MethodsX 7 (2020) 100774

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Protocol Article

A method to dissolve 3-MCPD mono- and di-esters in aqueous cell culture media

Mapa S.T. Mapa[a], Magali Araujo[a], Yang Zhao[a], Thomas Flynn[a], Jessica Sprando[b], Paddy Wiesenfeld[a], Robert L. Sprando[a], Miriam E. Mossoba[a,*]

[a] *Division of Applied Regulatory Toxicology (DART), Office of Applied Research and Safety Assessment (OARSA), Center for Food Safety and Applied Nutrition (CFSAN), U.S. Food and Drug Administration (US FDA), 8301 Muirkirk Rd., Laurel, MD 20708, United States*

[b] *Virginia-Maryland College of Veterinary Medicine, 205 Duck Pond Road, Blacksburg, VA 24061, United States*

A B S T R A C T

Fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPD) are chemical contaminants found in a wide range of edible oils that are thermally processed during industrial manufacturing of infant formula and other lipid-containing foods in the United States. Rodent studies have unequivocally demonstrated a plethora of *in vivo* toxicological effects including reproductive, neurological and renal dysfunction. To determine if similar effects are observed in human organ systems,*in vitro* studies using human cell lines are conducted to assess concordance of the data collected. One limitation to performing such *in vitro* research is the extremely high hydrophobicity of 3-MCPD esters; dissolving them into aqueous cell culture media is a tremendous challenge. To address this obstacle, we developed a simple protocol to circumvent the immiscibility of 3-MCPD esters and their corresponding free fatty acids into aqueous cell culture media in order to assess the effect of these esters on epithelial cells of kidney origin *in vitro*.

A R T I C L E I N F O

Protocol name: Three-step protocol to dissolve 3-MCPD esters and free fatty acids in aqueous cell culture media

Keywords: Solubilization, *In vitro* cell culture, 3-Monochloropropane-1,2-diol esters, Free fatty acids

Article history: Received 23 July 2019; Accepted 13 December 2019; Available online 17 December 2019

* Corresponding author.

E-mail addresses: MapaMudiyanselage.Mapa@fda.hhs.gov (Magali.DeAraujo@fda.hhs.gov (M. Araujo), Yang.Zhao1@fda.hhs.gov (Y. Zhao), Thomas.flynn@fda.hhs.gov (T. Flynn), sprando2@vt.edu (J. Sprando), paddy.wiesenfeld@fda.hhs.gov (P. Wiesenfeld), Robert.Sprando@fda.hhs.gov (R.L. Sprando), miriam.mossoba@fda.hhs.gov (M.E. Mossoba).

http://dx.doi.org/10.1016/j.mex.2019.100774

Specification Table

Subject Area:	Pharmacology, Toxicology and Pharmaceutical Science
More specific subject area:	*In vitro* Cellular Toxicology
Protocol name:	Three-step protocol to dissolve 3-MCPD esters and free fatty acids in aqueous cell culture media
Reagents/tools:	**Reagents:** Dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) Acetonitrile (Sigma, St. Louis, MO) 2-propanol (Sigma, St. Louis, MO) Ethanol (Sigma, St. Louis, MO) Hyclone fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) 3-Monochloropropane-1,2-diol (3-MCPD; Toronto Research Chemicals, Toronto, Canada) 1-Palmitoyl-3-chloropropanediol (1-Pa; Toronto Research Chemicals, Toronto, Canada) 1-Linoleoyl-3-chloropropanediol (1-Li; Toronto Research Chemicals, Toronto, Canada) 1-Oleoyl-3-chloropropanediol (1-Ol; Toronto Research Chemicals, Toronto, Canada) 1-Palmitoyl-2-linoleoyl-3-chloropropanediol (Pa-Li; Toronto Research Chemicals, Toronto, Canada) 1-Palmitoyl-2-oleoyl-3-chloropropanediol (Pa-Ol; Toronto Research Chemicals, Toronto, Canada) 1-Oleoyl-2-linoleoyl-3-chloropropanediol (Ol-Li; Toronto Research Chemicals, Toronto, Canada) 1,2-Di-palmitoyl-3-chloropropanediol (Pa-Pa; Toronto Research Chemicals, Toronto, Canada) 1,2-Di-oleoyl-3-chloropropanediol (Ol-Ol; Toronto Research Chemicals, Toronto, Canada) 1,2-Di-linoleoyl-3-chloropropanediol (Toronto Research Chemicals, Toronto, Canada) Palmitic Acid (Pa; Toronto Research Chemicals, Toronto, Canada) Oleic Acid (Ol; Toronto Research Chemicals, Toronto, Canada) Linoleic Acid (Li; Toronto Research Chemicals, Toronto, Canada) Human Kidney HK-2 Cell Line (ATCC, Manassas, VA) Keratinocyte SFM (Invitrogen, Carlsbad, CA) **Tools:** Fluostar Omega plate reader (BMG LabTech, Cary, NC) ACCU-SCOPE 3030 & 3030 PH inverted microscope with 10x Plan Phase, NA 0.25, WD 8.0 mm (Commack, NY) Eppendorf tubes (Eppendorf, Hamburg, Germany) Heat mat (Brisk, Columbus, OH) Isotemp hot plate (Fisher Scientific, Hampton, NH) Water bath (Fisher Scientific, Hampton, NH) Micro-pipettors with capacities of 2-1000 ul (Eppendorf, Hamburg, Germany)
Experimental design:	A range of concentrations of organic solvents was prepared in cell culture media and added to human epithelial cells of kidney origin (HK-2 cell line) cultured in vitro for 24 hours at 37 °C/5% CO_2. Cell viability was measured to identify solvent conditions that are nontoxic and the selected solvent was used to develop a cost-effective three-step protocol to easily dissolve hydrophobic compounds like 3-MCPD esters and free fatty acids that are relevant to the field of toxicology.
Trial registration:	Not applicable
Ethics:	Not applicable

