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Original Article

Development of a modified 3T3 Neutral Red Uptake Phototoxicity Test protocol for evaluation of poorly water-soluble substances

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ABSTRACT — The 3T3 neutral red uptake phototoxicity test (OECD TG432) is an alternative phototoxicity test method that is relatively easy and rapid to implement, with results obtainable in a short time, and is reported to have high reproducibility compared with *in vivo* assay methods. However, this method has been shown to be unsuitable for testing poorly water-soluble substances, which tend to separate out when mixed with the assay buffer solution. This causes difficulties in determining the dose dependency of substances and subsequent determination of the photoirritation factor because the ratio of cell viability, expressed as the half-maximal inhibitory concentration (IC_{50}) in the presence or absence of light, is not calculable. In this study, we investigated the optimum conditions for the evaluation of poorly water-soluble substances. In the conventional method, the final solvent concentration was 1% and the pre-incubation time was 60 min, but in the modified method, 10% and 5 min were used, respectively. Next, the results from the conventional method were compared with those of our modified method, which was found to be viable and comparable with the conventional method. Moreover, the false positive results frequently obtained with poorly water-soluble substances in the conventional method were not evident with the modified method, thus confirming its usefulness for the evaluation of such substances. We therefore propose that the modified method can be used for the *in vitro* testing of poorly water-soluble substances in phototoxicity evaluations.

Key words: 3T3 NRU PT, Phototoxicity, Photoirritation, Poorly water-soluble

INTRODUCTION

An important safety evaluation of the ingredients to be incorporated into cosmetics is photosafety screening. Phototoxicity is a chemically-induced toxic reaction that requires light to trigger the excitation of chemical substances. Chemical substances highly reactive to light are irradiated with ultraviolet (UV) A and B (320-400 nm and 290-320 nm, respectively) and visible (VIS) (400-700 nm) wavelengths to produce reactive oxygen species (ROS), causing photoirritation and photoallergies (Kong and Davison, 1980; Schafer and Buettner, 1999; Onoue *et al*., 2017).

Several *in vitro* photosafety test methods have recently been developed (Kim *et al*., 2015), and a detailed framework and guidance on photosafety evaluation of pharmaceuticals are described in the International Council on Harmonization (ICH) S10 guidelines (ICH, 2014). Furthermore, the 3T3 neutral red uptake phototoxicity test (3T3 NRU PT) (OECD, 2004), according to OECD TG 432 (Organization for Economic Cooperation and Development Test Guideline), has been developed as an *in vitro* method for photoirritant evaluation. 3T3 NRU PT is an alternative phototoxicity test method in a cell monolayer culture system that uses mouse fibroblast (3T3) cells, and has been well-established for some time as a convenient and highly sensitive method. Following the incubation of cells with test substances, cells are exposed to UV irradiation for a specific period of time, after which 50% cell viability (IC_{50}) is determined to ascertain the presence

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or absence of phototoxicity compared with nonirradiated cells (Spielmann *et al*., 1994b). The photo irritation factor (PIF) is calculated as the IC_{50} ratio of non-irradiated cells over irradiated cells, and the mean photo effect (MPE) is based on the comparison of the complete concentration response curve and is defined as the weighted average across a representative set of photo effect values. To determine whether a test substance is phototoxic using the 3T3 NRU PT method, PIF and MPE are calculated.

Among the ingredients used in cosmetics, many antibiotics, antibacterial substances, pigments, and natural substances such as perfumes and essential oils are reactive to light (Okamoto, 2001) and many are also characterized by poor water solubility. To accurately perform 3T3 NRU PT testing, the solubility of the test substance is an important factor, but cosmetic ingredients including, for example, an ultraviolet absorbent and a perfume, should be tested under 3T3 NRU PT test conditions using buffers, although precipitation and oil droplets may be observed upon preparation of the test substance. Therefore, the IC_{50} cannot be accurately obtained in certain circumstances due to a decreased amount of UV irradiation and its physical action on the cell. Furthermore, PIF cannot be evaluated unless the IC_{50} can be calculated.

Therefore, in this study, we sought to improve the evaluation accuracy of PIF in 3T3 NRU PT testing by examining the solvents and assay conditions to determine the optimum conditions for appropriately evaluating substances with poor water-solubility. Thirty test substances, including UV absorbents and perfumes, were evaluated under the optimized conditions to verify whether the study objective was achieved.

