Toxicol. Res.
Vol. 33, No. 1, pp. 43-48 (2017)
Open Access https://doi.org/10.5487/TR.2017.33.1.043
pISSN: 1976-8257 eISSN: 2234-2753 **Original Article**

Toxicological Research
Official Journal of
Korean Society of Toxicology
Available Online at http://www.ToxicolRes.org

Phototoxicity Evaluation of Pharmaceutical Substances with a Reactive Oxygen Species Assay Using Ultraviolet A

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(Received November 10, 2016; Revised November 28, 2016; Accepted December 2, 2016)

With ultraviolet and visible light exposure, some pharmaceutical substances applied systemically or topically may cause phototoxic skin irritation. The major factor in phototoxicity is the generation of reactive oxygen species (ROS) such as singlet oxygen and superoxide anion that cause oxidative damage to DNA, lipids and proteins. Thus, measuring the generation of ROS can predict the phototoxic potential of a given substance indirectly. For this reason, a standard ROS assay (ROS assay) was developed and validated and provides an alternative method for phototoxicity evaluation. However, negative substances are over-predicted by the assay. Except for ultraviolet A (UVA), other UV ranges are not a major factor in causing phototoxicity and may lead to incorrect labeling of some non-phototoxic substances as being phototoxic in the ROS assay when using a solar simulator. A UVA stimulator is also widely used to evaluate phototoxicity in various test substances. Consequently, we identified the applicability of a UVA simulator to the ROS assay for photoreactivity. In this study, we tested 60 pharmaceutical substances including 50 phototoxins and 10 non-phototoxins to predict their phototoxic potential via the ROS assay with a UVA simulator. Following the ROS protocol, all test substances were dissolved in dimethyl sulfoxide or sodium phosphate buffer. The final concentration of the test solutions in the reaction mixture was 20 to 200 μM. The exposure was with 2.0~2.2 mW/cm^2 irradiance and optimization for a relevant dose of UVA was performed. The generation of ROS was compared before and after UVA exposure and was measured by a microplate spectrophotometer. Sensitivity and specificity values were 85.7% and 100.0% respectively, and the accuracy was 88.1%. From this analysis, the ROS assay with a UVA simulator is suitable for testing the photoreactivity and estimating the phototoxic potential of various test pharmaceutical substances.

***Key words*:** Alternative testing method, Photosafety screening, Phototoxicity, Photoreactivity, Reactive oxygen species, Ultraviolet A

INTRODUCTION

Phototoxicity is an acute light-induced skin irritation when photoreactive chemicals are topically or systemically applied (1). Phototoxicity begins when photoreactive chemicals are excited by absorption of ultraviolet and visible light (UV/VIS). The excited chemicals can then transfer the absorbed energy and generate reactive oxygen species (ROS). The increased ROS levels provoke cytotoxicity through damage of DNA, lipids and proteins by oxidative stress (2,3). Several types of drugs, such as antibiotics, anticonvulsants, antimalarials, antipsychotics, thiazide diuretics, non-steroidal anti-inflammatory drugs and others, have phototoxic potential and can cause notable phototoxic reactions such as sunburn and hyperpigmentation (4-6). Because of drug-induced phototoxicity, regulatory agencies, US FDA, EU EMA and ICH, provide photosafety guidances, introducing test methods and evaluation strategies (1,7,8). Following the ICH guidance S10, few non-animal testing methods for phototoxicity are recommended and these include measure-

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ment of the molar extinction coefficient (MEC), a standard ROS assay, a 3T3 neutral red uptake phototoxicity assay and a reconstructed human skin model assay.

The ROS assay was developed to screen photoreactivity of drugs through generation of the superoxide anion (type 1 reaction) and singlet oxygen (type 2 reaction) and their generation is an early stage chemical reaction as part of the phototoxicity mechanism (6). The ROS assay protocol was established and the validation studies were conducted under the direction of the Japanese Center for the Validation of Alternative Methods (JaCVAM) (9-11). For the assay, high sensitivity and reproducibility were demonstrated and as part of the test, two solar simulators Suntest CPS series and SXL-2500V2 were evaluated. This assay, however, has low specificity, showing high false positive results. For this reason, we only focused on the effect of UVA (315~400 nm), which is much important than other ultraviolet in phototoxicity. In addition, a UVA simulator is commonly used for phototoxicity evaluations. In this study, we evaluated the performance of the ROS assay with UVA (UVA ROS assay) instead of sunlight (290~700 nm) using 50 phototoxins and 10 non-phototoxins.

