ARTICLE

Some Limits of Biocompatibility Testing for Lipophilic Leachates

Anne D. Lucas* and Edward A. Gordon

All Res. J. Biol., **2012**, 3, 1-5

All your results are good results!

THE ALL RESULTS JOURNALS

Issue 1, Vol. 3, 2012, 1-5

Some Limits of Biocompatibility Testing for Lipophilic Leachates

Anne D. Lucas* and Edward A. Gordon

*U.S. Food and Drug Administration, Center for Devices and Radiological Health, Office of Science and Engineering Laboratories (Division of Biology); 10903 New Hampshire Ave, Bldg 64 room 4010, Silver Spring MD 20903. *Corresponding Author: anne.lucas@fda.hhs.gov*

Abstract: Medical device standards recommend using both a polar and non-polar solvent to extract materials prior to *in vitro* testing. Testing lipophilic extracts in cell culture systems is limited by the toxicity of the lipophilic solvents used in extraction. Use of agar overlay and direct contact methods do not directly address the problem of testing for highly lipophilic leachates from device or material extracts. This particular problem was approached by 1) use of hydrotropes, and 2) by sealing the suspended cells in dialysis tubing and placing it directly in oil or media. The use of hydrotropes to eliminate micelle formation and increase the solubility of lipophilic compounds was not useful as the hydrotropes themselves were toxic to the cells at concentrations that significantly increased analyte solubility. Diffusion of hydrophobic compounds from either peanut oil or cell culture media into the dialysis tubing, where the test cells resided in media, was significantly higher for the cell culture media than the peanut oil. There were significant differences in toxicity for cells in dialysis tubing from devices extracted between peanut oil and media. This study illustrates the importance of examining if cell toxicity is due to micelle formation or to the soluble chemicals from lipophilic extracts.

Keywords: Lipophilic, biocompatibility, medical device extracts

Introduction

The Food and Drug Administration requires medical devices and materials be tested for safety. Biocompatibility testing is necessary for all devices requesting FDA clearance[1,2]. The standards used by the Agency for biocompatibility testing recommend using at least 2 different extraction solutions of different polarity[3,4]. Lipophilic solvents, such as DMSO and vegetable oil, are recommended extraction solvents for medical devices and materials prior to cell culture assay[5,6]. However, mammalian cells used in biocompatibility testing are affected by low concentrations (approximately 1% or less) of lipophilic solvents[7]. The ability to perform *in vitro* tests on lipophilic analytes is limited by the solubility of the analyte in aqueous solutions.

To attempt to address this problem, a system of indirect exposure of cells to oil was developed. By placing cells in dialysis tubing, then putting these cells directly in oil, it was expected that exposure to lipophilic compounds would increase. In addition, diffusion and solubility of the lipophilic analytes limits the amount of the analyte reaching the test cells. To attempt to increase exposure of the cells to hydrophobic leachates, 2 detergents were examined for their ability to increase the diffusion of lipophilic leachates into the cell culture test system. The use of hydrotropes to increase solubility has previously been applied to drugs[8] and its application to device leachate testing was a logical step.

Materials and Methods

Cell Cultures and Conditions

Jurkat cells, a human lymphoma cell, were obtained from ATCC (ATCC CRL 8163). The Jurkat cell line was chosen because it is a suspended cell line, not requiring surface attachment in order to grow. Although not commonly used for biocompatibility testing, this cell line is appropriate to use for toxicity testing because it is derived from human blood cells and blood cells have great potential to be exposed to and transport most drugs and leachates of devices. Cells were grown and maintained in CO_2 independent medium (Gibco Laboratories) supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum (FBS, Sigma Chemical Co.). Stock cells were maintained at 37°C.

Chemicals

Peanut oil and virgin olive oil were purchased from a local supermarket. Cottonseed oil, Dulbecco's phosphate buffed saline (DPBS), atrazine and tween-20 were purchased from Sigma-Aldrich. Polyethylene glycol 4000 was obtained from Mallinckrodt Chemicals, diethylhexyl-phthtalate (DEHP) from Fluka, and actonitrile from Fisher Scientific. A commercially available enzymatic cleaner for reprocessing medical devices was used to deliberately contaminate GI biopsy forceps.

Dialysis tubing was obtained from Spectra/Por (3500, 6000-8000, and 1200-14000 Dalton mwco, semipermiable regenerated cellulose); nominal flat width of both 32 mm ('large') and 10 mm ('small') were used. Tubing was rinsed, then boiled in distilled water to remove any glycerol residues and any other contaminates prior to use; the dialysis tubing could then be autoclaved.

