Quantitation and Human Monocyte Cytotoxicity of the Polymerization Agent 1-Hydroxycyclohexyl Phenyl Ketone (Irgacure 184) from Three Brands of Aqueous Injection Solution

Kazuhiko Yamaji,[a] Yoichi Kawasaki,[a] Kei Yoshitome,[b] Hisashi Matsunaga,[a] and Toshiaki Sendo*,[a]

[a] *Department of Pharmacy, Okayama University Hospital; and* [b] *Department of Legal Medicine, Graduate School of Medical, Dentistry and Pharmaceutical Sciences, Okayama University; 2–5–1 Shikata-cho, Kita-ku, Okayama 700–8558, Japan.* Received February 29, 2012; accepted June 22, 2012

In this study, levels of the photoinitiator 1-hydroxycyclohexyl phenyl ketone (1-HCHPK) in aqueous injection solutions were analyzed by GC-MS. In our previous studies, photoinitiators such as 2-methyl-4′-(methylthio)-2-morpholinopropiophenone (MTMP) were detected in intravenous (i.v.) injection bag solution, and they were found to be cytotoxic to human monocytes. Therefore, we hypothesized that 1-HCHPK might display similarly cytotoxicity. The purpose of this study was to quantitate the amount of contaminants from plastic containers such as those used for peripheral parenteral nutrition and to determine the cytotoxicity of such extracts on human monocytes. The sample extraction procedure for GC-MS analysis involved a liquid-phase extraction. The solvent was evaporated under a stream of nitrogen at 50°C to yield a residue, which was dissolved in *n*-hexane and injected into a GC-MS. Normal human peripheral blood mononuclear cells (PBMC), isolated from the buffy coat by centrifugation, were suspended in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay, cells (1×10^4) were treated with 1-HCHPK for 24 h or 48 h at 37°C. From the GC-MS analysis, 6.13–8.32 μg/mL of 1-HCHPK was found in 20 mL vials of water for injection solution. In the MTT assay, 1-HCHPK decreased cell viability for both the 24 h and 48 h incubation periods. In conclusion, our findings suggest that 1-HCHPK could promote adverse events in patients. Future studies will clarify the possible health risks of photoinitiator accumulation in human cells.

Key words 1-hydroxycyclohexyl phenyl ketone; cytotoxicity; monocyte; ampoule; photoinitiator

Plastic products are used broadly worldwide. Polyethylene (PE) is a common type of plastic material that is made from ethylene by the action of polymerization agents (photoinitiators). The production process for PE also includes the addition of antioxidants, slip agent, and anti-blocking agent. Because of the frequent, widespread use of plastic products, chemicals such as bisphenol A, nonylphenol, and phthalate esters, which are used as raw materials for polymerization or as plasticizers in the production of plastics, have been a major health concern. Indeed, bisphenol A is a well-known endocrine disruptor, which represents a major toxicological and public health concern due to its widespread exposure to humans.[1] Nonylphenol is also a persistent endocrine disruptor.[2] Recently, Matsumoto *et al.*[3] reported concerns that phthalate esters could be contributors to adverse reproductive and developmental effects in humans.

Photoinitiators are used in a broad range of commercial and biological applications,[4–8] and many research groups have analyzed the cytotoxicity of photoinitiators. Until now, the European Union has not enacted specific legislation on printing inks for food-packaging materials. In addition, a report published by the European Food Safety Authority (EFSA) stated that levels of the photoinitiator isopropylthioxanthone (ITX) ranged from 27 to 440 μg/L in milk and from <5 to 249 μg/L in juice.[9] According to the EFSA, although no fully convincing data on ITX toxicity is currently available, the presence of ITX in food is still considered undesirable. 9-Fluorenone has been found to induce hemolytic activity[10] and lipid peroxidation in erythrocytes.[11] A known component of marketed dental materials, the monomer bisphenol-A diglycidyl dimethacrylate (BISGMA) has been found to be cytotoxic in many cell culture systems.[12,13] Sabnis *et al.*[14] reported that Irgacure 2959 increased with increasing photoinitiator concentration (0.01–0.16%) and significantly affected cell survival. Eick *et al.*[15] reported that the photoinitiator Sarcat™ CD 1012 had a 50% cell survival (TC_{50}) of 14 μM, which is very cytotoxic. In another study, the photoinitiation agent 0.03% 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651) was found to completely kill all human fetal osteoblasts and bovine chondrocytes.[16]

In a recent study,[17] we reported for the first time detection of 2-methyl-4′-(methylthio)-2-morpholinopropiophenone (MTMP) from an intravenous (i.v.) injection bag solution by GC-MS, and the analysis quantified the amount of MTMP to be 5.62 ± 1.03 μg/mL. In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, MTMP decreased human monocyte viability in a dose-dependent manner for both 24 h and 48 h incubation periods. Our findings suggested that photoinitiators might promote adverse effects in patients. In this study, 1-hydroxycyclohexyl phenyl ketone (1-HCHPK) was detected in injection solution from polyethylene ampoules. Therefore, we hypothesized that 1-HCHPK was a potential source of cytotoxicity in patients.