MATERIALS AND METHODS

Chemicals and materials. Sixty test substances, including reference chemicals and phototoxic/non-phototoxic drugs, were selected for evaluation from the ROS assay protocol, a validation report of the ROS assay, package inserts and previous studies (6,9-16). 4-Aminobenzoic acid, 6-methylcoumarin, 8-methoxy psoralen, acridine, amiodarone, amlodipine, amoxapine, aspirin, atorvastatin, benzocaine, bezafibrate, bithionol, chlorothiazide, chlorpromazine HCl, ciprofloxacin, dapsone, demeclocycline, diclofenac, doxycycline, erythromycin, fenofibrate, flutamide, fluvastatin, furosemide, gliclazide, griseofulvin, hydrochlorothiazide, ibuprofen, ketoprofen, levofloxacin, losartan, lovastatin, methotrexate, nalidixic acid, naproxen, nifedipine, nitrofurantoin, norfloxacin, octyl salicylate, ofloxacin, omeprazole, oxytetracycline HCl, penicillin G, perphenazine, phenytoin, piroxicam, promethazine HCl, quinidine, quinine HCl, tetracycline, tiaprofenic acid, dimethyl sulfoxide (DMSO), sodium phosphate monobasic, sodium phosphate dibasic, p-nitroso-dimethylaniline (RNO), imidazole and nitroblue tetrazolium chloride (NBT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enoxacin, lomefloxacin, meloxicam, mequitazine, nitrendipine, pitavastatin and rosiglitazone were obtained from Santa Cruz (Dallas, TX, USA). L-Histidine and sulisobenzone were purchased from Tokyo Chemical Industry (Tokyo, Japan). Quartz reaction containers were obtained from Ozawa Science (Aichi, Japan). Spectrophotometer cuvettes were purchased from Eppendorf (Hamburg, Germany). The 96-well plates (clear, flat-bottom, without lid) were obtained Corning (Corning, NY, USA). Following the ROS assay protocol, 20 mM sodium phosphate buffer (NaPB, pH 7.4), 0.2 mM p-Nitrosodimethylaniline (RNO), 20 mM imidazole and 0.4 mM nitroblue tetrazolium chloride (NBT) were prepared (10). All test substances and reagents were protected from light.

UV spectral analysis. UV spectral analysis was conducted as described in a previous study (6). The test substances were dissolved in 20 mM sodium phosphate buffer (NaPB, pH 7.4) at 20 μM (final concentration). The UV/VIS absorption spectra were analyzed with a microplate spectrophotometer (Mecasys, Daejeon, Korea), and a spectrophotometer cuvette with a 10-mm pathlength was used. The MEC values were calculated using the highest absorption peaks from 290 to 700 nm.

Conditions of irradiation. A UVA simulator equipped with 40 W lamps was used (Vilbert-Lourmat, Marne-la-vallee, France). The UVA irradiation test was conducted at 25°C with 2.0 mW/cm^2 irradiance measured by a UVA detector (UVP, Cambridge, UK).

Reactive oxygen species (ROS) assay. ROS assay was performed as previously described in Onoue et al. (9,11) and the ROS assay protocol (10). Stock solutions of all test substances were prepared at 10 mM in DMSO or 20 mM sodium phosphate buffer (NaPB, pH 7.4) and used within the same day for accurate data. To detect the generation of singlet oxygen and superoxide anion, all prepared test substance stock solutions were mixed in reagents, containing 400 μM NBT in 20 mM NaPB (pH 7.4) for singlet oxygen (SO) and 200 μM RNO and 50 μM imidazole in 20 mM NaPB (pH 7.4) for superoxide anion (SA) detection, and the final concentration of test substances in the reaction mixture was at 200 μM. When precipitation was observed using a microscope (×100 magnification) in the reaction mixture, appropriate final concentrations (20, 50, 100 μM) were used. Two hundred microliters of each reaction mixture were put into three wells of a 96-well plate. Before UVA exposure, absorbance was measured at 440 nm for SO and 560 nm for SA by a microplate spectrophotometer and then a quartz reaction container was installed in the plate. The plate was irradiated with a UVA simulator. After irradiation, the absorbance at 440 nm and 560 nm for the plate was measured. Following the ROS assay protocol, ROS generation of SO and SA was calculated by mean absorbance before and after irradiation.