MATERIALS AND METHODS

Cell culture

Balb/3T3 clone A31 cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Corp., St Louis, MO, USA) supplemented with 10% newborn calf serum (NBCS; Sigma-Aldrich Corp.), 4 mM glutamine, penicillin (100 IU), and streptomycin (100 µg/mL) (Invitrogen Corp., Carlsbad, CA, USA).

Chemicals

Twenty phototoxic chemicals and 10 non-phototoxic chemicals were evaluated, including 6 drugs, 8 antimicrobials, 6 fragrances, 6 UV absorbers, and 4 other chemicals. Amiodarone HCl (1), anthracene (7), chlorpromazine HCl (9), 5-methoxypsoralen (15), 8-methoxypsoralen (16), 6-methylcoumarin (17), ofloxacin (18), hexachlorophene (21), ethylhexyl dimethyl p-aminobenzoate (23), chlorhexidine dihydrochloride (24), sodium lauryl sulfate (27), p-aminobenzoic acid (28), sulisobenzone (29), and lactic acid (30) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Rose bengal (2), bithionol (4), 3,3'4,5 tetrachlorosalicylanilide (5), fenofibrate (6), benzophenone (11), ketoprofen (13), methyl n-methylantranilate (14), and norfloxacin (19) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3,4',5-Tribromosalicylanilide (3), musk ambrette (8), promethazine (10), musk xylene (12), protoporphyrin IX, disodium (20), 2-octyl salicylate (22), 2-octyl methacrylate (25), and penicillin G (26) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

UV irradiation source

A mercury metal-halide lamp (SOL500, Dr. Hönle, Martinsried, Germany), which simulates the spectral distribution of natural sunlight, was used as the source of UV irradiation. The emitted energy was measured prior to each experiment using a calibrated UV-meter (part no. 0037, Dr. Hönle).

Test methods

Conventional method

Conventional testing was performed according to OECD TG 432 and the DB-ALM protocol no. 78 (EURL ECVAM DB-ALM; Spielmann *et al*., 1994a, 1994b). Balb/3T3 clone A31 cells in the logarithmic growth phase were seeded in a 96-well assay plate at 10^4 cells/well and cultured for 24 hr (37°C, 5% CO_2). Next, wells were washed with phosphate buffered saline (PBS) before test substances were added. Each test substance was dissolved in PBS or DMSO, and then diluted in PBS to 1% v/v. For each test substance, one culture plate was prepared for UV irradiation and one for non-UV irradiation, and the highest concentration of each plate was determined from the cytotoxicity of each test substance. After 100 µL of test substance was added to each well, the plates were incubated for 60 min before one plate was irradiated with UVA 1.7 mW/cm^2 for 50 min (5 J/cm^2) while the other plate was maintained at room temperature without irradiation. Following treatment, plates were washed with PBS and fresh medium was added to each well before incubation overnight. Next, the cells were washed with PBS, neutral red (NR) was added, and cells were incubated for 3 hr. Cytotoxicity was measured according to NR absorption at 540 nm using a microplate reader.

Modified method

For selection of solvents applicable to poorly soluble substances, solvent cytotoxicity was evaluated using DMSO, ethanol, acetonitrile, acetone, polyethylene glycol 400 (PEG400), 2-propanol, and mineral oil. The 80% cell survival rate following UVA irradiation (1.7 mW/cm^2 for 5 min [5 J/cm^2]) was determined. Next, the effects of solvent concentration and exposure time were examined to minimize the contact time between the solvent and the cells where high concentrations of solvent are required. After increasing the solvent concentration, the effect of incubation time prior to light exposure was evaluated by analyzing the changes in cell viability in the presence and absence of UV irradiation.

To establish the optimal dissolution conditions, each test substance was dissolved in DMSO, diluted in PBS at 10% v/v, and added to the cells in a 96-well plate (100 µL per well). Furthermore, after administration of the test substance to the cells, the incubation time prior to UV irradiation (pre-incubation time) was shortened to 5 min; otherwise, the assay was performed according to the OECD TG 432 protocol.

Evaluation and interpretation of results

The PIF relates the IC_{50}(UV−) value of the curve in darkness to the IC_{50}(UV+) value in the presence of light using the following formula: [PIF = IC_{50}(UV−)/IC_{50}(UV+)], where PIF ≥ 5 indicates photoirritation, 2 < PIF < 5 indicates likely photoirritation, and PIF ≤ 2 indicates no photoirritation. PIF was calculated using Phototox version 2 software for use with OECD TG432.