Although Chinese hamster V79 cells have generally been used to determine cytotoxicity effects,[18–20] in this study, we hypothesized that photoinitiators including from aqueous injection solutions would enter the blood stream. Therefore, normal human peripheral blood mononuclear cells (PBMC) were used because of the potential clear cause-effect from introducing photoinitiators into the human blood stream.

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: sendou@md.okayama-u.ac.jp

The purpose of this study was to quantitate the amount of 1-HCHPK in the injection solutions from three brands of polyethylene ampoules and to clarify the extent of the 1-HCHPK cytotoxic effect on human monocytes.

MATERIALS AND METHODS

Chemicals and Reagents For the GC-MS analysis, a reference standard of 1-HCHPK and *n*-hexane (for HPLC, ≥96%) were obtained from Sigma-Aldrich (U.S.A.). An internal standard, diphenyl was purchased from Katayama Chemical (Japan). Three brands of 20 mL vials of water for injection solution were purchased from Otsuka Pharmaceutical Factory Inc. (Product A), Nissin Pharmaceutical Co., Ltd. (Product B) and FUSO Pharmaceutical Industries, Ltd. (Product C) (Japan, respectively). 1-HCHPK and diphenyl were used in *n*-hexane solution.

For the MTT assay, MTT was purchased from Wako Pure Chemical Industries, Ltd. (Japan) and was dissolved in phosphate buffered saline (PBS). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. 1-HCHPK was used in methanol solution.

Instrumentation and Conditions GC-MS analysis was performed with a GCMS-QP2010 Plus (Shimadzu Co., Kyoto, Japan). The entire system was controlled by GC-MS solution® Software (Shimadzu Co.). For the GC analysis, an Ultra2 fused silica column (Shimadzu Co.) was used. Helium was used as the carrier gas at a flow rate of 35.0 mL/min and 49.0 kPa. The GC injection sample was split with a split ratio of 15. MS analysis was conducted in electron ionization mode with selected ion monitoring (81, 99, and 152 *m/z*). The maximum ion fragment mass was 99 *m/z*; therefore, it was used as the monitoring ion (Table 1).

Sample Extraction Procedure for GC-MS Analysis The sample extraction procedure for GC-MS analysis involved a liquid-phase extraction. Diphenyl (20 μg/100 mL *n*-hexane) was added as an internal standard. For each of the three brands, 100 mL of water for injection solution was extracted with 100 mL of *n*-hexane in a glass separatory funnel. The *n*-hexane layers were collected in glass bottles, and the combined extracts were concentrated under a stream of nitrogen while the bottles were kept at 50°C in a heating bath. The residue was dissolved in 1 mL of *n*-hexane, and a 1 μL aliquot was injected into the GC-MS. Calibration standards were prepared at four concentrations for the GC-MS analysis: 0 μg/mL, 2.5 μg/mL, 5.0 μg/mL and 10.0 μg/mL.

Table 1. The Instrumental Conditions on GC-MS

GC	
Instrument	GCMS-QP2010 Plus; Shimadzu
Internal standard	Diphenyl
Injection mode	Automatic split
Split ratio	15 : 1
Injection volume	1 μL
Injection port temperature	280°C
Carrier flow	135 kPa helium (constant pressure)
Oven program	150°C for 1 min, 19.5°C/min, 300°C final fold for 2 min
Column	Ultra 2, fused silica, 0.2 mm×12.5 m ×0.11 μm
MS	
Analysis mode	Electron ionization
Ion source temperature	250°C
Transfer temperature	280°C
Monitor ion	99 *m/z*

Isolation and Culture of PBMC Normal human PBMC were obtained from six healthy volunteers after informed consent. Thirty milliliters of peripheral blood was withdrawn from the vein in the forearm, and PBMC were isolated from the buffy coat by centrifugation on a Ficoll-Paque system (Amersham Biosciences AB, Uppsala, Sweden) followed by washing three times with RPMI 1640 medium (Nissui Co., Ltd., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma Chemical). PBMC were suspended at a final concentration of 1×10^4 cells/mL in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum.