Data judgment. Photoreactivity of the test substances was judged according to the following criteria (10,11). A test substance was classified as a photoreactive substance when an SO value 25 or more and/or an SA value 20 or more was measured; in turn, it was judged to be a non-photoreactive substance when values of less than 25 for SO and

less than 20 for SA were recorded.

RESULTS

Optimization of the irradiance dose. To use a UVA simulator, we performed a preliminary study in order to find the appropriate UVA dose with the reference chemicals listed in the ROS assay protocol (10). We exposed at 2.0 mW/cm^2 irradiance, which is in the irradiance range of Atlas Suntest CPS/CPS+ in the ROS assay (10). We irradiated at up to 18 J/cm^2 at intervals of 3 J/cm^2 and recorded the phototoxic information. The results produced at 9 or more J/cm^2 were matched with their phototoxic potential (Table 1). Also, values of positive and negative substances met the acceptance criteria (17,18). Considering the results obtained with the irradiation times, we selected the UVA dose of 9 J/cm^2 for the main study.

Results of ROS assay using UVA simulator. Using the selected UVA dose, we identified the performance of the ROS assay with 60 test substances including 50 phototoxins and 10 non-phototoxins (Table 2). Firstly, we measured an MEC for all the test substances if they were photoreactive. Four substances, ibuprofen, erythromycin, penicillin G and phenytoin, had an MEC of less than 1,000. Next, we tested their solubility and identified precipitation, coloration or any other interference at 200 μM in the reaction mixture. Amiodarone, demeclocycline, fenofibrate, piroxicam and rosiglitazone showed precipitation in the reaction mixture for SO and/or SA and an appropriate concentration was further explored for them. Except for amiodarone, the substances with the solubility issues were dissolvable at least at 20 μM. As such, the evaluable test substances were determined be 59 of the original 60. The test results indicated that the UVA ROS assay correctly classified 42 of 49 phototoxins and 10 of 10 non-phototoxins. Amoxapine, atorvastatin, flutamide, griseofulvin, hydrochlorothiazide, nifedipine and nitrendipine were falsely judged as being non-phototoxins. The predictive capacity showed an 85.7% sensitivity, 100.0% specificity and 88.1% accuracy (Table 3).

DISCUSSION

To use a UVA simulator instead of validated solar simulators, we established the irradiation condition of the ROS assay with UVA. We chose a UVA irradiance of 2.0 mW/cm^2, which has been used in Atlas Suntest CPS series and has shown the lowest variation in a previous study (6). We found an appropriate UVA intensity that was compatible with phototoxic information of reference chemicals (Table 1). Also, the selected intensity sufficiently generated ROS and in the range of 5~20 J/cm^2, which is widely used in *in vitro* and *in vivo* phototoxic assays (1).

Solubility evaluation of test substance solutions proceeded right before the assay, in order to prevent interference, such as precipitation and coloration that might affect

Table 1. Selection of UVA irradiation conditions using reference chemicals

No.	Substance name	CAS no.	Phototoxic information*			Concentration (μM)		ROS assay (J/cm^2)**					
			3T3 NRU	Animal	Human	SO	SA	3	6	9	12	15	18
Positive/negative controls													
1	Quinine HCl (PC)	6119-47-7	P	P	P	200	200	+	+	+	+	+	+
2	Sulisobenzone (NC)	4065-45-6	N		N	200	200	–	–	–	–	–	–
Reference chemicals													
3	4-Aminobenzoic acid	150-13-0	N	N		200	200	–	–	–	–	–	–
4	8-Methoxy psoralen	298-81-7	P	P	P	200	200	–	–	+	+	+	+
5	Acridine	260-94-6	P	P	P	200	200	+	+	+	+	+	+
6	Benzocaine	94-09-7	N			200	200	–	–	–	–	–	–
7	Chlorpromazine	69-09-0	P	P	P	200	200	–	+	+	+	+	+
8	Diclofenac	15307-79-6			P	200	200	+	+	+	+	+	+
9	Doxycycline	10592-13-9	P	P	P	200	200	+	+	+	+	+	+
10	Erythromycin	114-07-8	N			200	200	–	–	–	–	–	–
11	Fenofibrate	49562-28-9	P		P	20	20	–	+	+	+	+	+
12	Furosemide	54-31-9	P/N		P	200	200	–	+	+	+	+	+
13	L-Histidine	71-00-1	P			200	200	–	–	–	–	–	–
14	Ketoprofen	22071-15-4	P	N	P	200	200	+	+	+	+	+	+
15	Nalidixic acid	389-08-2	P	P	P	200	200	+	+	+	+	+	+
16	Norfloxacin	70458-96-7	P	P	P	200	200	+	+	+	+	+	+
17	Omeprazole	73590-58-6			P	200	200	–	+	+	+	+	+
18	Promethazine HCl	58-33-3	P		P	200	200	+	+	+	+	+	+