Evaluation of reproducibility

Eight chemicals with a logK_{ow} value > 4, as calculated using KOWWIN software ver. 1.68 by EPI suite™ (Environmental Protection Agency, Washington, DC, USA), were considered dissoluble chemicals and were examined for assay reproducibility. Experiments were repeated in triplicate for each chemical, and the use of standard deviation (SD) or coefficient of variation (CV) as the index of variation was decided based on the dispersion tendency of the PIF values. Following calculation of the difference in the selected index for the eight chemicals, the medians of the differences and 95% confidence intervals (CIs) were estimated.

RESULTS

Screening of vehicle and optimization of the modified method

Fig. 1 shows the results of cytotoxicity of the various solvents following UV irradiation, except for mineral oil. The solvent found to have the least cytotoxic effect on cells was DMSO, with 80% cell viability (IC_{80}) at a con-

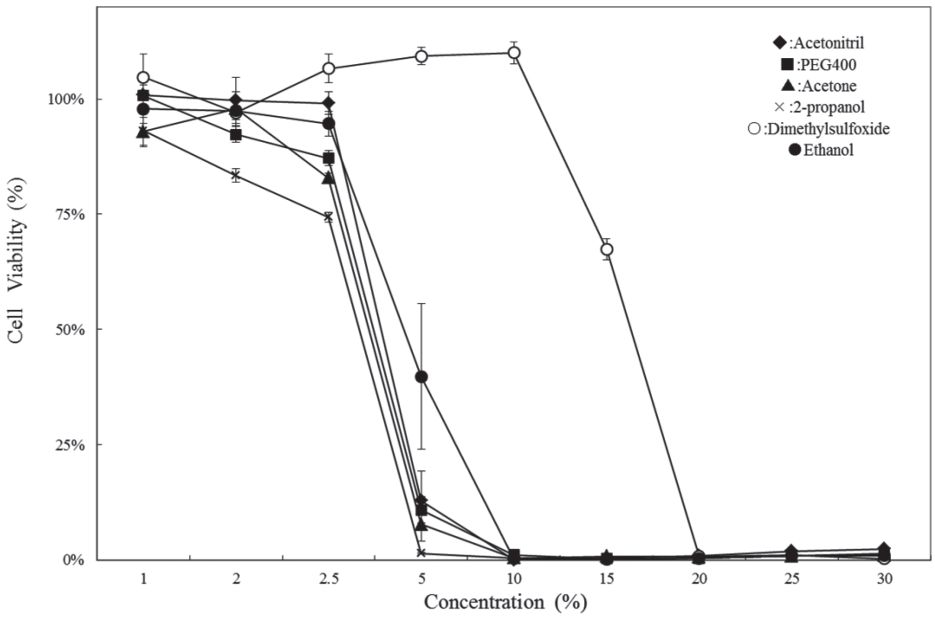

Fig. 1. Changes in cell viability by solvent under UVA irradiation (5 min, 5 J/cm^2)

centration of 13%. The other solvents were highly cytotoxic with IC_{80} values < 5%. However, mineral oil showed no toxicity to cells, even at 100% concentration, but was excluded from the data due to poor testing operability during administration and removal of the test substance.

Figure 2 shows the results of the cell viability assay using DMSO as the solvent at various concentrations and pre-incubation times. Favorable cell viability and operability results were obtained after specific periods of time (5, 30, and 60 min), as described above. In this experiment, cells were pre-incubated with the solvent prior to UV irradiation for 60 min or 30 min, and increased cell toxicity was observed for the UV irradiation plate (UV+) with increasing DMSO concentration. However, where the pre-incubation time was set to 5 min, a 10% DMSO solution had little effect on cells subjected to UV irradiation conditions. Therefore, in the modified assay method, DMSO was used as a solvent at a concentration of 10% v/v in PBS and the preincubation time was set to 5 min.

Evaluation of 30 substances using the conventional and modified methods

Table 1 shows the chemical information and results of 3T3 NRU-PT using the conventional and modified methods. Where cytotoxicity did not occur and the IC_{50} could therefore not be obtained, PIF could not be determined. For the conventional method, PIF could not be calculated for PIF evaluation in the modified method for eight of the substances (musk ambrette; benzophenone; methyl N-methylantranilate; 6-methylcoumarin; protoporphyrin IX, disodium; 2-octyl salicylate; ethylhexyl dimethyl p-aminobenzoate; and 2-octyl methacrylate). A further eight substances (anthracene, ketoprofen, 5-methoxypsoralen, 8-methoxypsoralen, ofloxacin, norfloxacin, p-aminobenzoic acid, and sulisobenzone) could not be evaluated by either the conventional or the modified method. Finally, two substances could not be evaluated using the modified method (3, 4', 5-tribromosalicylanilide and musk xylene).

