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Research paper

In situ measurement of spectral changes in the anterior eye following application of ultraviolet-absorbing compounds

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ABSTRACT

The ocular structures are very sensitive to damage from ultraviolet (UV) radiation, exposure is linked to corneal and conjunctival damage, cataract formation and may also be implicated in the aetiology of age-related macular degeneration. These structures are usually protected by wearing suitable eyeglasses and goggles. An alternative to conventional eyeglasses/goggles is the concept of "liquid sunglasses" which involve the topical application of eye drops that are designed to block harmful UV radiation reaching the sensitive ocular surfaces. The evaluation of such compounds directly applied to the eye surface requires *in situ* measurements to compare the efficacy of different formulations. A novel ocular spectrometer system has been used to evaluate changes in the transmission of ultraviolet (UV) radiation through the anterior eye following topical application of candidate UV-absorbing formulations. The key feature of the system is the ability to propagate a beam of light tangentially through the anterior eye using a compact, hand-held lens assembly incorporating UV-transmitting optical fibres. A range of formulations containing UV-absorbing compounds were topically applied to *ex vivo* rabbit eyes. Significant increases in the absorption of the UV spectrum were detected in seven of the eight formulations studied, demonstrating the potential of this measurement technique in the evaluation of formulations developed as potential topical ocular sunscreens.

1. Introduction

The intense sunlight usually associated with leisure activities at the beach, visits to tropical locations or at altitude on the snow slopes is an underestimated hazard. Nearly all the ultraviolet components of sunlight are absorbed by the anterior tissues of the unprotected eye, and even short exposure to the UV portion of the solar spectrum can lead to burning of the cornea, otherwise termed acute corneal photokeratitis. Longer term exposure has been linked to chronic corneal photokeratitis as well as conjunctival tumours such as pterygium [1] and also to cataract formation in the lens [2–4].

The solar UV spectrum is usually divided into UVA (320–400 nm) and UVB (290–320 nm). The human cornea absorbs virtually all the incident UVB and a portion of the UVA, the lens absorbs practically all of the UVA as well as the portion of UVB that is transmitted through the cornea [5–7]. Thus, the retina is effectively shielded from exposure to UV light incident on the eye [8]. Long-term exposure to the blue portion of the visible solar spectrum has been linked with an increased incidence of age-related macular degeneration [9,10], the leading cause of blindness in the over-50 age group in the international developed economies.

These data highlight the need to reduce the ocular exposure to both UV and visible solar radiation. Sunglasses with lenses which both reduce the transmission of visible radiation and completely block UV radiation are commonly used. However, there are some situations where the wearing of sunglasses is not necessarily practical, but where protection of the eye from solar UV is desirable, good examples being water and contact sports. Additionally, it has been shown that even when wearing sunglasses between 2% and 20% of the UV dose can still reach the cornea [11]. This led us to the concept of a topically applied ocular sunscreen alternatively described as "liquid sunglasses". The basic principle has been evaluated previously, for example Daxer et al. [12] report the *in vitro* evaluation of a commercially available product. Their protocol involved the measurement of the UV absorption spectrum for a range of thin film thicknesses and concentrations which had been spread on top of a quartz plate. They concluded that the product would not absorb any significant solar UV unless an unrealistic thickness of 0.5 mm was to reside on the surface of the cornea. The limitation of this type of evaluation method is that although

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it gives a thorough evaluation of the UV spectral absorption of the compound, the method is not adaptable to *in situ* measurements in the eye.

We have developed an ocular spectrometer which has successfully obtained measurements of changes in the absorption spectrum from a beam of light traversing the anterior eye [13], with the initial application being the detection of topically applied drugs in the *in vivo* human cornea and anterior chamber [14]. The data and experience obtained using our ocular spectrometer have indicated that the system can be applied to the *in situ* characterisation of products formulated for ocular use, such as topically applied ocular sunscreens.