*Phototoxic information was from JaCVAM (17) and Onoue *et al.* (11); P, phototoxic; N, non-phototoxic.
**+, Positive result; –, Negative result.

Table 2. Results of the UVA ROS assay

No.	Substance name	CAS no.	UV absorption*		Concentration (μM)		UVA ROS assay**			Phototoxic information
			λ_{max} (nm)	MEC ($M^{-1}cm^{-1}$)	SO	SA	SO	SA	Result	
Positive/negative controls										
1	Quinine HCl (PC)	6119-47-7	331	5250	200	200	279.6 ± 24.5	190.3 ± 31.3	+	(11), (17)
2	Sulisobenzone (NC)	4065-45-6	(290)	9200	200	200	0.6 ± 4.9	N.D.	−	(11)
Phototoxic substances										
3	6-Methylcoumarin	92-48-8	(290)	8750	200	200	39.4 ± 1.8	53.7 ± 4.5	+	(11), (17)
4	8-Methoxy psoralen	298-81-7	300	12250	200	200	27.4 ± 7.3	18.6 ± 3.5	+	(11), (17)
5	Acridine	260-94-6	355	9250	200	200	169.1 ± 10.5	95.1 ± 4.0	+	(11), (17)
6	Amiodarone	19774-82-4	358/371	10150	20	20	N.A.(P)	N.A.(P)	X	(11), (17)
7	Amlodipine	111470-99-6	365	20900	200	200	9.9 ± 12.2	67.9 ± 34.0	+	(18)
8	Amoxapine	14028-44-5	298	9000	200	200	N.D.	14.2 ± 10.3	−	(6)
9	Atorvastatin	134523-00-5	(290)	9200	200	200	13.1 ± 6.9	10.6 ± 7.3	−	(18)
10	Bezafibrate	41859-67-0	(290)	345	200	200	3.6 ± 3.4	24.8 ± 3.9	+	(18)
11	Bithionol	97-18-7	322	7750	200	200	80.7 ± 7.2	21.1 ± 7.5	+	(11)
12	Chlorothiazide	58-94-6	293	11950	200	200	3.6 ± 1.2	38.6 ± 15.3	+	(6)
13	Chlorpromazine HCl	69-09-0	293	4600	200	200	N.D.	55.0 ± 17.6	+	(11), (17)
14	Ciprofloxacin	85721-33-1	322	15300	200	200	198.3 ± 54.0	86.4 ± 12.2	+	(15)
15	Demeclocyline	64-73-3	375	15700	200	50	143.7 ± 12.8	46.1 ± 2.0	+	(12)
16	Diclofenac	15307-79-6	(290)	7850	200	200	149.5 ± 10.3	150.8 ± 32.6	+	(6)
17	Doxycycline	10592-13-9	348	11650	200	200	67.2 ± 9.1	80.6 ± 4.0	+	(11), (17)
18	Enoxacin	74011-58-8	334	13500	200	200	239.5 ± 5.1	357.6 ± 2.3	+	(18)
19	Fenofibrate	49562-28-9	294	11300	20	20	67.4 ± 15.8	N.D.	+	(11), (17)
20	Flutamide	13311-84-7	291	7800	200	200	12.7 ± 4.1	7.3 ± 2.6	−	(14)
21	Fluvastatin	93957-55-2	303	11050	200	200	190.4 ± 3.4	155.6 ± 12.6	+	(18)
22	Furosemide	54-31-9	(290)	2850	200	200	74.3 ± 21.2	17.7 ± 8.6	+	(11)
