 9.

## DISCUSSION

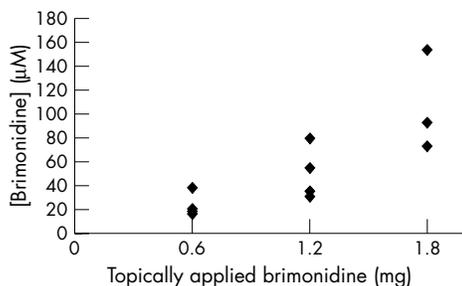
Details of the in vivo light absorptive properties of the anterior human and rabbit eye are useful for many applications; here it provides us with a baseline to which subsequent measurements can be compared. To our knowledge this is the first demonstration of an instrument that is capable of performing in situ absorption spectroscopy measurements of the anterior human eye. The absorption spectrum in the cornea shows a generally featureless increase in absorption with decreasing wavelength with a cut off in transmission at approximately 300 nm in both humans and rabbit. Our data (fig 3) are consistent with reported ex vivo<sup>12-16</sup> and in vivo studies.<sup>6</sup> In our experiments, the cornea and aqueous humour of the rabbit were found to transmit significantly more UV and blue wavelength than the human eye. This may be exaggerated by the longer corneal



**Figure 6** Time course of spectroscopically measured brimonidine concentration in the human anterior eye after topical application of 30 µl of brimonidine (2%).



**Figure 7** Time course for brimonidine concentration in the anterior rabbit eye; 30 µl of brimonidine (2%) was topically applied twice with a 20 minute interval.

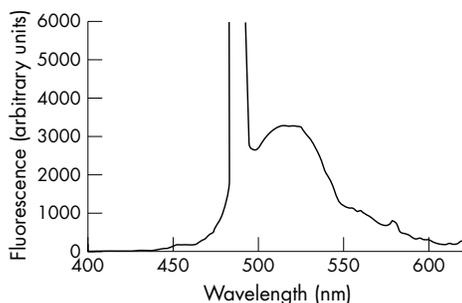


**Figure 8** Dose dependence of brimonidine concentration in the anterior rabbit eye. One (0.6 mg), two (1.2 mg), or three (1.8 mg) drops of brimonidine (2% in isotonic saline) were topically applied with a 20 minute interval between drops. All measurements were taken 25 minutes after application of the last drop.

component for the path length in human eyes. However, even normalising for this factor, there remains a difference between human and rabbit absorption curves.

Topical fluorescein has been detected previously in the rabbit aqueous and cornea.<sup>17</sup> Poor absorption of topical fluorescein in humans has been noted before. Tonjum and Green<sup>18</sup> resorted to iontophoresis to achieve measurable corneal penetration of fluorescein. In humans, blinking and reflex tearing remove an applied drug so quickly that even within 20 seconds of administration the residual drug has dropped to 20% or less of the original tear film content.<sup>19</sup> Hence, though the device was able to detect fluorescein in the rabbit, the sensitivity was insufficient to detect the very small fraction remaining in the human tear film.

The absorption spectrum in figure 5 and the time course in figure 6 demonstrate that using our technique, it is clearly possible to detect the presence and monitor the time course of a topically applied drug in the in vivo human eye. The minimally invasive nature of the measurement should enable



**Figure 9** Fluorescence peak for 5.3 nM fluorescein in ex vivo rabbit eye. The fluorescence peak is the broad peak at 515 nm. The peak at 488 nm is scattered light from the excitation beam.

application to a wide range of situations. To be clinically useful the technique needs to have the sensitivity to detect therapeutic drug concentrations. While the results described for brimonidine in humans were measured after application of 10 times the standard therapeutic dose,<sup>20</sup> the measured brimonidine concentration did not fall below 60  $\mu\text{M}$  in the 3 hours following application; this is 20 times greater than the 3  $\mu\text{M}$  brimonidine detection limit.<sup>10</sup> Determination of the time course of absorption following a clinical dose of brimonidine should be possible with only a small increase in instrument sensitivity by incorporating a continuous reference sample, changing what is currently a single beam system into a double beam system.

The data shown represent light absorption by brimonidine in cornea and aqueous humour combined. Although the corneal segment constitutes only 18% of the total light path, the cornea clearly contains most of the measured brimonidine, at least in the early stages of absorption. Absorption kinetics of radiolabelled brimonidine in the rabbit indicate that the ratio of corneal to aqueous concentrations is approximately 15:1 at 20 minutes following topical administration.<sup>2</sup> If we apply this ratio to the present data recorded 25 minutes following application of 0.6 mg brimonidine, the cornea would account for 77% of the total measured absorption. Therefore, at this 25 minute time point the brimonidine concentrations in cornea and aqueous were approximately 100  $\mu\text{M}$  and 6.7  $\mu\text{M}$ , respectively. The cornea serves as a substantial depot for topically absorbed drugs. With continual loss of drug in the aqueous humour, concentrations in cornea are likely to remain higher than aqueous levels until eventually they cease to be measurable.<sup>21</sup>

The detection of 5.3 nM fluorescein via fluorescence expands the sensitivity of the technique by two orders of magnitude from our previously reported detection limit of 0.3  $\mu\text{M}$  fluorescein, obtained using absorption spectroscopy.<sup>10</sup> While regionally specific drug concentrations are interesting, repeated non-invasive estimation of total drug in cornea and aqueous is useful since the aqueous bathes all the structures of the anterior eye. The fact that our device can give repeated measurements without trauma in the same subject, in animals or in humans, makes it potentially very useful in the development of ocular formulations and of novel methods of drug delivery.

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