For practical application, any candidate formulation intended for use as an ocular sunscreen needs to meet three basic criteria. The first is a broad absorption of light through the UVB and UVA bands using a photostable pigment. Secondly, there should be corneal uptake and retention following topical application, and finally, the formulation must be non-irritant. There are important caveats to these remarks: the compounds used here are not those which might be ultimate choices for formulation since dissipation of the energy may result in corneal toxicity; moreover, an 'ideal' agent would be restricted to the most superficial corneal layers. The work reported here describes the proof of concept for the first part of this development. Here, we present results obtained using the ocular spectrometer, where the *in situ* measurements have been derived from fresh rabbit eyes.

2. Methods and materials

2.1. Selection of UV-absorbing compounds

For the purpose of this proof of concept, seven compounds were selected to include both an oil- and water-soluble compound from each of the following: a well-characterised sunscreen, a vitamin K-based compound, and a compound that should be a strong absorber as suggested from the chemical structure. Using these seven compounds, a total of eight simple formulations were manufactured which are summarised in Table 1. The oil-soluble compounds were incorporated into a castor oil emulsion (formulations 1–6) and the water-soluble ones (formulations 7 and 8) into conventional phosphate buffered saline (PBS) solution.

The following UV compounds were selected: oxybenzone, dioxybenzone, hycanthone, menadione sodium bisulphate, 3-phytylmenadione, sulisobenzone (Sigma Aldrich, UK) and salicylideneaniline (Fisher Scientific, UK). Other reagents used were castor oil, polysorbate 80, glycerin, sodium hydroxide and PBS tablets (Sigma Aldrich, UK), ethanol (Merck, UK) and pemulen TR-2, NF (kindly donated by BF Goodrich, Belgium).

Table 1
Concentration of UV-absorbing compounds in each formulation and an estimate of the applied dose absorbed in the cornea and aqueous humour following topical application of 60 μl of each formulation to *ex vivo* rabbit eyes.

Formulation number	UV-absorbing compound(s)	Concentration applied to *ex vivo* eye % (w/w)	Estimate of penetration into the cornea and aqueous humour eye (% dose)
1	Hycanthone	0.33	10.8
2	Hycanthone and Salicylideneaniline	0.3 and 1.05	7.3
3	Oxybenzone	0.51	2.9
4	Oxybenzone	1.07	1.4
5	Dioxybenzone	1.02	2.2
6	3-Phytylmenadione	1.92	<0.01
7	Menadione sodium bisulphate	20.00	1.1
8	Sulisobenzone	1.99	2.7

2.2. Preparation of water-soluble formulations

Phosphate buffered saline (PBS) solution was prepared as per conventional formulae. An appropriate amount of the active ingredient under test was weighed and PBS added until the desired concentration was obtained.

2.3. Preparation of emulsion formulations

Stock solutions of 10% w/w polysorbate 80, 0.25% w/w pemulen concentrate and 1.0 M sodium hydroxide were prepared with distilled water. An aqueous phase was prepared, this was composed of glycerin (0.22 g), polysorbate 80 stock solution (1.0 g) and 6.47 g of distilled water (or 5.47 g of distilled water if ethanol was added to the oil phase). An oil phase was prepared. The maximum concentration of UV-absorbing compounds was not >5% w/v with a concentration of surfactants not >1.0% w/v and a pH 6.6–7.6, in the belief that these parameters were appropriate for ocular use. For each preparation, an appropriate amount of the UV-absorbing compound was weighed into a glass vial followed by 0.25 g of castor oil: 1 ml of ethanol was then added to dissolve compounds with low oil solubility. The vial was then placed in a water bath at 60 °C while being gently shaken for 20 min. Care was taken to ensure the mixture remained as one phase. The vial was then sonicated for 10 min, followed by a further 5 min in the water bath.

The oil phase, the aqueous phase, pemulen concentrate and 1.0 M of sodium hydroxide were separately warmed at 60 °C in a water bath before use. The physical appearance of the oil phase and the aqueous phase were as clear solutions. Two millilitres of pemulen concentrate was added to the aqueous phase and the resulting solution immediately vortex mixed at 1400 rpm for 30 s. The oil phase was then added and vortex mixed at 2500 rpm in four separate 30-s pulses. Forty microlitres of 1 M NaOH was added to the mixture to adjust the pH to 6.8–7.4. This final mixture was vortex mixed in ten 30-s pulses at 2500 rpm. The emulsion was then allowed to cool to room temperature before use.

