Communication

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New Therapeutic Approaches for Fibrosing Skin Diseases: An Ecofriendly UVA-1 Permissive Sunscreen Formulation Which Displays Activity at the Aryl Hydrocarbon Receptor. A Potential Cost Effective and Convenient Therapeutic Approach to Fibrosing Skin Di

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New Therapeutic Approaches for Fibrosing Skin Diseases: An Ecofriendly UVA-1 Permissive Sunscreen Formulation Which Displays Activity at the Aryl Hydrocarbon Receptor: A Potential Cost Effective and Convenient Therapeutic Approach to Fibrosing Skin Diseases

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Abstract: UVA1 phototherapy has been reported to mediate some of its effects via FICZ/AHR/MAPK signaling. A proof-of-concept study was performed to assess whether a topical solution of L tryptophan formulated to favor the generation 6-formylindolo[3,2-b] carbazole (FICZ) demonstrated activity at the aryl hydrocarbon receptor (AHR) as assessed by the intensity of cytochrome P450 1A2 staining. Ten participants applied a trial agent consisting of L- tryptophan, lecithin, polyvinyl alcohol and ethanol followed by 36 sessions of graduated sun exposure increasing to a total of 20 Joules per square centimetre. At the end of the trial, biopsies were taken from a treated site with photo-protected buttock skin used as a control. Assessment was via intensity of cytochrome P450 1A2 staining. An average baseline value of 53488923 units was obtained at the control site and 972214294 at the treatment site supporting the concept that this preparation displays activity at the AHR. As the components of this preparation are known to have UV absorbing properties and are potentially permissive of UVA-1 transmission, UV absorbance was assessed for natural sunlight and at 365 and 395nm confirming strong absorption in the UVB and UVA-2 spectra with partial transmission in the UVA-1 spectrum.

Keywords fibrosing skin disorder; topical tryptophan; FICZ; Aryl hydrocarbon receptor; UVA-1; sunscreen

1. Introduction

The Aryl hydrocarbon receptor (AHR) is a cytosolic receptor for low molecular weight molecules which acts as an environmental sensor and influences several signalling pathways, most significantly the cytochrome P450 1A1, 1A2, 1B1 monooxygenases which introduce functional groups prior to conjugation with water soluble molecules by the phase 2 detoxification enzymes [1]. In addition, it has immunomodulatory effects and plays an important role in the cutaneous response to UV light and pigmentation.

It is emerging as an important therapeutic target. Tar preparations are an established dermatological modality known to be active at the AHR [2] and tapinarof is an AHR agonist currently available for atopic dermatitis [3] and psoriasis [4].

6-formylindolo[3,2-*b*] carbazole (FICZ) is a potent endogenous ligand of the AHR produced by the photo-oxidation of L-tryptophan. It is immunoinhibitory at high concentrations and immunostimulatory at low concentrations [5].

L-tryptophan is metabolized to nicotinamide via the kynurenine pathway (KP). Several of the metabolic intermediates are active at the AHR [1] and contribute to AHR modulation.

Fibrosing cutaneous disorders such as morphea and scleroderma remain incompletely understood. The initial or inflammatory phase begins with vascular endothelial damage and the upregulation of adhesion molecules such as E-cadherin and VCAM-1[6] and the subsequent recruitment of pro-inflammatory Th1 and TH 17 cells. At this stage the cytokine signature profile is TH 1 weighted [7]. In the second or fibrotic phase, a TH2 cytokine signature predominates [8]

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mediating tissue fibrosis. In morphea fibrosis resolves over 2-5 years but may be followed by persistent tissue atrophy [9].

The primary aim of this study was to establish that a graduated increase in natural ultraviolet light (UV) exposure combined with a topical preparation of L-tryptophan formulated to favor the generation of FICZ demonstrated activity at the AHR.

As sunflower lecithin was incorporated in the preparation and lecithin-based multilamellar liposomes have been reported to offer a biodegradable alternative to traditional sunscreen preparations with equal efficacy [10] the UV absorbance of natural sunlight was determined via UV integrator. Additional values were also determined utilizing 365 and 395 UV light sources to assess UV absorbance in the ultraviolet A1 spectrum (340-400nm) as UVA1 phototherapy is recommended for fibrosing cutaneous disorders. An ideal agent would demonstrate strong activity at the AHR and UV protection comparable to traditional sunscreens whilst permissive of transmission within the UVA 1 spectrum.