Using the conventional method, PIF evaluation was therefore possible for 12 out of 30 substances, but with the modified method, 18 substances could be evaluated. In the modified method, the number of substances for which PIF evaluation could be made was more than that in the conventional method. In particular, the confirmed substances were in the phototoxic chemicals group.

Reproducibility

Table 2 shows the results of reproducibility testing of the eight substances with logKow values > 4. Tests were repeated in triplicate for both the conventional and mod-

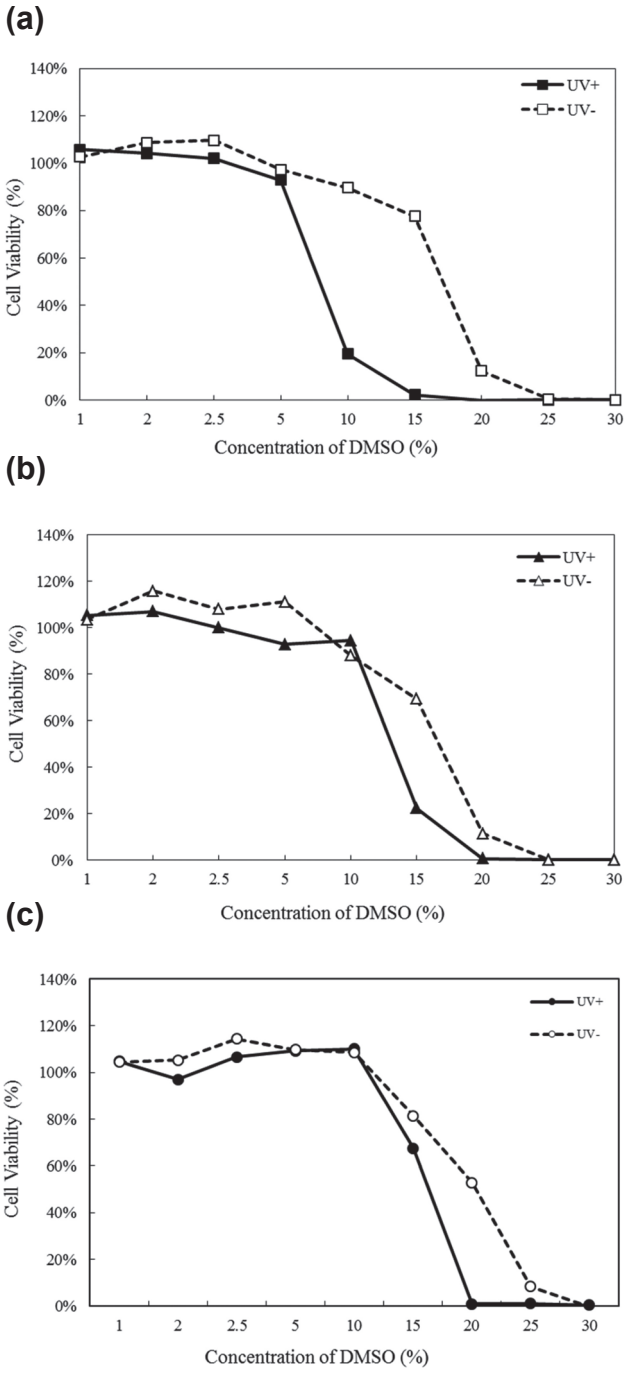

Fig. 2. Changes in cell viability by pre-incubation time. (a) 60 min pre-incubation. (b) 30 min pre-incubation. (c) 5 min pre-incubation. Considering the influence of cytotoxicity, the preincubation time was examined With DMSO as the solvent. After preincubation with each time point, UV+ was performed at 50 min, 5 J/cm^2, and UV- was incubated for 50 min in the dark.

Table 1. 3T3 NRU-PT under conventional and modified methods.