2.4. Spectroscopic evaluation

The absorption spectra of the formulations were measured using 10 and 100 μm path length cuvettes (Starna, Optiglass, Essex, UK) placed in a UV–VIS spectrometer (UV300, Unicam, Cambridge, UK). For the oil-soluble compounds, the absorption spectra of the both the complete emulsion and the spectra of only the oil phase of the oil-soluble formulations were measured. For clarity, the spectra of the oil phase are shown, thus avoiding the confounding effect of light scattering from the oil/aqueous interface which is present within the emulsion, but not present in the anterior eye following topical application and absorption. Over such a short path length both the oil and the PBS buffer had minimal absorption in the spectral region of interest.

2.5. Ex vivo evaluation of absorption spectra

Formulations containing the UV-absorbing compounds were tested on *ex vivo* rabbit eyes using the ocular spectrometer [13,14]. The spectrometer works by effectively turning the anterior eye into a cuvette. It achieves this via a custom designed sensor head which, when placed in contact with the cornea enables a beam of light to be transmitted laterally across the anterior eye as shown in Fig. 1. Measurements were performed on intact rabbit cadavers, commencing between 30 and 45 min after death. The test was standardised as follows. The cornea and conjunctiva were

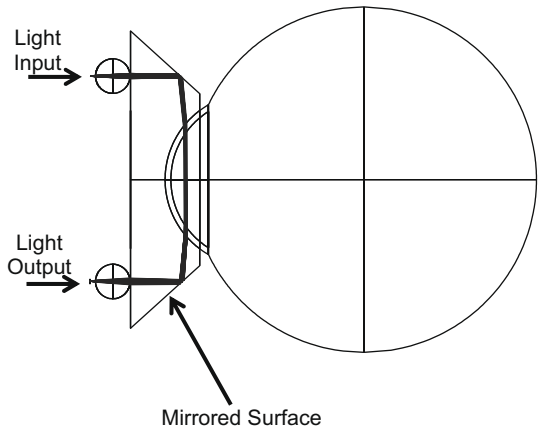

Fig. 1. Schematic of light path through the anterior eye. When the lens is in contact with the cornea, light is directed in, across and out of the anterior eye, enabling changes in the absorption spectrum of the anterior eye to be measured.

rinsed with PBS solution, then the sensor head was placed on the cornea and a baseline absorption spectrum $A(\lambda)_{baseline}$ measured. One 30-μl drop of the target formulation was topically applied and the eye manually blinked four or five times to spread the formulation evenly over the corneal surface. After 5 min, another 30-μl drop was added and the blinking repeated. Five minutes after addition of the second drop, the peri-ocular area was thoroughly rinsed with PBS solution. Finally, the absorption spectra was re-measured giving $A(\lambda)_{measurement}$. The subtraction of the baseline spectrum from the measurement spectrum gives the difference in absorption after the topical application of the UV-absorbing formulation where $A(\lambda)_{difference} = A(\lambda)_{measurement} - A(\lambda)_{baseline}$.

2.6. Spectral detection range

The instrument has a spectral range of 250–700 nm with a resolution of 1 nm. Previously, we have established that it can resolve absorption changes of 0.01 AU, enabling detection, via absorption spectroscopy, of 0.3 and 3.0 μM concentrations of fluorescein and brimonidine, respectively [13]. Both the rabbit and human cornea have a transmission cut-off at 300 nm [5,6,14,15]. This cut-off means that in practice we can obtain useful ocular spectral measurements for wavelengths greater than 310 nm. However, for the purpose of this study, it is desirable to determine the effect of the formulations for the whole solar UV region, i.e. 290–400 nm. This was achieved by comparing the full range spectra obtained using the UV300 spectrometer (250–700 nm) with the *ex vivo* spectra (310–700 nm) and extrapolating the latter for the 290–310 nm spectral region. This extrapolation was straightforward as the UV absorption spectra of all candidates had broad and characteristic spectral features.