2. Results

Ten participants applied a trial agent consisting of L- tryptophan, lecithin, polyvinyl alcohol and ethanol followed by 36 sessions of graduated sun exposure increasing to a total of 20 Joules per square centimetre. At the end of the trial, biopsies were taken from a treated site with photo-protected buttock skin used as a control. Assessment was via intensity of cytochrome P450 1A2 staining.

The results are outlined in Figure 1. The Kolmogorov-Smirnov Test confirms the distribution as normal with a mean of 60319716 at the control site and 972214294 at the treatment site with a p value of 0.047 supporting a statistically significant result. Sample slides are shown in Figure 2 a, b, and c. Although all cells in the integument express the AHR [1], Cytochrome P450 induction was noted to be strongest amongst fibroblasts and infiltrating mononuclear cells (Figure 2a).

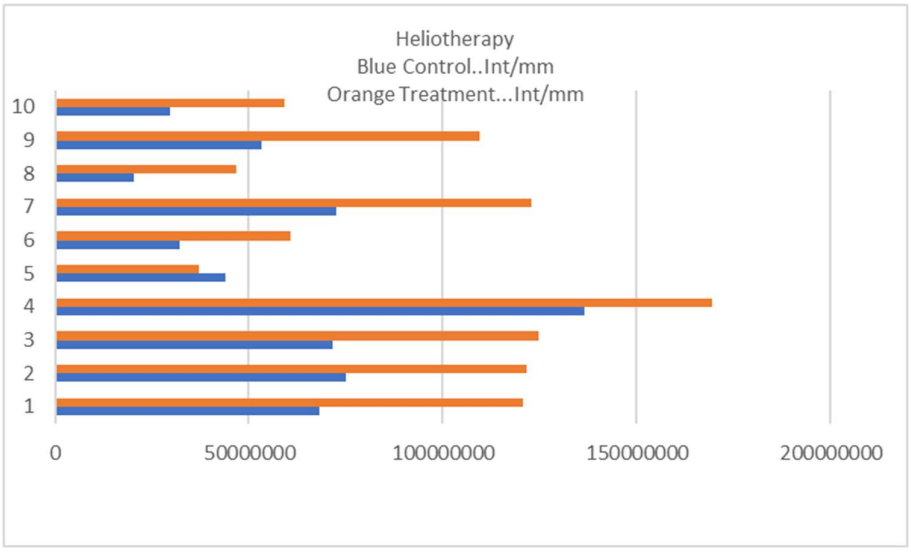

Figure 1. Immunohistochemistry intensity/mm^2 Heliotherapy/Trial agent limb.

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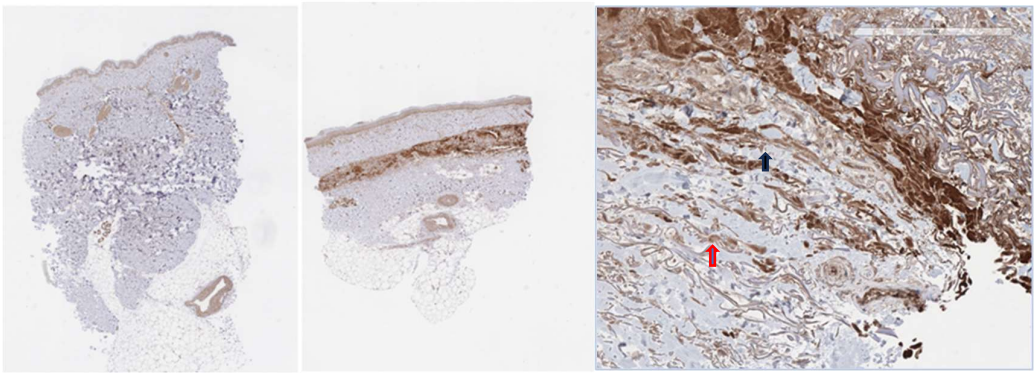
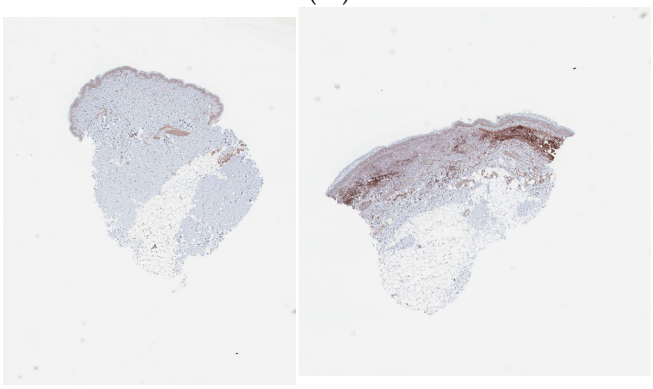
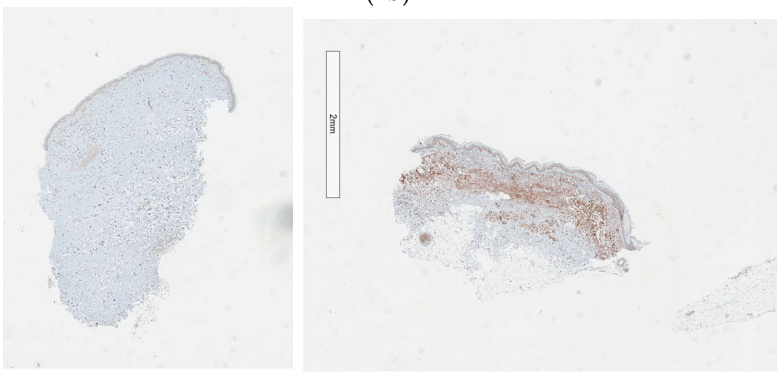