	Category	Chemical	CAS	Purity	$LogK_{ow}$ <KOWWIN by EPI suite>	Conventional Method			Modified Method			Source of Photosafety Information
						IC_{50} (UV+)	IC_{50} (UV-)	PIF	IC_{50} (UV+)	IC_{50} (UV-)	PIF	
Phototoxic Chemicals												
1	Drugs	Amiodarone HCl	19774-82-4	>98%	7.29	3.661	25.63	7.124	2.157	28.34	13.33	Spielmann *et al.* (1998a, 1998b)
2	Other Chemicals	Rose Bengal	632-69-9	-	6.58	0.247	6.420	25.82	0.171	20.31	127.9	Peter and Holzhütter (2002), Spielmann *et al.* (1995, 1998a)
3	Antimicrobials	3,4',5-Tribromosalicylanilide	87-10-5	>98%	5.97	2.021	40.34	19.73	3.786	>100	*N.C.*	
4	Antimicrobials	Bithionol	97-18-7	>98%	5.91	1.132	7.055	6.727	0.701	16.76	24.11	Spielmann *et al.* (1995 and 1998a, 1998b)
5	Antimicrobials	3,3'4',5-Tetrachlorosalicylanilide	1154-59-2	-	5.87	1.347	25.40	18.70	1.541	66.89	43.30	Spielmann *et al.* (1995)
6	Drugs	Fenofibrate	49562-28-9	>98%	5.19	1.111	117.4	108.4	1.026	76.64	76.04	Peter and Holzhütter (2002), Spielmann *et al.* (1998a)
7	Other Chemicals	Anthracene	120-12-7	>99%	4.35	0.024	>100	*N.C.*	0.021	>100	*N.C.*	Spielmann *et al.* (1998a, 1998b)
8	Fragrances	Musk Ambrette	83-66-9	>99%	4.17	3.333	>500	*N.C.*	1.972	18.32	10.53	Peter and Holzhütter (2002), Spielmann *et al.* (1998a, 1998b)
9	Drugs	Chloropromazine HCl	69-09-0	>98%	3.69	0.621	26.00	41.94	0.423	36.90	87.29	Spielmann *et al.* (1998a, 1998b)
10	Drugs	Promethazine HCl	58-33-3	>98%	3.15	0.598	70.19	129.3	0.870	85.79	110.9	Spielmann *et al.* (1995, 1998a)
11	UV absorbers	Benzophenone	119-61-9	>98%	3.15	11.53	>1000	*N.C.*	8.107	134.2	18.11	Sugiura *et al.* (2002)
12	Fragrances	Musk Xylene	81-15-2	>99%	3.09	2.365	302.0	143.1	1.847	>500	*N.C.*	
13	Drugs	Ketoprofen	22071-15-4	>98%	3.00	6.647	>1000	*N.C.*	4.054	>1000	*N.C.*	Spielmann *et al.* (1998a, 1998b)
14	Fragrances	Methyl N-methylanthranilate	85-91-6	>95%	2.81	2.857	>1000	*N.C.*	4.061	348.3	89.46	
15	Fragrances	5-Methoxypsoralen	484-20-8	>99%	2.14	2.542	>1000	*N.C.*	2.209	>1000	*N.C.*	Spielmann *et al.* (1998a)
16	Fragrances	8-Methoxypsoralen	298-81-7	>98%	2.14	12.29	>100	*N.C.*	6.186	>100	*N.C.*	Spielmann *et al.* (1995)
17	Fragrances	6-Methylcoumarin	92-48-8	>99%	2.06	29.14	>500	*N.C.*	15.62	307.7	18.10	Peter and Holzhütter (2002) and Spielmann *et al.* (1998a)
18	Antimicrobials	Ofloxacine	82419-36-1	>99.8%	-0.20	32.29	>500	*N.C.*	15.78	>500	*N.C.*	Spielmann *et al.* (1998a)
19	Antimicrobials	Norfloxacin	70458-96-7	>98%	-0.31	34.36	>100	*N.C.*	16.20	>100	*N.C.*	Peter and Holzhütter (2002), Spielmann *et al.* (1998a)
20	Drugs	Protoporphyrin IX, Disodium	50865-01-5	>95%	-	0.104	>1000	*N.C.*	0.011	20.80	1929	Peter and Holzhütter (2002), Spielmann *et al.* (1998a, 1998b)
Non-phototoxic Chemicals												
21	Antimicrobials	Hexachlorophene	70-30-4	>99%	6.92	3.923	7.216	1.898	3.060	11.14	4.854	Peter and Holzhütter (2002), Spielmann *et al.* (1998a, 1998b)
22	UV Absorbers	2-Octyl Salicylate	118-60-5	>98%	5.97	12.37	>1000	*N.C.*	5.107	9.399	1.931	Peter and Holzhütter (2002), Spielmann *et al.* (1998b)
23	UV Absorbers	Ethylhexyl Dimethyl p-Aminobenzoate	21245-02-3	>98%	5.77	10.20	>500	*N.C.*	5.044	10.19	1.943	
24	Antimicrobials	Chlorhexidine Dihydrochloride	3697-42-5	>98%	4.85	7.213	18.38	2.591	13.97	36.60	2.621	Peter and Holzhütter (2002), Spielmann *et al.* (1998a)
25	UV Absorbers	2-Octyl Methacrylate	688-84-6	>99%	4.64	18.54	>1000	*N.C.*	12.80	17.59	1.377	Peter and Holzhütter (2002), Spielmann *et al.* (1998b)
26	Antimicrobials	Peniciline G	113-98-4	>98%	1.85	>1000	>1000	1*	>1000	>1000	1*	Peter and Holzhütter (2002), Spielmann *et al.* (1998a)
27	Other Chemicals	Sodium Lauryl Sulfate	151-21-3	>99%	1.69	16.37	16.66	1.018	23.56	26.70	1.154	Spielmann *et al.* (1998a, 1998b)
28	UV Absorbers	p-Aminobenzoic Acid	150-13-0	>99%	0.96	913.8	>1000	*N.C.*	697.3	>1000	*N.C.*	Peter and Holzhütter (2002), Spielmann *et al.* (1998a)
29	UV Absorbers	Sulisobenzone	4065-45-6	>97%	0.37	>1000	>1000	1*	669.0	>1000	*N.C.*	Onoue *et al.* (2010), Peter and Holzhütter (2002)
30	Other Chemicals	Lactic Acid	50-21-5	>98%	-0.65	>1000	>1000	1*	>1000	>1000	1*	