In addition to light lost through absorption will be light loss due to scattering. This scattering, from collagen fibrils within the cornea, differs from absorption in that it does not have an absorption peak rather it is a broad spectral loss, decreasing with increasing wavelength [5]. This scattering has been reported to increase post-mortem in excised corneas [16]; however, we have measured these changes to be negligible during the time frame used in this study.

2.7. Absorbed dose calculation

The percentage of the applied dose lost to the cornea and aqueous humour was calculated using Beer's law, $A = \varepsilon cl$, where A is the measured absorption change, ε is the molar extinction coefficient, c is the molar concentration, and l is the path length. The molar extinction coefficient is determined from the short path length spectra, the ocular concentration is determined from the measured difference spectra following topical application, the dose calculated then compared to the applied dose to determine the and the dose lost to the ocular structures.

3. Results

3.1. Comparison of short path length and ex vivo spectral evaluation of UV-absorbing compounds

A comparison of the short path length spectral evaluation and the *ex vivo* ocular spectral evaluation is given in Fig. 2. The short path length spectral evaluation demonstrated that all of the compounds have strong absorption in the UVB and a significant portion of the UVA spectra regions. These are compared to the difference spectra obtained following topical ocular application of each of the eight formulations. The baseline and measurement spectra measured before and after the application of formulation 1 are also shown (Fig. 2.1b). Seven of the eight difference spectra showed a significant increase in UV absorption following application of the

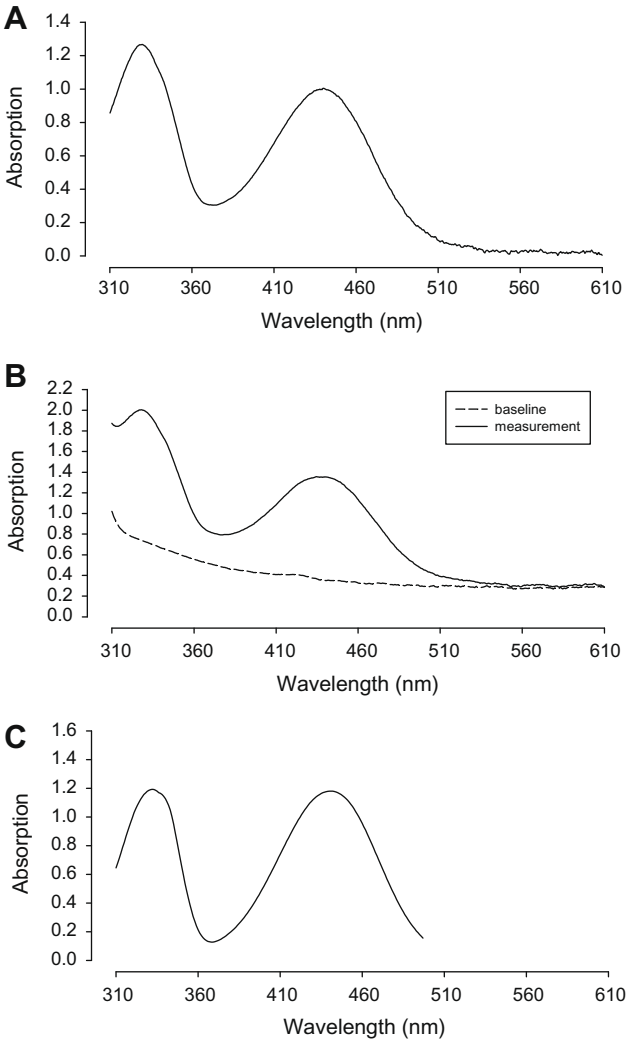

Fig. 2.1. (A) Change in absorption of the anterior eye following application of formulation 1 (0.33% hycanthone). (B) Absorption spectra of the anterior rabbit eye before (baseline) and after (measurement) the topical application of formulation 1 (0.33% hycanthone). (C) Absorption spectrum of 0.54% hycanthone in castor oil, path length is 100 μm.