(2**a**)



(2**b**)



(2**c**)

Figure 2. 2a. CytP450 IHC. Trial agent. Control left. Treatment centre. Right Maximal induction is seen in the fibroblasts (blue arrow) and infiltrating mononuclear cells (red arrow). 2b. CytP450 IHC. Trial agent. Control left. Treatment right. 2c. CytP450 IHC. Trial agent. Control left. Treatment right.

Due to the strong induction of Cytochrome P4501A2 the Hematoxylin and Eosin sections were examined for evidence of cellular apoptosis. No significant increase was noted.

2.1. Tryptophan Toxicity

The ingestion of contaminated L tryptophan has been reported to have an association with eosinophilia myalgia syndrome although other authors have suggested that kynurenine metabolites themselves may have been responsible for this disorder [11]. Eosinophilia myalgia syndrome was reported with daily doses of L tryptophan of 1.2-2.4 G [12] equating to the use of 60-120G of the trial preparation daily, well beyond the recommended maximum daily dose of 20G based on the fingertip unit scale. No participants reported symptoms or displayed and signs of eosinophilia myalgia syndrome.

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2.2. UV Absorbance Natural Sunlight

The Sun Protection factor (SPF) of a sunscreens is traditionally determined using ten human volunteers and is based on the UV dose required to produce cutaneous erythema on skin without the photoprotective agent compared to skin with the photoprotective agent, the ratio providing the value. In this study, as an alternative, ten in vitro measurements were made using a UV integrator. The trial agent was applied to adhesive tape mounted over the sensor component UV-SPEEDRE UV Integrator immediately prior to sun exposure at a concentration of 2.5mg per cm^2 and the UV dose measured after 1 hour of sun exposure. A second UV-SPEEDRE UV Integrator with the sensor covered with adhesive tape was used as a control. The results are outlined in Table 1 and the setup in Figure 3. They are consistent with a 50 plus value previously reported for a lecithin-based sunscreen [10].

Figure 3. Determination of UV absorbance. Natural sunlight.

Table 1. UV absorbance natural sunlight. Measurements were taken at various times during the day at a latitude of 26 degrees south during the winter months. There were variable periods of cloud cover.

Time	*Trial Agent*	*Control*
Sunlight 1200-1300	*31*	2193
Sunlight 0900-1000	5	1012
Sunlight 1130-1230	0	2360
Sunlight 1400-1500	0	805
Sunlight 1100-1200	0	1818
Sunlight 1200-1300	0	1592
Sunlight 1500-1600	0	542
Sunlight 1000-1000	0	868
Sunlight 1100-1200	0	623
Sunlight 1100-1200	66	5099

2.3. UV Absorbance 395 nm

This protocol involved a 60 second illumination via a 395nm 3-watt light source applied directly over the sensor of a UV-SPEEDRE UV Integrator covered with adhesive tape to which the trial agent had been applied at a concentration of 2.5mg per cm^2. The control value was determined utilizing the same sensor covered with adhesive tape without the trial agent. The results are outlined in Table 2 and demonstrate approximately a 75 percent reduction in UV transmission at 395nm consistent with

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the known absorbance spectrum of lecithin [13] and supportive of the concept that this agent permits partial transmission in the UVA 1 spectrum.

