Transfer and Refinement of the Reactive Oxygen Species (ROS) Assay to Evaluate Photoreactivity Potential

Kwiatek, J., Tran, K., Supit, T., Hilberer, A.
Institute for *In Vitro* Sciences, Inc., Gaithersburg, MD USA

4841/N695

Introduction

Photoreactivity evaluation is one of the initial screenings in a photosafety assessment that can identify compounds which may have the potential to produce various types of adverse photo reactions, including photoirritation (or phototoxicity), photoallergy, and photogenotoxicity. The Reactive Oxygen Species (ROS) Photoreactivity Assay, adopted under OECD Test Guideline (TG) 495, is an *in chemico* test system that measures the amount of ROS generated by a test compound upon exposure to simulated sunlight. The generation of ROS is determined through measurement of singlet oxygen (SO) and superoxide anion (SA) produced through the bleaching of *p*-nitrosodimethylaniline (RNO) and the reduction of nitroblue tetrazolium (NBT) in respective reaction mixtures.

The transfer of the ROS Photoreactivity assay in our laboratory required establishing a framework for testing by procuring specific reagents and equipment, determining acceptable exposure conditions, and generating proper documentation and historical control databases with the goal of conducting the assay under Good Laboratory Practices (GLPs). Seventeen proficiency chemicals, including the positive (quinine hydrochloride) and negative (sulisobenzone) controls, were used to evaluate the impact of several variables including light source, irradiance exposure conditions, and reagent supplier. The assay was successfully transferred after thorough investigation and optimizations of several variables that had the potential to affect assay performance and results.

Materials & Methods

The ROS assay was performed in accordance with OECD TG 495 (**Figure 1**). Singlet oxygen (SO) and superoxide anion (SA) values were calculated as presented in **Figure 2**. The average of the 3 replicate wells for each SO and SA value was used to evaluate the photoreactivity potential using the prediction model described in the OECD TG 495 (**Figure 3**). In our assay transfer and optimizations, we evaluated the Impacts of the light source (**Figures 4-5**), irradiance exposure conditions (**Figures 6-9**), and reagent suppliers (**Figures 10-11**). Since the light sources used in our experiments, SOL 3 (Dermalight, UVATech) and SOL500 (Honle), were different than those presented in TG 495, optimization and extensive evaluations were needed to determine the appropriate exposure conditions. The final irradiation exposure conditions, conducted in quartz box, were determined for the SOL3 equipped with H2 filter to allow UVB, UVA, visible light exposure with UVA intensity of 4.25 ± 0.1 mW/cm^2 for 80 minutes, creating a total irradiation of ~20.4 J/cm^2. Potential supplier impacts of mixture reagents, DMSO and imidazole, were also evaluated for a subset of compounds. After exposure conditions and selected reagents were established, 17 proficiency compounds were evaluated (**Table 1**).

Figure 1: Outline of ROS Photoreactivity Assay procedure as described in OECD Test Guideline 495

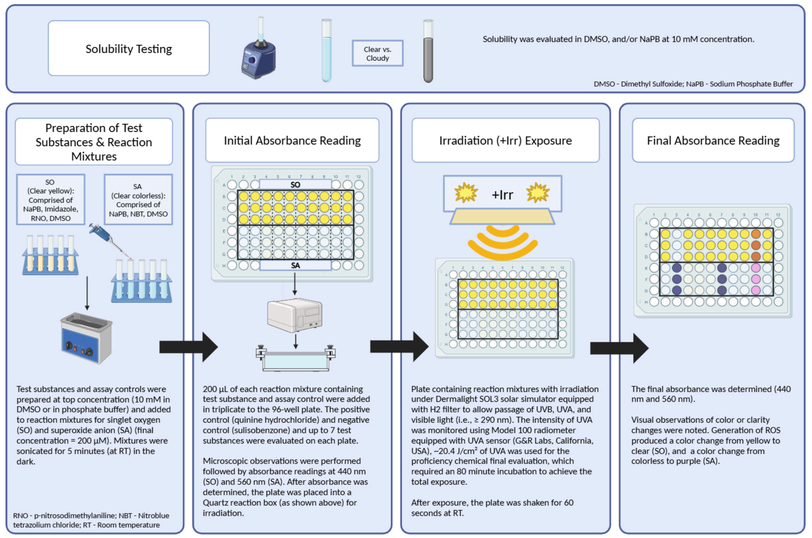


Figure 2: Equations used to calculate singlet oxygen (SO) and superoxide anion (SA) values