Value of the Protocol

- Having a protocol to easily dissolve hydrophobic compounds such as 3-MCPD esters and free fatty acids into aqueous cell culture medium is valuable for its downstream applications. Once compounds are rendered soluble in cell culture media, then a plethora of *in vitro* cellular toxicology assays can be performed to evaluate their potential toxicity levels and mechanisms of action. Such evaluations are especially important for establishing *in vitro* to *in vivo* concordance in the field of predictive toxicology.

Background

Successful *in vitro* cellular toxicity testing is predicated on how easily and stably test compounds can dissolve into cell culture media, but when compounds are inherently hydrophobic or non-polar, then dissolving them into aqueous media limits their achievable concentration range. Ester derivatives of 3-monochloropropane-1,2-diol (3-MCPD) are low-level contaminants found in infant formula [1,2] and have been shown in rodents to be toxic to the renal, testicular, and nervous systems [3–6]. They are too hydrophobic to easily dissolve into cell culture media and the use of organic solvents to solubilize these hydrophobic compounds is limited by the solvents inherent toxicity to mammalian cells. In our attempts to circumvent these limitations, we established a simple three-step protocol to overcome their immiscibility in human epithelial cells of kidney origin (HK-2) cell culture medium and thus enable *in vitro* cellular toxicity testing.

Organic solvent pre-screening

Although organic solvents are generally very toxic to mammalian cells, they have been useful in many cases to help dissolve hydrophobic compounds, such as free fatty acids [7–9]. We hypothesized that their cellular toxicity could be mitigated by (a) careful selection of the organic solvent and (b) using very low concentrations on cells. We selected ethanol, 2-propanol, acetonitrile, and dimethylsulfoxide (DMSO) as four candidate organic solvents (all from Sigma, St. Louis, MO). We then tested the toxicity of each one at concentrations ranging from 0 to 10% (v/v) in HK-2 cell culture media (Keratinocyte media supplemented with 10% fetal bovine serum, 0.3% bovine pituitary extract, and 2.5 ng/ml of epidermal growth factor; Invitrogen, Carlsbad, CA). Following the manufacturer's instructions, we measured HK-2 cell viability following a 24 -h exposure to each solvent using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). Luminescent signal representing cell viability levels were measuring using the Fluostar Omega plate reader (BMG LabTech, Cary, NC). As shown in Fig. 1, we found that HK-2 cells could be cultured without inducing significant loss of cell viability in the presence of all tested solvents if, these solvents comprised approximately 1% (v/v) of the cell culture medium. For solvent levels above 1%, only acetonitrile and DMSO in cell culture medium for 24 h at 37 °C/5%CO_2 could maintain basal cell viability. DMSO, however, is better suited for use in cell culture systems than acetonitrile because its vapor pressure at 20 °C is much lower (DMSO: 0.417 mm Hg *vs.* acetonitrile: 72.8 mm Hg) [10,11], making it considerably less volatile and easier to work with.