Table 2. UV absorbance 395nm (Manufacturer's stated fluence at source 1500mJ/cm^2).

Cycle	*Trial Agent*	*Control*
1	264	1231
2	427	1356
3	239	1011
4	329	1179
5	251	1158
6	477	1185
7	200	1110
8	317	1151
9	406	1083
10	129	1127

2.4. UV Absorbance 365 nm

This protocol involved a 60 second illumination via a 365 nm light source applied directly over the sensor of a UV-SPEEDRE UV Integrator covered with adhesive tape to which the trial agent had been applied at a concentration of 2.5mg per cm^2. The control value was determined utilizing the same sensor covered with adhesive tape without the trial agent. The results are outlined in Table 3.

Table 3. UV absorbance at 365nm (Manufacturers stated fluence at source 455 mJ/cm^2).

Cycle	Trial Agent	Control
1	64	340
2	64	398
3	44	371
4	44	378
5	42	362
6	44	347
7	42	341
8	46	381
9	42	335
10	41	362

3. Discussion

Fibrosis is characterized by the aberrant deposition of extracellular matrix leading to organ dysfunction. Although the exact pathogenesis is yet to be elucidated transforming growth factor beta (TGF beta) signalling, myofibroblast activation and immunological disequilibrium play key roles.

As AHR signalling is involved in all the above processes, it is an attractive therapeutic target.

Myofibroblast differentiation is central to the fibrotic process. Progenitor cells undergoing epithelial to mesenchymal transition (EMT), mesothelial to mesenchymal transition (MMT) and endothelial to mesenchymal transition (EndoMT) in response to cytokines released by infiltrating immune cells differentiate into active myofibroblasts. TGF beta is pivotal in promoting EMT, MMT and EndoMT, the secretion of various extracellular matrix (ECM) components and matrix metalloproteinase (MMP) activity. TGF beta expression is negatively influenced by AHR signalling [14].

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The AHR and KP intermediates serve several important functions in myofibroblast activation. The KP metabolite kynurenine has been reported to modulate the expression of several ECM components via AHR signalling [15-16] and the archetypical AHR ligand TCDD induces the induction of MMP-1 in keratinocytes [17]. In a similar fashion the tryptophan photoproduct and AHR ligand FICZ has been demonstrated to upregulate MMP-1 expression in dermal fibroblasts offering an insight into the therapeutic mechanism of phototherapy in scleroderma [18].

An imbalance between TH17 cells and Treg cells has been reported in systemic sclerosis [19] and the AHR is known to promote T reg differentiation [20] which supresses the profibrotic immune response [21].

The inclusion of lecithin in this preparation is significant. Lecithin absorbs UV light in a broad band from 200-380nm with peak absorbance at 235, 271 and 355 nanometres [13] and thus has potential as a biodegradable and ecofriendly sunscreen with an efficacy equivalent to traditional sunscreens [10]. As such, preparations of the kind used in this trial may form the basis for therapeutic sunscreens, that is preparations which offer both photoprotection and the immunomodulatory benefits of ultraviolet light-based therapy without the harmful effects associated with ultraviolet (UV) irradiation of the skin [22].

L-tryptophan absorbs maximally at 280nm with absorbance falling rapidly to 310nm and is thus expected to provide strong photoprotection in the UVB spectrum.

UVA1 phototherapy utilizing UV radiation in the 340-400nm band is considered a promising treatment for cutaneous fibrosing disorders [23], although it is unlikely that all the antifibrotic effects of UVA 1 therapy are mediated via the AHR [24]. Specifically, immediate T cell apoptosis through the activation of the FAS/FAS ligand system appears to be a specific property of UVA1 phototherapy, since it is not observed with UVB [25] and thus likely to be AHR independent. As such an agent which is permissive of partial UV transmission in the 340-400nm range would be advantageous.

The results are consistent with previous studies that lecithin displays a 50 plus SPF [10] (98% UV absorption) in the UVB and UVA-2 spectrum with absorbance falling to 87% at 365 nm and 80% at 395nm.

In summary this is a promising preparation which provides strong UV protection via filtering ultraviolet B (290-310 nm} and ultraviolet A2 (320-340 nm) light yet retains strong activity at the AHR, a potential therapeutic target in fibrosing skin disorders. In addition, it allows partial UVA 1 transmission (340-400nm) theoretically providing convenient and cost-effective antifibrotic therapy. Finally, it is biodegradable and ecofriendly with a lower potential impact on coral reefs and marine plankton [10]. The availability of UVA-1 units is often restricted to major population centres and remain non accessible to rural and remote populations and an alternative means of delivering UVA-1 therapy would represent meaningful progress.