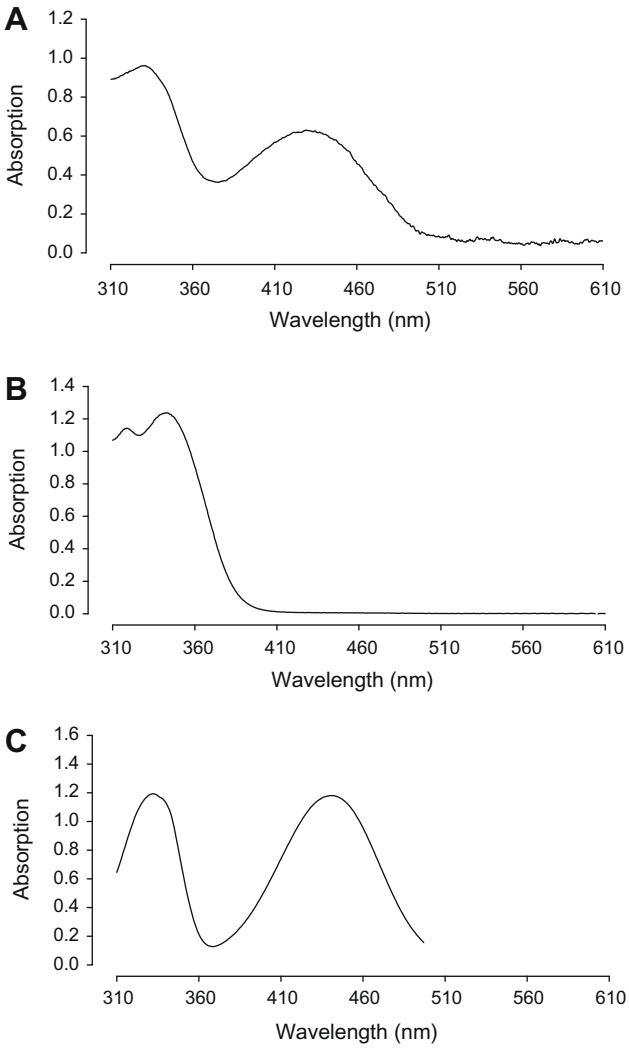

Fig. 2.2. (A) Change in absorption of the anterior eye following application of formulation 2 (0.30% hycanthone and 1.05% salicylideneaniline). (B) Absorption spectrum of 1.12% salicylideneaniline in castor oil, path length is 10 μm. (C) Absorption spectrum of 0.54% hycanthone in castor oil, path length is 100 μm. Note this is a repeat of Fig. 2.1C and is included here for convenience of comparison.

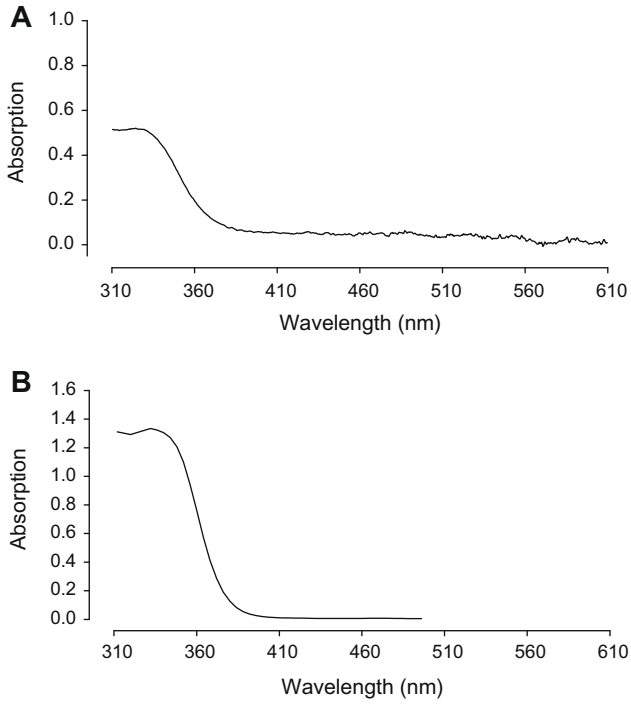

Fig. 2.3. (A) Change in absorption of the anterior eye following application of formulation 3 (0.51% oxybenzone). (B) Absorption spectrum of 4.86% oxybenzone in castor oil, path length is 10 μm.