Cell Exposure

Exposure of the cells to various oils was accomplished by placing usually 5 ml of cells in media directly into the dialysis tubing with the ends of the tubing tied tightly and any excess material was trimmed off. Cells were then put directly into a 50 ml polypropylene test tube with the appropriate extraction solution (Figure 1). Initially, 25 ml of the extraction oils (cottonseed, olive, or peanut oil) were tested to select the best oil with cell culture media as a point of comparison. After selecting the oil best tolerated by the cells, chemicals and extracts of medical devices were then assessed for cytotoxic response. The test device or materials was placed directly in the extraction solution, then 5 to 10 ml of cells enclosed in the large (32 mm) dialysis tubing was added. The potential effect of the dialysis tubing on the cells themselves was explored by placing a 16 cm long piece of tubing in a 50 ml polypropylene test tube with 10 ml of media. To this 10 ml of cells in media were added; the control for this experiment was 10 ml of media and 10 ml of cells in a 50 ml polypropylene test tube. Different pore sizes and nominal flat widths of various dialysis membranes were also evaluated.

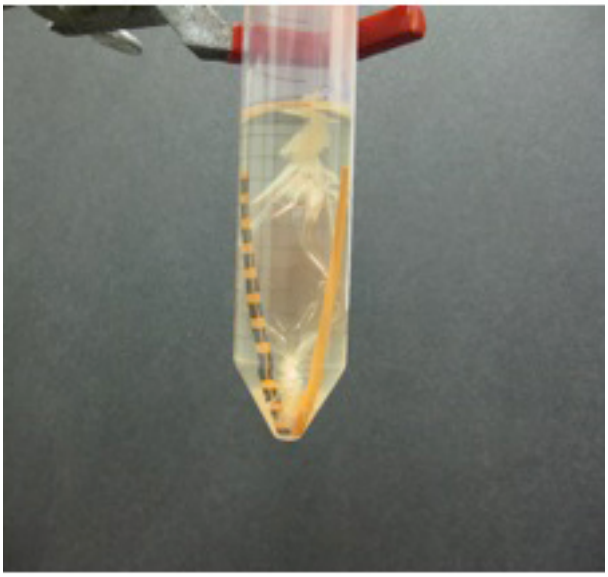

Figure 1: Picture of the test system: cells enclosed in dialysis tubing, placed in peanut oil with a device.

Analysis of Cells

Cytotoxic affects were analyzed using a flow cytometer (FACScan, Becton Dickenson) as previously described[9, 10]. Cells were analyzed according to the side-scattering profile (proportional to cellular granularity) versus the forward-scattering profile (proportional to the cellular cross sectional area). Changes in cell populations were evaluated by gating on the normal cell population and comparing to the test groups over time. The use of propidium iodide dye (propidium iodide is actively excluded from viable, healthy cells) to determine viability was not possible because of the fluorescent background observed when 10 ml of media was placed in with oil and analyzed. There was no background observed in the gated region for dead cells. Experiments were run in triplicate with the exception of range finding experiments. Data are expressed as an average of the percentage of dead cells, and the variability of the assay is expressed as the standard error of the mean[11]. Student t-test was used to evaluate significance.

Devices

Devices used for extraction were latex gloves and GI biopsy forceps. For the latex, 3 different natural rubber latex (NRL) standard examination gloves from 3 different manufacturers were cut into pieces and placed in a polypropylene test tube with oil or media (1 g/10 ml). 5 ml of cells in dialysis tubing were placed directly in the oil or media with the glove pieces. The test tubes were incubated overnight at 37^{o}C. In the preparation of the biopsy forceps, 6 cm pieces of a GI biopsy forceps were placed in either water or a commercially available cleaning agent for devices and diluted as per manufacturer's instructions. 5 pieces of the forceps soaked in

water were placed in a 50 ml polypropylene test tube with peanut oil; 2 pieces were placed in culture media. 5 ml of cells in dialysis tubing were directly added to the polypropylene test tube with the pieces of forceps in oil or media. The identical procedure was followed with pieces of the biopsy forceps soaked in the cleaning agent; the cleaning agent soaked forceps were not rinsed before use.

Evaluation of Hydrotropes and Hydrophilic Compound Diffusion

Tween-20 and PEG were serially diluted in DPBS with 1% acetonitrile to determine any increase in solubility of DEHP. DEHP was initially examined as the test lipophilic analyte due to its low solubility in aqueous solutions and its prevalence in medical device materials. However, because peanut oil came in a plastic container and many plasticizers are ubiquitous low level contaminates, the herbicide atrazine was also used as a model compound to evaluate the movement of a lipophilic analyte from oil into the test cell culture system. The use of acetonitrile with the detergent was essential as stock solutions of DEHP and atrazine needed to be made in organic solvents. The tolerance of the test cells for the various concentration of Tween-20 or PEG in 1% acetonitrile was also examined. To assess the actual concentration of small analytes to pass from either the peanut oil or the media into the dialysis tubing with cells, DEHP and atrazine were spiked into the oil or media and measured using HPLC[12, 13].