Limitations of this study include the fact that this is a proof-of-concept study only, designed to confirm this agent displays activity at the AHR whilst offering photoprotection and partial transmission within the UVA 1 spectrum and may thus act as an antifibrotic agent. Although displaying statistically significant activity at the AHR, the small cohort would necessitate repeat studies in a larger trial group.

Additionally, a demonstration of efficacy in the management of fibrosing cutaneous disorders would need to be made and outcomes would need to be assessed against UVA-I therapy. The localized scleroderma cutaneous assessment tool (LoSCAT) is used to measure the therapeutic response in morphea [26] and the modified Rodnan skin score in scleroderma [27]. No scoring tool exists for nephrogenic systemic fibrosis and the clinical scoring of chronic graft versus host disease incorporates visceral disease.

Assessing this agent against UVA-1 phototherapy is difficult as fibrosing skin conditions are uncommon making it difficult to source a large cohort and previous studies with phototherapy have been reported to be based on low quality evidence [28]. None the less improvement is reported [28].

Another limiting factor is the relative contributions of AHR activation and immediate T cell apoptosis in the antifibrotic effect. UVA-1 therapy is traditionally divided into low dose (10-20J/cm^2), medium dose (50-60J/cm^2) and high dose (up to 130J/cm^2) and although various protocols have been used the Cochrane metanalysis has been reported as showing no difference in outcomes [28]. The summer (December to February), the peak daily UVA exposure has been reported as 205 J/cm^2 in the subtropics [29]. UVA1-absorption of 87% at 365nm and 80% at 395 nm would equate to a full day's

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sun exposure at subtropical latitudes in summer utilizing this agent as a sunscreen to achieve low dose UVA-1 equivalence and thus this preparation may be of less value in the winter months at lower latitudes, but this would require further evaluation.

4. Materials and Methods

4.1. The Generation of FICZ from Tryptophan

This is an oxidative deamination reaction mediated by either an oxidizing agent or UV light. The intermediate is indole 3 acetaldehyde (I3A), two molecules of which undergo condensation under acid or alkaline conditions [30].

Lecithin, a mixture of the glycerophospholipids phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid was included in the trial agent. Ethanol was used in the preparation to promote the aggregation of the phosphatidylcholine molecules as they are more soluble in ethanol [31]. L-Tryptophan plays an important role in anchoring proteins to cell membranes [32] and it was proposed that aggregation of phosphatidyl choline, the principal component of cell membranes would bring individual L tryptophan molecules into proximity facilitating the deamination reaction.

The UV exposure protocol employed is outlined in Table 4. Dosage was determined by a UV-SPEEDRE UV Integrator. If the recommended dose had not been achieved at 2 hrs sun exposure was ceased. The dose escalation paralleled that used in Narrowband UVB (NBUVB) and was designed to allow "hardening" of the skin to minimize the risk of UV burns. The final total dose achieved corresponded to a summative low dose NBUVB (1000mJ) and low dose UVA 1 therapy (~ 20J).

Table 4. Heliotherapy protocol. FST refers to theFitzpatrick skin type.

Cycle	FST 1 (mJ)	FST 2-3 (mJ)	FST4-5 (mJ)	FST 6 (mJ)
1	400	600	1000	1200
2	480	720	1200	1440
3	576	864	1440	1728
4	691	1037	1728	2074
5	829	1244	2074	2488
6	995	1493	2488	2986
7	1194	1792	2986	3583
8	1433	2150	3583	4300
9	1720	2580	4300	5160
10	2064	3096	5160	6192
11	2477	3715	6192	7430
12	2972	4458	8916	8916
13	3566	5350	8916	10699
14	4280	6420	10699	12839
15	5136	7704	12839	15407
16	6163	9244	15407	18488
17	7395	11093	18488	20000
18	8874	13312	20000	20000
19	10649	15974	20000	20000
20	12779	19169	20000	20000
21	15335	20000	20000	20000
22	18402	20000	20000	20000

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23-26	20000	20000	20000	20000

The trial agent was applied to involved sites immediately prior to sun exposure using the fingertip unit (250mg covering a palm sized area with the fingers together).

The decision to limit sun exposure to 2hrs was based on considerations of practicality and evidence from studies on "Dead Sea therapy" which it was felt may have shared sufficient similarities to allow data to be extrapolated [33].