The study protocol was approved by the Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences Ethics Committee (No.1026).

Cell Survival Assay with MTT Reduction Cell survival was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenase.[21] Cells (1×10^3) immediately after isolation were treated with 1-HCHPK in 96-well plates for 24 h or 48 h at 37°C. The medium, which included 2.5% methanol (100 μL), was then incubated with 20 μL of 5 mg/mL MTT solution for 1 h at 37°C. After centrifugation at 1500 rpm for 10 min, the culture medium was removed, and 100 μL of DMSO was added to each well to dissolve the formazan. The absorbance at 590 nm was measured using a micro-plate reader (Model 680 microplate reader; Bio-Rad Laboratories, Inc., Japan). Cell survival was expressed as a percentage of the absorbance determined for control cultures.

Statistical Analysis The data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey test or Dunnett's test. The significance level was set at $p<0.05$.

RESULTS

Analysis of 1-HCHPK by GC-MS Among the detected compounds, the photoinitiator 1-HCHPK (T_R: 4.95 min) was found in the aqueous injection solutions from three to four samples with the same lot number (Figs. 1, 2). Linearity was assessed by a weighted least squares regression analysis. The GC-MS calibration curve for 1-HCHPK had a correlation coefficient (r^2) of ≥0.99 (Fig. 3). The recovery rate was 91.57±4.44% (mean±S.D.), and the coefficient of variation for the three brands of solution was 1.612%, 1.157%, and 18.97%, respectively. A calibration curve for the GC-MS analysis was constructed using 1-HCHPK concentrations ranging between 0 μg/mL and 10.0 μg/mL. As presented in Fig. 4, 1-HCHPK concentrations of 6.33±0.10 μg/mL (mean±S.D.), 8.32±1.58 μg/mL, and 6.13±0.07 μg/mL were determined from Products A, B and C, respectively. *Post hoc* comparisons demonstrated no significant difference for each brand [$F(2,8)=5.095$, $p=0.0374$].

Cytotoxicity Testing by the MTT Assay In cell viability studies with the MTT assay, lower values indicated increased cytotoxicity. A negative control of 2.5% methanol without 1-HCHPK added to the medium gave an average cell survival of 100±19.59% and 100±17.57% for the 24 h and 48 h

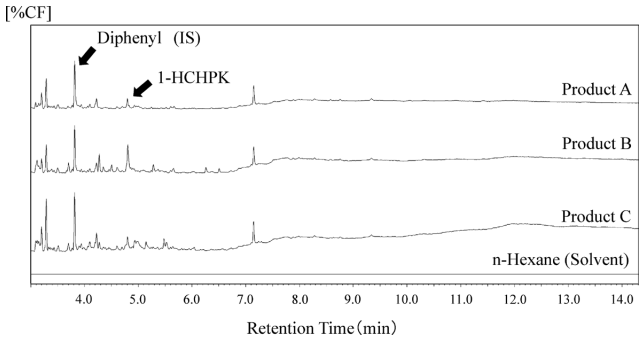

Fig. 1. GC Chromatograms of Extracts from 20 mL Vials of Water for Injection Solution

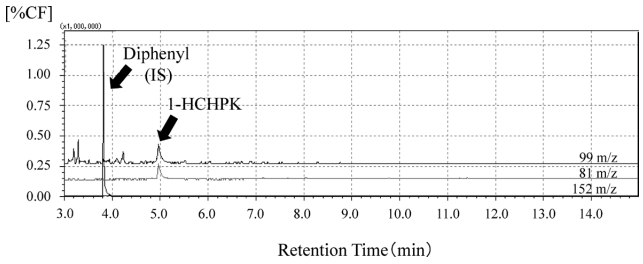

Fig. 2. GC-MS Chromatograms of Extracts from 20 mL Vials of Water for Injection Solution