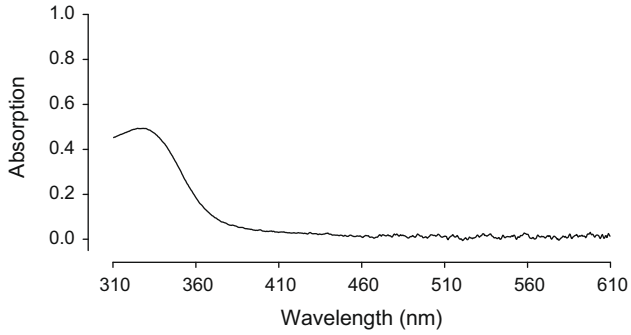

Fig. 2.4. Change in absorption of the anterior eye following application of formulation 4 (1.07% oxybenzone).

formulations and one, formulation 6 (Fig. 2.6), did not. For formulations 1, 3–5, 7, and 8, the shape and peak position of the short path length spectra matched the difference spectra obtained following *ex vivo* application. Formulation 2 (Fig. 2.2) is a combination of two absorbing compounds and the difference spectra can be interpreted as a combination of the short path length spectra for both of these. The absorption spectra measured from a short path length spectrum containing the oil phase of formulation 6 has a broad peak centred at 330 nm, this peak is not present in the corresponding difference spectra, which shows very little change in ocular absorption following topical application. This minimal change also demonstrates the lack of spectral changes due to scattering during the measurement period.

The absorbing compound in formulations 3 and 4 was oxybenzone. Following ocular application, two similar difference spectra were obtained (Figs. 2.3 and 2.4), despite formulation 4 having more than twice the oxybenzone concentration of formulation 3.

3.2. Absorbed dose

The percentage of the applied dose lost to the ocular structures (specifically the cornea and aqueous humour) was estimated as described earlier and summarised in Table 1. Seven of the eight formulations showed an applied dose of a few percent, the value of <0.01 given for formulation 6 is our calculated threshold sensitivity for this measurement technique.

4. Discussion

4.1. Evaluation of ocular spectrometer

The ocular spectrometer has been shown to readily detect any changes in UV absorption spectra of the anterior eye following topical application of the formulations studied. Using the absorption spectra, it is also possible to estimate the percentage of the ocular dose that has been absorbed into the cornea and aqueous humour. To our knowledge, this is the first ocular spectrometer capable of such *in situ* measurements. Previously, this instrument has successfully been demonstrated *in vivo* in human eyes [14] and, given appropriate formulations, could be used for clinical evaluation. An advantage of this type of instrument would be the direct measure-

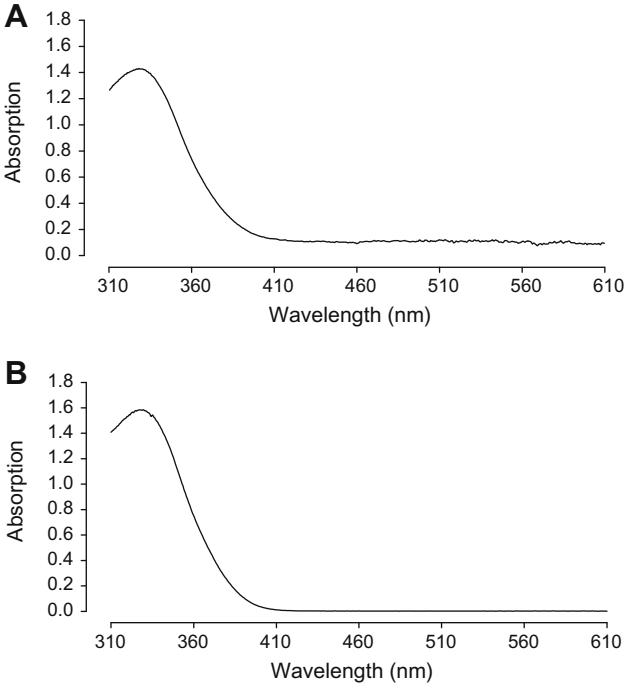

Fig. 2.5. (A) Change in absorption of the anterior eye following application of formulation 5 (1.02% dioxybenzone). (B) Absorption spectrum of 2.87% dioxybenzone in castor oil, path length is 10 μm.