Three-step solubilization protocol for 3-MCPD esters

Hydrophobic compound solutions in aqueous media are often performed in a two-step manner, wherein compounds of interest are dissolved into an organic solvent and subsequently diluted in

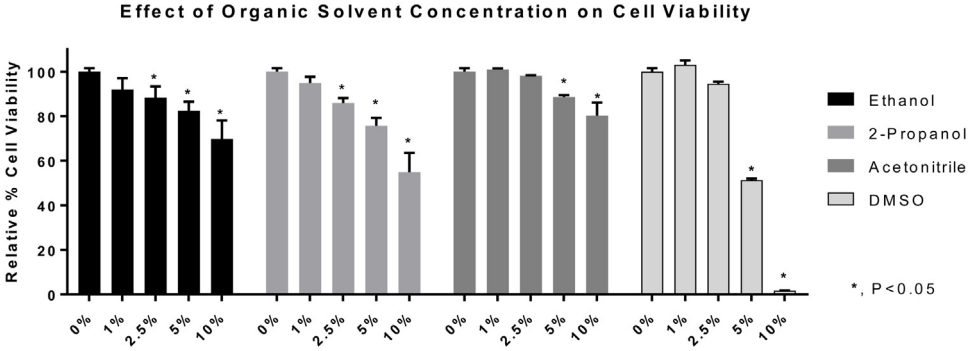

Fig. 1. Relative percentages of cell viability levels of human kidney HK-2 cells in 0–10% (v/v) organic solvents following exposure for 24 h at 37 °C/5%CO_2.

aqueous media to reduce the organic solvent concentration down to 1% or less [7–9]. However, some compounds, such as 3-MCPD esters, are not amenable to this protocol; instead of completely dissolving, they undergo 'hydrophobic effects' [12] and precipitate out as globules/spheres/droplets of varying sizes. This effect was verified using several compounds (all from Toronto Research Chemicals, Toronto, Canada): nine esters of 3-MCPD, including three mono-esters of 3-MCPD (1-Pa, 1-Li, and 1-Ol), six di-esters of 3-MCPD (Pa-Li, Pa-Ol, Ol-Li, Pa-Pa, Li-Li, and Ol-Ol), and three free fatty acids (Pa, Li, and Ol). As shown in Fig. 2A, whereas light microscopy images of PBS, cell culture media, and water-soluble free 3-MCPD were free of droplet precipitate, the majority of 3-MCPD esters and free fatty acids contained obvious droplets or globules of various sizes. It should be noted that although this two-step method could not achieve good solubility outcomes for all tested compounds, a few of their 100 μM solutions (1-Pa, Pa-Li, and Pa) exhibited only small droplets, rather than large globules of precipitate (Fig. 2A). When evaluating the effects of any solution on cells, it is best to achieve the most homogenous and finest stable solute emulsion possible across all compounds undergoing toxicological evaluation. Having large globules or droplets is not ideal for *in vitro* cellular toxicity assays, since differences in solubility between compounds would translate to insufficient and/or inconsistent exposures, which in turn would confound the interpretation of toxicological data.

To overcome this unwanted effect, a three-step solubilization protocol was developed to achieve stable homogenous solutions of each compound of interest.

Step 1: Prepare 10 mM stock solutions of each compound of interest in pure DMSO (Sigma, St. Louis, MO) at room temperature. In some cases, if a compound is unusually immiscible, briefly vortex it at the maximum setting for five seconds in DMSO, then warm it to 37 °C in a water bath before moving on to the next step of the protocol.