$SO = [OD_{440}\,(initial) - OD_{440}\,(final) - (mean\ blank\ initial - mean\ blank\ final)] \times 1000$

$SA = [OD_{560}\,(final) - OD_{560}\,(initial) - (mean\ blank\ final - mean\ blank\ initial)] \times 1000$

Figure 3: Prediction model for photoreactivity potential as described in OECD TG 495 (2019)

Judgement	Concentration	SO (mean of 3 wells)		SA (mean of 3 wells)
Photoreactive	200 µM	≥25	and/or	≥70
		<25 and/or interference	and/or	≥70
		≥25	and/or	<70 and/or interference
Weakly Photoreactive	200 µM	<25	and	≥20, <70
Photoreactive	20 µM	≥25	and/or	≥20
Non-Photoreactive	200 µM	<25	and	<20
Inconclusive	The results do not meet any of the above-mentioned criteria			

Note: Interference above indicates precipitation or coloration was noted

Results

Impact of Light Source

Quinine hydrochloride (positive control), sulisobenzone (negative control), and four proficiency chemicals evaluated at UVA intensity of 5.25 mW/cm^2 for 80 minutes (total UVA of 25.2 J/cm^2) with plate lid using the SOL 3 and SOL 500 solar simulators. The SOL 3 simulator was equipped with H2 filter, allowing passage of UVB, UVA, and visible light (> 290 nm), while the SOL 500 simulator was equipped with H1 filter, allowing passage of UVA and visible light (> 320 nm). Calculated SO and SA values (see Figure 2), presented graphically comparing values obtained from the SOL 3 (green bars) and SOL500 (orange bars).

Figure 4

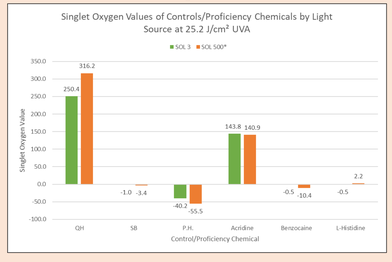
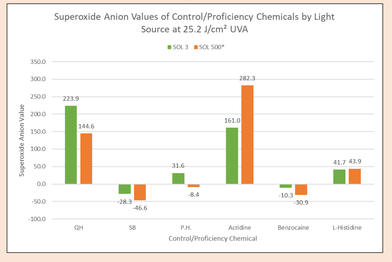


Figure 5



Legend: QH: Quinine hydrochloride, SB: Sulisobenzone, P.H.: Promethazine hydrochloride

Note: Black precipitate was observed in some wells after light exposure when using the SOL500

Impact of Irradiance Exposure Conditions

Quinine hydrochloride (positive control) and sulisobenzone (negative control) evaluated using the SOL3 equipped with H2 filter and UVA intensities of 1.7 – 7.0 mW/cm^2 at varying exposure times, resulting in total UVA irradiation of 11.2 - 25.2 J/cm^2. Identical plates containing reaction mixtures were tested concurrently with the polystyrene plate lid on (green bars), plate lid off (blue bars), and housed in a quartz reaction box (yellow bars) and results shown graphically with the lowest acceptable (solid horizontal line) and highest acceptable (dotted horizontal line) SO and SA values, as presented in TG 495.