N.C.: not calculated. Achieving IC_{50} is a prerequisite for PIF evaluation in both light irradiation and non-light irradiation. If one or the other does not reach IC_{50}, phototoxicity is not determinable.
*: If neither IC_{50} (UV-) nor IC_{50} (UV+) can be calculated, because a chemical exhibits no cytotoxicity up to the highest test concentration, this indicates no phototoxicity.
In such cases, a formal "PIF=1*" is used to characterize the result.

Table 2. Reproducibility of water-insoluble substances ($LogK_{ow} > 4$).

		Conventional Method			Modified Method		
		IC_{50}(UV+)	IC_{50}(UV-)	PIF	IC_{50}(UV+)	IC_{50}(UV-)	PIF
Amiodarone HCI	Average	3.661	25.63	7.124	2.157	28.34	13.33
	SD	0.728	2.684	0.852	0.691	8.613	1.601
	CV	20%	10%	12%	32%	30%	12%
Rose Bengal	Average	0.247	6.420	25.82	0.171	20.31	127.9
	SD	0.063	2.526	4.587	0.030	6.282	63.33
	CV	26%	39%	18%	18%	31%	50%
3,4',5-Tribromosalicylanilide	Average	2.021	40.34	19.73	3.786	>100	N.C.
	SD	0.388	11.36	2.185	1.756	N.C.	N.C.
	CV	19%	28%	11%	46%	N.C.	N.C.
Bitionol	Average	1.132	7.055	6.727	0.701	16.76	24.11
	SD	0.489	1.112	1.635	0.070	0.668	2.258
	CV	43%	16%	24%	10%	4%	9%
3,3',4',5-Tetrachlorosalicylanilide	Average	1.347	25.40	18.70	1.541	66.89	43.30
	SD	0.249	10.58	7.02	0.350	16.671	3.276
	CV	19%	42%	38%	23%	25%	8%
Fenofibrate	Average	1.111	117.4	108.4	1.026	76.64	76.04
	SD	0.249	47.01	40.68	0.253	16.29	11.72
	CV	22%	40%	38%	25%	21%	15%
Anthracene	Average	0.024	>100	N.C.	0.021	>100	N.C.
	SD	0.009	N.C.	N.C.	0.005	N.C.	N.C.
	CV	36%	N.C.	N.C.	25%	N.C.	N.C.
Musk Ambrette	Average	3.333	>500	N.C.	1.972	18.32	10.53
	SD	0.712	N.C.	N.C.	0.504	2.604	1.346
	CV	21%	N.C.	N.C.	26%	14%	13%

N.C.: not calculated. Achieving IC_{50} is a prerequisite for PIF evaluation in both light irradiation and non-light irradiation. If one or the other does not reach IC_{50}, phototoxicity is not determinable.

ified methods. The SD and CV for each result showed an apparent tendency toward a greater variance with an increasing PIF and, therefore, the CV was used as the index of variation. The estimated median (95% CI) for the PIF values was 18.47 [range -8.50-26.50], based on the differences between the CV and SD values of the conventional and modified methods, respectively. Based on this result, the CI for both the CV and SD included the null value and, therefore, it could be concluded that the two methods had the same level of reproducibility.