4.2. Histological Analysis

At the completion of therapy, a 4mm punch biopsy was taken form a treated site and another which acted as a control from photo-protected non treated site (buttock). Specimens were stained for hematoxylin and eosin. Cytochrome P450 1A2 staining was via immunohistochemistry. Cytochrome P450 1A2 (Cytochrome P450 1A2 Polyclonal Antibody, Biotin Conjugated) was used as the marker for AHR activity.

Slides were dewaxed sequentially with xylene (one 3-minute cycle followed by two 1-minute cycles) and ethanol (two 1minute cycles using 100% ethanol, one 1-minute cycle using 90% ethanol and one 1-minute cycle using a 70% ethanol) before being washed under running water for 3 minutes.

Endogenous peroxide activity was blocked by incubation with 3% hydrogen peroxide for 5 minutes before being washed in running water for 2 minutes.

The slides were placed Dako Epitope Retrieval buffer (pH 9.0) and heat induced epitope retrieval performed in a Biocare Medical Decloaker for 5 minutes at 125C before being allowed to cool for 20 minutes.

The slides were then washed three times in Tris Buffer Saline and 0.02% Tween 20 (TBSTW) for 2 minutes before applying Vector Biotin Blocking solutions (Streptavidin and Biotin) for 10 minutes each, washing between applications. The slides were than washed three times in TBSTW for 2 minutes.

The tissue was then covered with the blocking solution, Biocare Medical Background Sniper + 2% BSA for 15 min. The blocking solution was than aspirated and the primary antibody, Bioss Rabbit anti-Cytochrome P450 1A2 diluted 1:300 in Da Vinci Green antibody diluent added to each slide and incubated for 2 hours at room temperature. The slides were than washed three times in TBSTW for 2 minutes.

Following this the tissue was incubated with Jackson Immunoresearch streptavidin conjugated HRP diluted 1:600 in TBSTW for 60 minutes before being washed three times in TBSTW for 2 minutes.

Biocare DAB was applied for 5 mins and the slides washed with water for 5 minutes after which a coverslip was applied.

Analysis was via Aperio Imagescope using Positive Pixel Count v9. Values for total intensity/mm^2 were used to assess enzyme activity.

4.3. Preparation of the Trial Agent

3 grams of L-tryptophan were dissolved in 100 mls of sterile water at pH 2.8 via continuous stirring at room temperature. The solution was then heated to 50C, and 45 grams of sunflower lecithin slowly added whilst stirring. 5G of polyvinylalcohol were then added with continued stirring until dissolved.

The solution was then allowed to cool to room temperature and100% ethanol added to 20% of the final volume, stirring until into solution. The pH was than adjusted to 5.8. Final volume 150mls.

The preparation was prepared by a commercial compounding pharmacy (Formulae Compounding Lab Albion) utilizing pharmaceutical grade ingredients.

4.4. Determination of UV Absorption (Natural Sunlight)

The trial agent was applied to adhesive tape mounted over the sensor component UV-SPEEDRE UV Integrator immediately prior to sun exposure at a concentration of 2.5mg per cm^2 and the UV dose measured after 1 hour of sun exposure. A second UV-SPEEDRE UV Integrator with the sensor covered with adhesive tape was used as a control. Measurements were taken at various times during

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the day as outlined in Table 1. Cloud cover was variable. Measurements were taken at latitude was 26 degrees south during the winter months. The setup used is shown in Figure 3.

4.5. Determination of UV Absorption at 395 nm

The trial agent was applied to adhesive tape mounted over the sensor component UV-SPEEDRE UV Integrator immediately prior to exposure at a concentration of 2.5mg per cm^2 and the UV dose measured after 60 seconds of UVB exposure. Illumination was via a 395nm light source applied directly over the sensor. The manufacturers reported fluence was 1500mJ/cm^2 at the source. The control value was determined utilizing the same sensor covered with adhesive tape without the trial agent.

4.6. UV Absorbance 365 nm

This protocol involved a 60 second illumination via a 365 nm light source applied directly over the sensor of a UV-SPEEDRE UV Integrator covered with adhesive tape to which the trial agent had been applied at a concentration of 2.5mg per cm^2. The manufacturers reported fluence at the source was 455 mJ/cm^2. The control value was determined utilizing the same sensor covered with adhesive tape without the trial agent.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee the Ramsay Health Care QLD HREC, Newdegate St, Greenslopes QLD 4120.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The author reports no conflicts of interest.

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