Figure 6

Figure 7

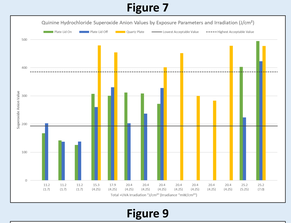


Figure 8

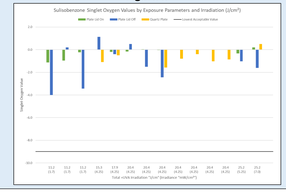
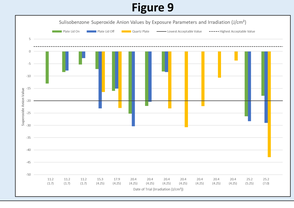


Figure 9



Impact of Reagent Supplier

Reagent mixture reagents, DMSO and imidazole, from suppliers Fujifilm-Wako (Japan) and Sigma-Aldrich (St. Louis, USA) were used to create reaction mixtures containing controls and subset of proficiency chemicals. Both supplier reagents were tested on the same dates using the SOL3 equipped with H2 filter with plate in quartz box. The experiments with DMSO were evaluated using 17.9 and 25.2 J/cm^2 of UVA, and the experiments with imidazole using 20.4 J/cm^2 of UVA. The SO values for each chemical with DMSO from Sigma (green bars) or Fujifilm (blue bars), and the SA values from Sigma (orange bars) and Fujifilm (yellow bars) presented graphically.

Figure 10

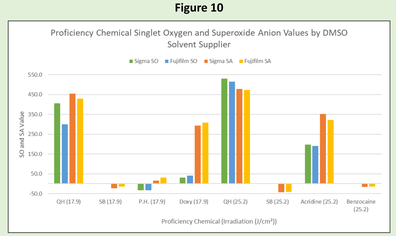
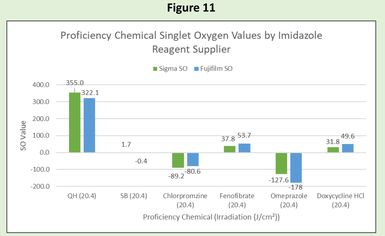


Figure 11



Legend: QH: Quinine hydrochloride, SB: Sulisobenzone, Doxy: Doxycycline hydrochloride

Assay Transfer Summary Results

After the optimization process, 17 proficiency chemicals and controls, were evaluated using the SOL3 solar simulator with H2 filter and plate held in quartz box for irradiation exposure with UVA intensity of 4.25 mW/cm^2 for 80 minutes (total UVA of 20.4 J/cm^2) with Sigma-Aldrich DMSO and Fujifilm-Wako Imidazole. The SO and SA values (representing mean of triplicate wells), as well as the prediction based off TG 495 is presented in **Table 1** below, with acceptable SO and SA ranges, according to TG 495 presented in **Table 2**.

Table 1: Proficiency Chemicals Summary Results

Proficiency Chemical	SO Value	SA Value	Prediction
Quinine hydrochloride	358	283	Photoreactive
Sulisobenzone	-1.03	-10.7	Non-Photoreactive
p -Aminobenzoic acid	0.467	-8.80	Non-Photoreactive
Benzocaine	0.033	2.67	Non-Photoreactive
Doxycycline hydrochloride	49.6*	325	Photoreactive
Erythromycin	-4.47	18.4	Non-Photoreactive
Fenofibrate	53.7*	0.03	Photoreactive*
L-Histidine	0.300	55.0	Weakly Photoreactive
Norfloxacin	76.8*	124	Photoreactive
8-Methoxy psoralen	34.9	97.8	Photoreactive
Octyl salicylate	2.47	12.5	Inconclusive
Acridine	161*	228	Photoreactive
Chlorpromazine hydrochloride	-80.6*	67.6	Weakly Photoreactive
Diclofenac	205	258	Photoreactive
Furosemide	75.0	74.1	Photoreactive
Ketoprofen	162	110	Photoreactive
Nalidixic acid	129	449	Photoreactive
Omeprazole	-178	158	Photoreactive^
Promethazine hydrochloride	-17.4*	34.8	Weakly Photoreactive

In accordance with Table 2, green indicates a value inside of the acceptable range; red indicates a value outside of the acceptable range;* see Table 2 for acceptable range; + only the SO value was used for the prediction; ^ only the SA value was used for the prediction

Table 2: Acceptable range of proficiency chemicals (OECD TG 495)