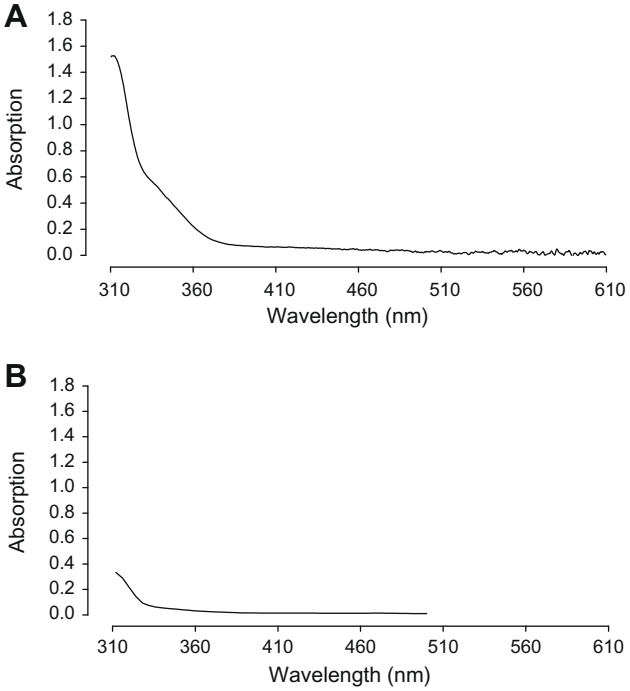

Fig. 2.7. (A) Change in absorption of the anterior eye following application of formulation 7 (20.0% menadione sodium bisulphate). (B) Absorption spectrum of 0.50% menadione sodium bisulphate in phosphate buffered saline. Path length is 100 μm.

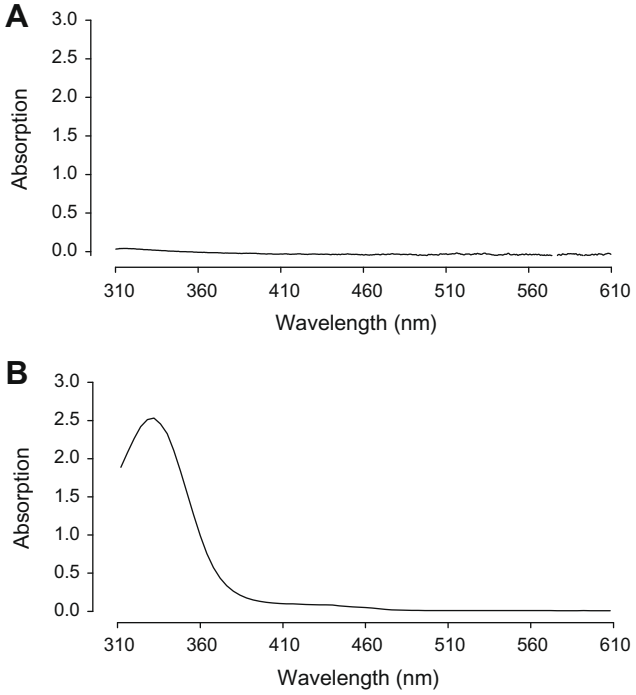

Fig. 2.6. (A) Change in absorption of the anterior eye following application of formulation 6 (1.92% 3-phytylmenadione). (B) Absorption spectrum of 4.5% 3-phytylmenadione in castor oil. Path length is 100 μm.