DISCUSSION

3T3 NRU PT *in vitro* testing is convenient and reproducible for evaluating phototoxicity and is therefore widely used. Given that false negatives are particularly infrequent when using this method, it can be considered suitable for the evaluation of phototoxicity. One application of 3T3 NRU PT testing is the evaluation of poorly water-soluble substances, for which an alternative method, a 3-dimensional cultured skin model, has also been proposed (Ceridono *et al*., 2012; Portes *et al*., 2002; Netzaff *et al*., 2005). Since 3T3 NRU PT can characteristically identify phototoxicological hazards with almost 100% specificity, it can be considered highly economical and easier to use than the 3-dimensional cultured skin model and was therefore used in this study to evaluate poorly water-soluble substances.

When conducting 3T3 NRU PT testing, concerns about variations in IC_{50} values obtained for poorly water-soluble substances can arise. In addition to the dissolution challenges in evaluating these substances, cytotoxicity may vary due to the UV irradiation in the test state. Therefore, to reduce variations in IC_{50} values following UV irradiation, it is necessary to administer poorly water-soluble substances to cells in a dissolved state. We therefore evaluated the optimal dissolution conditions for poorly water-soluble substances. Among the solvents studied, DMSO was selected for further experiments as it exerted the lowest toxicity in cells under the prescribed UV irradiation conditions. Next, the effect of incubation time (preincubation) prior to UV irradiation after administration of the

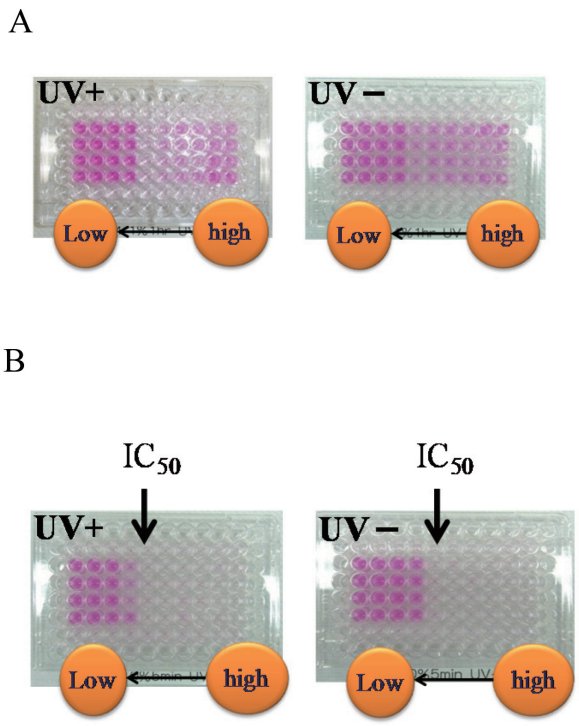

Fig. 3. Cytotoxicity of 2-octyl salicylate after addition of Neutral Red. The plates represent the results of 2-octyl salicylate, a non-phototoxic substance, and indicate a false positive result by the conventional method (upper) and a negative result by the modified method (lower). [A] Conventional Method (Final concentration of 1% DMSO) Test substances in high doses form oil droplets, making the concentration non-uniform. Therefore, it is not possible to verify cell viability in a dose-dependent evaluation. [B] Modified Method (Final concentration of 10% DMSO). This method allows for poorly water-soluble substances to be dissolved and administered to cells. The results demonstrated cell viability in dose-dependent testing, and allowed IC_{50} to be determined.

test substance was examined. As the final concentration of solvent added to the cells increased, the cytotoxicity tended to increase accordingly, but it was not significantly affected by setting the preincubation time to 5 min. Therefore, in order to minimize the influence of solvent on the cells, the final concentration of DMSO was set to 10% v/v (in PBS) and the incubation time to 5 min. We subsequently adopted these conditions for the modified test method. However, we believe that compliance with the preincubation time is a key factor for the maintenance of reproducibility. When DMSO was used as a solvent, cytotoxicity tended to be lower over a prolonged period of time. Therefore, in order to ensure good reproducibility, it was considered necessary to adhere to a preincubation time of 5 min.