Proficiency Chemical	SO Acceptable Range	SA Acceptable Range
Quinine hydrochloride (QH)	319 to 583	193 to 385
Sulisobenzone (SB)	-9 to 11	-20 to 2
p -Aminobenzoic acid	-8 to 12	-11 to 7
Benzocaine	-7 to 9	-7 to 17
Doxycycline hydrochloride (Doxy)	115 to 429	230 to 468
Erythromycin	-15 to 11	-9 to 21
Fenofibrate	77 to 203	-31 to 11
L-Histidine	-8 to 12	8 to 120
Norfloxacin	131 to 271	57 to 161
8-Methoxy psoralen	31 to 137	0 to 126
Octyl salicylate	-5 to 11	-8 to 20
Acridine	182 to 328	121 to 243
Chlorpromazine hydrochloride	-56 to 70	66 to 106
Diclofenac	34 to 416	47 to 437
Furosemide	31 to 225	-7 to 109
Ketoprofen	120 to 346	77 to 151
Nalidixic acid	54 to 246	88 to 470
Omeprazole	-221 to 103	30 to 216
Promethazine hydrochloride (P.H.)	20 to 168	-3 to 77

Conclusions

The results of the experiments showed that several variables require careful investigation and optimization during transfer of a validated test method. The irradiation exposure conditions had the greatest potential impacts to experimental results. The solar simulators and irradiation conditions used in our experiments (SOL3 and SOL500) differed from those presented in OECD TG 495 (Suntest CPS+ or CPS (Atlas) (6.5-7.9 J/cm^2 UVA with filter allowing light >290 nm) or SXL-2500V2 (Seric) (11-18 J/cm^2 UVA with filter allowing light >300 nm), and required several optimizations to produce SO and SA values within ranges presented in TG 495. The quartz reaction box was necessary as higher experimental values were produced in a shorter irradiation time period, resulting in SO values, in particular, falling within the expected ranges.

Vendor specific reagents used in this *in chemico* test system, imidazole and DMSO, did not impact results, as the SO and SA values used in respective experiments showed minimal, if any differences.

Six proficiency chemicals, docycycline hydrochloride, fenofibrate, norfloxacin, acridine, chlorpromazine hydrochloride, and promethazine hydrochloride produced SO values (**Table 1**) that were not within the ranges specified within TG 495 (**Table 2**). The SO and SA ranges presented in the TG 495 were determined using two different solar simulators than our experiments, which could explain the differences. Further, some of the SO and SA ranges presented in TG 495 cover different prediction potential, e.g., chlorpromazine hydrochloride SO range of -56 to 70 falls into all three categories of photoreactive (≥25) and weakly or non-photoreactive (<25). Although not within the range, the use of SO or SA value, independently, allowed for the correct predictions for photoreactivity, and on the other hand, a non-photoreactive prediction requires both SO and SA values to fall under the thresholds (See also **Figure 3**).

One proficiency chemical, octyl salicylate, produced SO and SA values in the ranges presented in TG 495, however, the prediction was inconclusive because the maximum stock concentration evaluated was lowered from 200 µM to 20 µM due to solubility, as recommended in TG 495. The only conclusive prediction allowed at 20 µM concentration is photoreactive if SO and SA values are ≥25 and/or ≥20, respectively (**Figure 3**).

In our transfer of the ROS assay for photoreactivity, after establishing appropriate exposure conditions with experimental controls and small subset of reference chemicals, proficiency in the test method and the successful transfer was supported by appropriate results of the proficiency chemicals as presented in **Table 1** in comparison to the expected results presented in OECD TG 495 (**Table 2**).

References

OECD (2019), Test No. 495; Reactive Oxygen Species (ROS) Assay for Photoreactivity, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/915e00ac-en.

Food and Drug Administration, HHS. International Conference on Harmonisation; S10 Photosafety Evaluation of Pharmaceuticals; guidance for industry; availability. Notice. Fed Regist. 2015;80(17):4282-4283.

Onoue, S., Igarashi, N., Yamada, S. and Tsuda, Y. (2008). High-throughput reactive oxygen species (ROS) assay: an enabling technology for screening the phototoxic potential of pharmaceutical substances. J Pharm Biomed Anal, 46, 187-93.

Acknowledgements

IIVS would like to recognize Georgia Price, Megan Madrid, and the IIVS Lab Operations Team for their contributions in helping generate data for this poster.