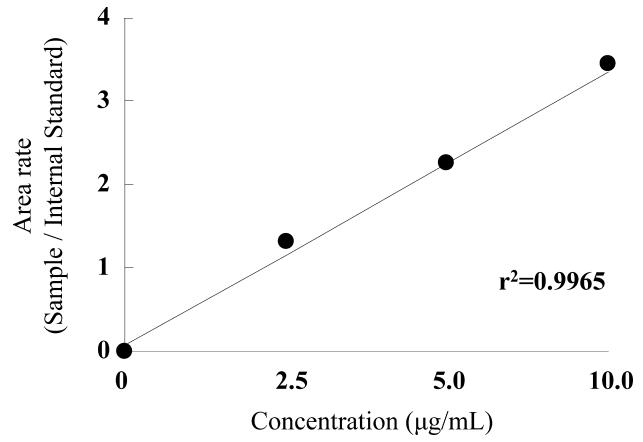

Fig. 3. Calibration Curve of 1-HCHPK

incubation periods. These cell survival percentages indicated non-cytotoxicity. Incubation with 1-HCHPK for 24 h resulted in a dramatic attenuation of cell viability [$F(9,50)=19.275$, $p<0.01$]. *Post hoc* comparisons showed a significant effect was observed at a dose of 250 μg/mL ($p<0.01$). Similarly, incubation with 1-HCHPK for 48 h resulted in a dramatic attenuation of cell viability [$F(9,50)=59.485$, $p<0.01$]. Following the *post hoc* comparisons, a significant effect was observed at doses of 125 μg/mL and 250 μg/mL ($p<0.01$, each) (Fig 5). In addition, the 1-HCHPK TC_{50} was determined to be 885 μM and 730 μM after incubation for 24 h and 48 h, respectively.

DISCUSSION

In this study, we detected compounds including approximately 6 to 8 μg/mL of the photoinitiator 1-HCHPK in

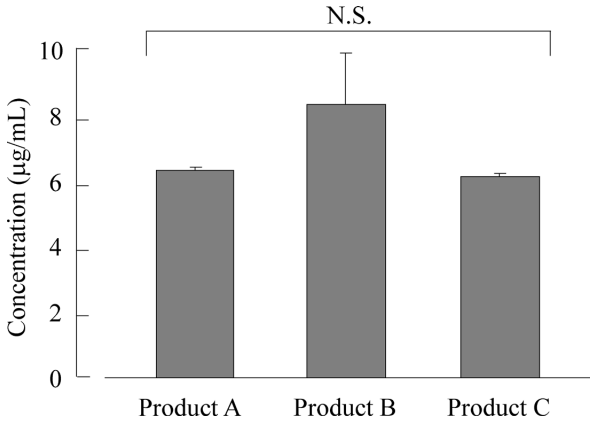

Fig. 4. Concentration of 1-HCHPK in Three Brands of Injection Solution

N.S.: Non-significant $n=3$–4

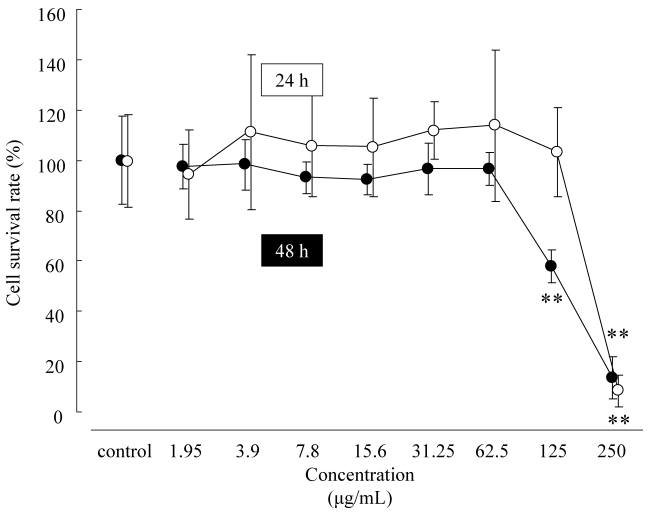

Fig. 5. Effect of 1-HCHPK on Cell Viability of Human Monocytes

** $p<0.01$ *vs.* control group. $n=6$

extracts from 20 mL vials of water for injection solution, which amounts to approximately 120 to 160 μg of 1-HCHPK per vial. Additionally, we found that 1-HCHPK was cytotoxic to human monocytes.

Photoinitiators are used in a broad range of commercial and biological applications such as printing,[4,22] dentistry,[5] encapsulation of pancreatic islet cells,[6,7] and blood vessel adhesives.[8] Thus, a high level of daily exposure to photoinitiators is possible. However, safety criteria for photoinitiators have not been decided in the European Union, similar to the situation in Japan. In the European Union, the directive states that "to protect the health of the consumer, direct contact between foodstuffs and the printed surfaces of regenerated cellulose film should be avoided."[23–25] Therefore, photoinitiators are generally considered to be a human health concern. However, it would appear that photoinitiators have a more significant effect with injection solutions as compared to skin and oral cavity usage in humans because of their direct administration into the body. In this study, 1-HCHPK was detected in injection solution; however, with the exception of our previous study, we have not found any other literature reviews of this