23	Gliclazide	21187-98-4	-	-	200	200	4.3 ± 10.2	91.7 ± 12.2	+	(18)
24	Griseofulvin	126-07-8	295	24200	200	200	4.4 ± 2.8	10.6 ± 2.9	−	(18)
25	Hydrochlorothiazide	58-93-5	318	3350	200	200	1.9 ± 3.3	N.D.	−	(18), (19)
26	Ibuprofen	15687-27-1	294	60	200	200	1.8 ± 2.3	62.7 ± 10.7	+	(6)
27	Ketoprofen	22071-15-4	(290)	6450	200	200	123.5 ± 12.8	76.4 ± 6.9	+	(11), (17)
28	Levofloxacin	138199-71-0	(290)	27150	200	200	107.3 ± 28.6	367.3 ± 14.2	+	(18)
29	Lomefloxacin	98079-52-8	326	13350	200	200	693.9 ± 22.1	64.5 ± 1.5	+	(13)
30	Losartan	124750-99-8	(290)	925	200	200	N.D.	50.3 ± 3.2	+	(18)
31	Lovastatin	75330-75-5	325/333	1950	200	200	27.0 ± 8.9	N.D.	+	(18)
32	Meloxicam	71125-38-7	(290)	8350	200	200	9.2 ± 1.9	31.9 ± 9.7	+	(18)
33	Mequitazine	29216-28-2	303	5850	200	200	114.3 ± 11.9	15.4 ± 2.2	+	(18)
34	Methotrexate	59-05-2	303	26100	200	200	N.D.	195.1 ± 20.6	+	Package insert
35	Nalidixic acid	389-08-2	335	12100	200	200	73.2 ± 6.4	307.0 ± 16.8	+	(11), (17)
36	Naproxen	22204-53-1	293	3550	200	200	39.6 ± 3.2	77.9 ± 8.2	+	(18)
37	Nifedipine	21829-25-4	342	5850	200	200	8.8 ± 3.2	N.D.	−	(6)
38	Nitrendifine	39562-70-4	357	5000	200	200	N.D.	11.6 ± 1.5	−	(6)
39	Nitrofurantoin	67-20-9	381	20800	200	200	62.4 ± 6.6	N.D.	+	(6)
40	Norfloxacin	70458-96-7	323	14450	200	200	167.3 ± 24.1	82.3 ± 34.6	+	(11), (17)
41	Ofloxacin	82419-36-1	(290)	28250	200	200	66.9 ± 6.4	349.6 ± 11.6	+	(11), (17)
42	Omeprazole	73590-58-6	299	14400	200	200	N.D.	92.0 ± 6.3	+	(6)
43	Oxytetracycline HCl	2058-46-0	358	16100	200	200	88.4 ± 25.0	66.8 ± 9.4	+	(6)
44	Perphenazine	58-39-9	309	3600	200	200	N.D.	47.8 ± 9.5	+	(18)
45	Piroxicam	36322-90-4	355	17900	200	50	99.9 ± 5.8	37.2 ± 28.0	+	(11), (17)
46	Pitavastatin	147526-32-7	291	10900	200	200	N.A.(P)	47.9 ± 19.5	+	(18)
47	Promethazine HCl	58-33-3	300	3500	200	200	59.5 ± 4.0	6.4 ± 4.5	+	(11), (17)
48	Quinidine	56-54-2	331	5200	200	200	154.6 ± 14.9	87.6 ± 16.1	+	(19)
49	Rosiglitazone	122320-73-4	311	4900	200	20	31.9 ± 5.9	10.1 ± 1.5	+	(11), (17)
50	Tetracycline	60-54-8	363	15950	200	200	39.5 ± 5.4	49.0 ± 3.3	+	(11), (17)