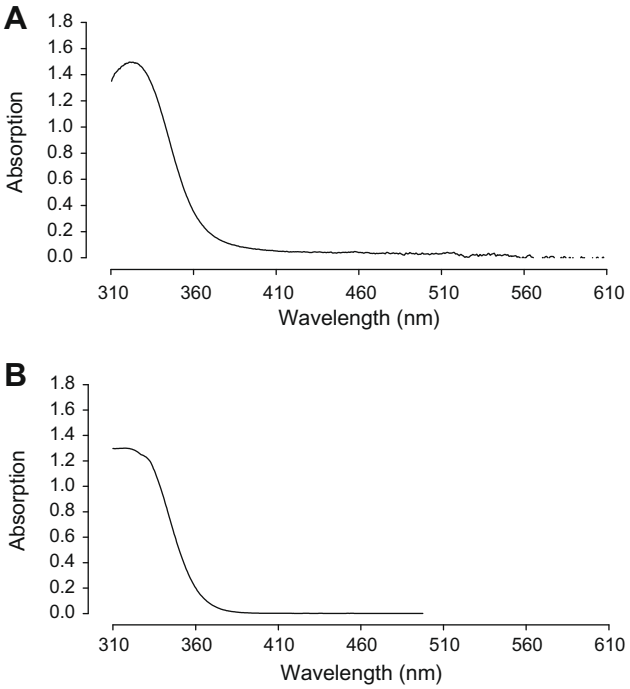

Fig. 2.8. (A) Change in absorption of the anterior eye following application of formulation 8 (1.99% sulisobenzone). (B) Absorption spectrum of 0.50% sulisobenzone in phosphate buffered saline. Path length is 100 μm.

ment of persistence of the target formulations. Previous reports studying the effect of applying UV-absorbing formulation to the eye have only be inferred the UV spectral ocular absorption by measurement of the spectra of thin films using a bench-top spectrometer [12].

4.2. Evaluation of formulation spectra

With the exception of formulation 6, topical application of the formulations resulted in significant increases in the UV absorption spectra of the anterior eye. As these increases matched the absorp-

tion spectra of the formulations measured using the bench-top spectrometer, we conclude that the formulations have been absorbed into the anterior eye. The formulations used in this evaluation all strongly absorbed in the UVB band, with the absorption also extending into part of the UVA band. These formulations potentially provide UV absorption characteristics that would enable the determination of an ocular equivalent of the dermatological sun protection factor (SPF) [17]. To produce a formulation that provides a more complete coverage of the UVB and UVA spectrum, it is likely that, as for dermatological sunscreens, a multi-component formulation would be required. As well as providing attenuation in the UV, formulations 1 and 2 also resulted in significant attenuation in the blue part of the visible spectrum. Studies have shown that exposure to this part of the visible spectrum may be a factor in age-related macular degeneration [9,10]. In the short term, attenuation of these blue wavelengths could offer the retina protection against photochemical injury. Taking the concept of protective eye drops to its ideal conclusion would be the pursuit of a formulation which would mimic the function of sunglasses and result in a broad spectral blocking of the UV spectra combined with uniform attenuation of the visible spectrum. As with sunglasses the colour perception of the eye would have to be considered when manufacturing such topical formulations.

Figs. 2.3 and 2.4 show near identical absorption spectra, despite the fact that oxybenzone concentration and therefore applied dose, differed by a factor of 2. It is known that permeation of the stroma is a rate-limiting step for very lipophilic compounds [18].

The absorption spectra of formulation 6 did not show any significant change after application. We therefore assume that this formulation was not absorbed in any significant quantity by the cornea and was washed off before the measurement spectra were taken. For validation of this assumption, we calculated the expected size of the absorption peak if a reasonable percentage of the applied dose had been absorbed by the cornea. Using the values in Table 1 as a guide, we calculated that if 2% of the dose had been absorbed, the difference absorption spectrum would have had a peak of 0.16 at a wavelength of 330 nm. As can be seen from Fig. 2.6a, this was not the case.

4.3. Increased loading

The residency time and percentage of applied dose loaded into the target tissue will be a critical factor in the effectiveness of a UV-absorbing formulation. Increased loading could be achieved by increasing tear film thickness using hyaluronate [19]. Alternatively, the incorporation of cyclodextrin and other oligosaccharides into the ophthalmic formulations offers the potential to increase the aqueous solubility of candidate drugs, improve aqueous stability and importantly reduce any irritation associated with the drug [20].

5. Conclusion

We have demonstrated the ability of the ocular spectrometer to detect absorption changes in the anterior eye following topical application of UV-absorbing formulations. Additionally, the percentage of the applied formulation absorbed by the cornea and aqueous humour was estimated. The measurement technique is minimally invasive and has previously been applied to the unanaesthetised human eye. Thus proving the feasibility of this technique for the evaluation of potential ophthalmic sunscreens.

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