Next, a panel of 30 phototoxic/non-phototoxic substances was evaluated by 3T3 NRU PT assay. Using the conventional method, M ambrette, benzophenone, methyl N-methylantranilate, 6-methyl coumarin, protoporphyrin IX disodium, 2-octyl salicylate, ethylhexyl dimethyl p-aminobenzoate, and 2-octyl methacrylate were diluted in PBS (1:100) and added to the cells, whereby precipitation or oil droplet dispersion was observed, a characteristic of substances classified as having logKow > 4. These substances therefore could not be used to evaluate dose-dependent cytotoxicity. Of note, 2-octyl salicylate, a non-phototoxic substance, was observed to disperse oil droplets particularly at high concentrations when mixed with a buffer solution or added to cells under the conventional assay method. Fig. 3a shows the cytotoxicity results for 2-octyl salicylate. After cytotoxicity was confirmed by NR staining following UV irradiation, IC_{50} values could be calculated for the UV irradiated plate but little cytotoxicity was observed for the UV nonirradiated plate. However, given that a lower than 50% survival rate was not obtained, the IC_{50} value could not be determined and PIF evaluation could not be performed. However, the oil droplet dispersion observed in the conventional method could not confirmed visually or by microscopy in the modified method. As a result, cells in both the UV irradiated and UV nonirradiated plates showed dose-dependent changes in cell viability, with clear IC_{50} values allowing PIF determination to be possible. 2-octyl salicylate was evaluated as PIF = 1.931 (nonphototoxic), and this was in agreement with previous *in vivo* results (Spielmann *et al*., 1998b; Peters and Holzhütter, 2002). Ethylhexyl dimethyl p-aminobenzoate and 2-octyl methacrylate also showed similar results.

For the phototoxic substance musk ambrette, the IC_{50} (UV−) was ≥ 500 µg/mL using the conventional method, whereas the modified method showed a 20-fold dissolution rate of IC_{50} (UV−) of 18.32 µg/mL. Protoporphyrin IX disodium also showed a 50-fold dissociation rate of IC_{50} (UV−) of 100 µg/mL using the conventional method and IC_{50} (UV−) of 20.80 µg/mL in the modified method. As described previously, the solubility of poorly water-soluble substances was a limitation and there was a possibility that an erroneous IC_{50} was calculated under the UV− conditions, but this was not the case in the modified method.

Next, for the two substances (3,4', 5-tribromosalicylanilide and musk xylene) that could not be evaluated by

the modified method, precipitation was not observed with either the conventional or the modified method. These are different from the case where the non-homogeneity of the dissolution state was affected and an accurate IC_{50} could not be calculated. One of the reasons that the two substances could not be detected is thought to be the weakening of cytotoxicity of UV (−) due to the short preincubation time.

For DMSO and other organic solvents, a final concentration of approximately 1% v/v (but no more than 10% v/v) is recommended for administration to cells (Spielmann *et al*., 1998a) to avoid solvent-induced toxicity. Therefore, with a poorly water-soluble substance, improved dissolution could be obtained using 10% v/v DMSO, and in the case of 2-octyl salicylate, it therefore became possible to correctly evaluate false positive results.

Generally, 3T3 NRU-PT is considered a method unlikely to produce false negative results (European Medicine Agency, 2011). However, for substances with extremely poor solubility, care must be taken to avoid negative interpretation of results, which may be falsely negative in some cases. Here, we showed that dissolution was improved in the modified method compared with the conventional method, and that the substance precipitated at a high concentration was in contact with the cell in a solubilized state. Therefore, we consider that the modified method can be applied even to substances with LogKow> 4.

Next, the reproducibility of the conventional method and the modified method was verified with a poorly water-soluble substance having logKow > 4. The difference between the CVs of both the methods was calculated and a 95% CI in PIF evaluation was obtained. In the PIF evaluation, the 95% confidence interval of the median in the CV difference is [−8.5, 26.5], and since 0 is included in between and the lower limit is −8.5, the modified method is comparable with the conventional method. The possibility that CV is inferior by 10% is considered low. The estimated median had a positive value of 18.47, indicating that the CV of the conventional method tended to be larger than the CV of the modified method. From these results, it was shown that the conventional method and the modified method retained the same degree of reproducibility.

Our findings demonstrate that the modified method described here allows for the evaluation of not only highly water-soluble but also water-soluble substances with good repeatability, as with the conventional method. In addition, use of the modified method increased PIF evaluability, and could be used in particular to ensure adequate dissolution conditions when administering poorly water-soluble substances to cells, especially substances showing false positives *in vivo*, and a similar *in vivo* result was obtained. As many water-insoluble substances are used as cosmetic ingredients and in drug development, the possibility of further improving this modified method to evaluate phototoxicity for substances that are difficult to evaluate using the conventional method should be considered. Therefore, this method can be considered an improvement on the 3T3 NRU PT assay method.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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