Table 2. Continued

No.	Substance name	CAS no.	UV absorption*		Concentration (μM)		UVA ROS assay**			Phototoxic information
			λ_{max} (nm)	MEC ($M^{-1}cm^{-1}$)	SO	SA	SO	SA	Result	
51	Tiaprofenic acid	33005-95-7	316	15500	200	200	654.5 ± 23.0	201.4 ± 15.4	+	(12)
Non-phototoxic substances										
52	4-Aminobenzoic acid	150-13-0	(290)	7300	200	200	−0.2 ± 0.8	−7.2 ± 0.5	−	(11), (17)
53	Aspirin	50-78-2	(290)	2050	200	200	1.5 ± 2.8	N.D.	−	(11), (17)
54	Benzocaine	94-09-7	(290)	16850	200	200	N.D.	N.D.	−	(11), (17)
55	Dapsone	80-08-0	294	26250	200	200	10.7 ± 2.3	N.D.	−	(19)
56	Erythromycin	114-07-8	-	-	200	200	N.D.	15.1 ± 0.4	−	(11), (17)
57	L-Histidine	71-00-1	291	3000	200	200	0.7 ± 0.6	6.3 ± 0.6	−	(11), (17)
58	Octyl salicylate	118-60-5	(290)	12500	20	20	3.5 ± 8.0	4.6 ± 3.8	−	(11), (17)
59	Penicillin G	113-98-4	-	-	200	200	0.2 ± 0.3	9.8 ± 0.7	−	(11), (17)
60	Phenytoin	57-41-0	295	900	200	200	N.D.	15.4 ± 0.9	−	(11), (17)

*When the maximum wavelengths were under 290 nm, these are marked 290 nm with parentheses.
**SO: Singlet oxygen, SA: Superoxide anion, N.A. (P): Not available due to precipitation, N.D.: Not detected because SA or SO value was below zero.

the test results. To overcome a solubility problem, the ROS assay was modified to use the micelle system using Tween 20 (18). Thus, low solubility substances could be evaluated for their phototoxic potential by adapting the micellar system to the UVA ROS assay.

The study results showed that the 7 phototoxic substances, amoxapine, atorvastatin, flutamide, griseofulvin, hydrochlorothiazide, nifedipine and nitrendipine, were classified as non-phototoxins (Table 2). These substances showed a UVA absorption and MEC of over 1,000 but did not generate ROS, either singlet oxygen or superoxide anion species. Moreover, irradiation of UVA at up to 18 J/cm^2 did not generate ROS (data not shown). However, these substances were correctly classified in previous studies, which used a solar simulator (6,18). Different results between a UVA simulator and a solar simulator could be related with UVB wavelength. UVB may be an essential factor to generate ROS of these chemicals, even they absorbed UVA wavelength. Therefore, misclassified chemicals may not generate ROS. On the other hand, the final concentration of a test chemical, 200 μM, could be limited to generate ROS in the UVA ROS assay system. If using more higher concentration than the final concentration, these chemicals would be generated ROS and met the criteria of photoreactivity. L-Histidine, penicillin G and phenytoin that were false positive substances in the ROS assay were correctly classified as non-phototoxins (Table 2) (6,9,11). Surprisingly, bezafibrate, gliclazide, ibuprofen and losartan generated superoxide anion and were thus classified as phototoxins even though they had low MEC values in the UV/VIS range (290~700 nm). These results were consistent with those of the previous study, and MEC values cannot be always used to evaluate-phototoxic potential of chemicals (6). We conducted the UVA ROS assay with 60 test substances to identify their phototoxic potential. The results showed that this assay could adequately evaluate phototoxicity of the test substances analyzed. In addition, the UVA ROS assay has higher specificity and lower sensitivity than the ROS assay, and the performance of the UVA ROS assay is comparable with that of the ROS assay (Table 3). Our findings suggest that the UVA ROS assay could be used as a method for phototoxicity evaluation of pharmaceutical substances.

Table 3. Comparison of predictive capacity of the UVA ROS assay with the standard ROS assay using two solar simulators

	Standard ROS assay*		UVA ROS assay
	Suntest CPS series	SXL-2500V2	Biospectra
Sensitivity (%)	100.0	100.0	85.7
Specificity (%)	61.6	55.9	100.0
Accuracy (%)	86.8	88.7	88.1

*Performance capacity was from Onoue *et al.* (11) and sensitivity, specificity and accuracy of each solar simulator in the ROS assay indicate the average of each participated lab results.

ACKNOWLEDGMENTS

This research was supported by a grant (15181MFDS458) from the Ministry of Food and Drug Safety of Korea in 2015.

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