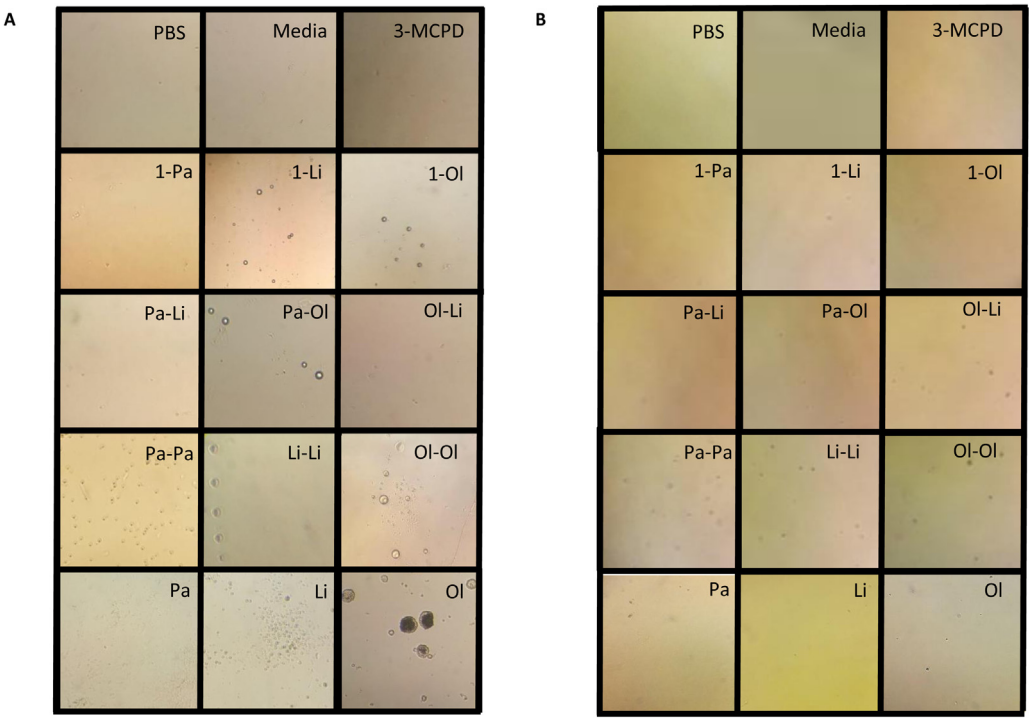

Fig. 2. Light microscopy images at 10× magnification of 100 μM test compound stock solutions prepared using the common two-step (A) or newly developed three-step protocol (B) with final solvent concentrations of 1% DMSO in HK-2 cell culture medium.

Step 2: Dilute all compounds by10-fold using fetal bovine serum that has been pre-warmed to ~50 °C in a water bath. Keep all solutions warm using a Brisk heat pad set to ~40 °C.

Step 3: Perform a final dilution in pre-warmed cell culture media (containing only 1% FBS) to achieve a final test compound concentration of 100 μM.

To verify the success of this three-step protocol, we prepared 100 μM solutions of the same 13 compounds that had previously been shown in Fig. 2A to have solubility issues using the conventional two-step method. As shown in Fig. 2B, when we imaged each of the 13 solutions, we found that the three-step protocol achieved excellent solubility in aqueous cell culture media. Notably, only Pa-Pa needed to be briefly vortexed at step 1 and warmed to 37 °C in a water bath before moving on to step 2 of the protocol. Using this three-step protocol, our research group has been able to perform comprehensive *in vitro* cellular toxicological evaluations of the effects of these compounds of interest [13]. Prior to the creation of this protocol, however, the lack of 3-MCPD ester solubility hampered *in vitro* cellular studies until now. We expect that its continued application to the field of *in vitro* cellular toxicology will allow other researchers to evaluate a great number of hydrophobic compounds that need to be evaluated for toxicity and mechanistic effects relevant to human health and disease.

Acknowledgements

Authors would like to acknowledge Dr. Brenna Flannery (FDA) for helpful discussions.

Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1 J.H. Spungen, et al., Estimated US infant exposures to 3-MCPD esters and glycidyl esters from consumption of infant formula, Food Addit. Contam. Part A 35 (2018) 1085–1092.
2 S. MacMahon, T.H. Begley, G.W. Diachenko, Occurrence of 3-MCPD and glycidyl esters in edible oils in the United States, Food Addit. Contam. Part A 30 (2013) 2081–2092.
3 M. Liu, G. Huang, T.T.Y. Wang, X. Sun, L. (Lucy) Yu, 3-MCPD 1-Palmitate induced tubular cell apoptosis in vivo via JNK/p53 pathways, Toxicol. Sci. 151 (2016) 181–192.
4 E. Barocelli, A. Corradi, A. Mutti, P.G. Petronini, Comparison between 3-MCPD and its palmitic esters in a 90-day toxicological study, EFSA Support. Publ. 8 (2011) 187E.
5 M. Liu, et al., Preparation of five 3-MCPD fatty acid esters, and the effects of their chemical structures on acute oral toxicity in Swiss mice, J. Sci. Food Agric. 97 (2017) 841–848.
6 G. Huang, et al., Suppression of T lymphocyte activation by 3-chloro-1,2-propanediol mono- and di-palmitate esters in vitro, Toxicol. In Vitro 51 (2018) 54–62.
7 P.T. Jain, J.T. Pento, A vehicle for the evaluation of hydrophobic compounds in cell culture, Res. Commun. Chem. Pathol. Pharmacol. 74 (1991) 105–116.
8 A.F. Oliveira, et al., In vitro use of free fatty acids bound to albumin: a comparison of protocols, BioTechniques 58 (2015) 228–233.
9 J. Rivers-Auty, J.C. Ashton, Vehicles for lipophilic drugs: implications for experimental design, neuroprotection, and drug discovery, Curr. Neurovasc. Res. 10 (2013) 356–360.
10 Pubchem. Dimethyl sulfoxide. https://pubchem.ncbi.nlm.nih.gov/compound/679.
11 M.B. Ewing, J.C.S. Ochoa, Vapor pressures of acetonitrile determined by comparative ebulliometry, J. Chem. Eng. Data 49 (2004) 486–491.
12 N.T. Southall, K.A. Dill, A.D.J. Haymet, A view of the hydrophobic effect, J. Phys. Chem. B 3 (2002) 521–533.
13 M. Mossoba, et al., In vitro toxicological assessment of free 3-MCPD and select 3-MCPD esters on human proximal tubule HK-2 cells, Cell Biol. Toxicol. (2019) 1–13.