area. In malignant lymphoma therapy, an aqueous formulation of doxorubicin is administered with a single injection, as described in the package insert in Japan. Similarly, Uyama *et al.*[26] reported a pilot study in which doxorubicin was administered in a one-shot protocol. With an intravenous drip (DIV), 1-HCHPK slowly enters the body in small amounts over an extended period of time. In contrast, administration of a single bolus results in human monocytes contacting a much higher dose of 1-HCHPK as the entire drug solution rapidly enters the blood stream. Therefore, MTT assay results of this study suggest *in vivo* cytotoxicity of 1-HCHPK although its amounts detected in three injection solutions are lower than those observed *in vitro* cytotoxicity. Eick *et al.*[15] reported that the photoinitiator Sarcat™ CD1012 was very cytotoxic (TC_{50}=14 μM). In addition, they showed that other dental composites including Araldite™ GY281 (TC_{50}=17 μM), BISGMA (TC_{50}=36 μM), and Epon™ 825 (TC_{50}=50 μM) were also very cytotoxic, while ethyl 4-dimethylaminobenzoate (TC_{50}=540 μM) and camphorquinone (TC_{50}=779 μM) had lower levels of cytotoxicity. In our previous study, MTMP after a 48 h incubation had a TC_{50}=730 μM. Williams *et al.*[16] reported that 1-HCHPK was significantly cytotoxic to human fetal osteoblasts and bovine chondrocytes in the concentration range of 0.05–0.1%. In this study, 1-HCHPK had a similar cytotoxic effect after incubation with PBMC cells for 24 h or 48 h, with significant cytotoxicity to human monocytes in the concentration range of 125 μg/mL (0.0125%) to 250 μg/mL (0.025%) for the 48 h incubation. Therefore, we suggest that different cell types react similarly to the same concentrations of 1-HCHPK.

There is little information available regarding the analysis of photoinitiators in food items. One of the most common photoinitiators is benzophenone, which has been shown to migrate from packaging through the packaging layer in direct contact with food and into the food itself.[27] A few research groups have found photoinitiators such as isopropylthioxanthone (ITX) in milk[22,28,29] and other beverages.[30] In addition, photoinitiators[31] (benzophenone, Irgacure 184, benzylketal BDK, Irgacure 907, ITX) have been detected in aqueous stimulants. In recent studies, key parameters of this chemical migration were determined for different food matrices.[32,33] Sanches-Silva *et al.*[34] reported migration levels for six photoinitiators (Irgacure 184, benzophenone, Irgacure 651, Irugacure 907, ITX, and 2-ethylhexyl-(4-dimethylamino)-benzoate) into different food stimulants compared after a 30 d contact period with additivated plastics. These photoinitiators are used in printing.[1,18] Therefore, we propose that 1-HCHPK is derived from the printing ink on injection packages and has different migration behavior in different injection components.

Shen *et al.*[35] showed that photoinitiators can easily migrate from packaging to foods with a high fat content. The cytotoxicity of compounds like photoinitiators has been thought to be due in part to their hydrophobicity[36] since permeability through the phospholipid bilayer of cellular membranes increases with compound hydrophobicity. Mast cells, a component of the immune system generated from marrow pluripotent haematopoietic cells, influence both innate and adaptive immunity. Brown *et al.*[37] documented mast cells as being a critical effector in allergic reactions and histamine release as well as many other mediators of the immune response including cytokines and chemokines. Clinically, relationships between anaphylactic shock and histamine,[38] cytokines, and chemokines have been reported.[39] In addition, it has been reported that histamine may be a factor in pruritus[40] and the erythemal response.[41,42] Therefore, we believe that photoinitiators accumulate in cells, in particular mast cells, that sequentially release histamine, cytokines, and chemokines,[43] which leads to various adverse events. In fact, Momo *et al.*[44] reported that ITX, a highly lipophilic photoinitiator, was involved in possible interactions with the lipid moieties of biological membranes to increase the fluidity of the bilayer. Therefore, the hydrophobicity of photoinitiators such as 1-HCHPK might also promote their accumulation in adipocytes.

In conclusion, we have shown that 1-HCHPK, including from aqueous injection solutions, may be cytotoxic to human cells and suggest it could accumulate and promote adverse events in patients. Indeed, Fedorovich *et al.*[45] indicated adverse events from photopolymerization on the viability and cell cycle progression of exposed bone marrow derived multipotent stromal cells. In future studies, we plan to clarify possible health risks associated with photoinitiator accumulation in human cells.

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