Results and Discussion

Initially, the ability of cells to tolerate various oils was examined. 10 ml of Jurkat cells were put inside the dialysis tubing, then the cells were placed in a 50 ml test tube containing 25 ml of cottonseed, olive, or peanut oil (Figure 1). Media was used as the negative control. In addition, cottonseed, olive, or peanut oil was partitioned against an equal volume of DPBS ('washed') to examine if any aqueous components were responsible for any negative effects on the cells. Both the cottonseed and the olive oil were toxic to the cells. "Washing" the oil with DPBS did not acceptably reduce the toxicity of the cottonseed or olive oil to the cells (data not shown). The peanut oil showed minimal toxicity to the cells at 24 hours, but toxicity insignificantly increased after that, $p<0.02$ at 48 hrs and $p<0.002$ at 72 hrs (Figure 2). There was a small, but significant, increase in the percentage of dead cells in the PPE control when compared to that in the PS flasks (data not shown). For the media samples, the increase in dead cells was significant at 72 hrs ($p<0.02$). When comparing the different size dialysis tubing to the PPE control, there was not a significant difference in cell viability.

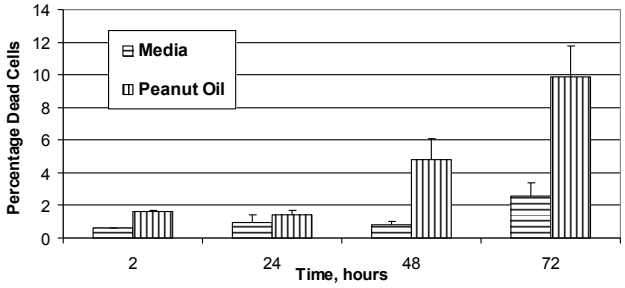

Figure 2: The effect of extraction media on cells over time in the test system. Student t-test indicated statistical significance between the media and oil at 48 hrs ($p<0.01$) and 72 hrs ($p<0.001$).

To evaluate any background fluorescence or micelle formation that would interfere with the flow cytometer, cottonseed, olive, peanut oil, or media were placed in 50 ml test tubes; however, 10 ml of media only was placed in the dialysis tubing. Analysis of these background samples indicated a large fluorescent background in the area where live cells are gated. Nominal background (less than 0.25%) was seen in the area where dead cells were gated, therefore, the percentage of dead cells was used as an indication of toxicity or the forward vs. side scattering profile. Addition of dialysis tubing alone to cells did not exert a toxic affect on the cells (data not shown).

Application of this interface of cells and oil in order to evaluate the cytotoxicity of natural rubber latex gloves is depicted in Figure 3. The gloves extracting in the media showed higher toxicity than the same gloves extracting in peanut oil. Cutting the gloves in pieces probably had no effect on the cell toxicity, and was done to provide a uniform size sample and to prevent air pockets. This system may be useful in looking at surface contamination of devices. Occasionally, reused devices contain residues of the cleaning solution if the devices are not rinsed thoroughly. GI biopsy forceps deliberately contaminated with a cleaning agent then extracted in media showed significantly higher toxicity than those extracted with peanut oil (Figure 4). GI biopsy forceps soaked in water showed no toxicity using either peanut oil or media to extract the device. However, when acetonitrile was added to either media or peanut oil and the toxicity of the cells in dialysis tubing was examined the next day, the cells in peanut oil showed a much larger increase in the percentage of dead cells (Figure 5).

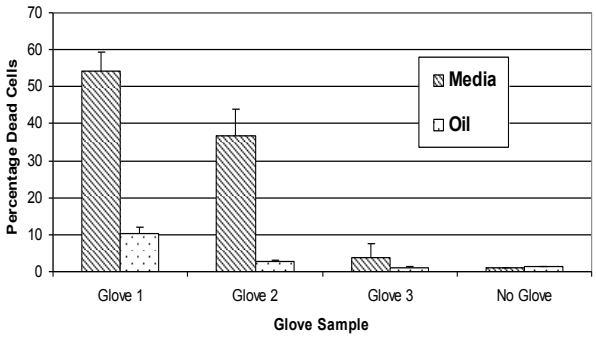

Figure 3: The toxicity of 3 different gloves on the cells in dialysis tubing using either oil or media in extraction. Significant difference was noted between extraction solutions for glove 1 ($p<0.004$) and glove 2 ($p<0.007$).

The use of detergents to aid in the diffusion of lipophilic analytes into the cell culture system was not successful; the solubility of DEHP can be estimated by using the flow cytometer to visualize micelle formation[14]. Toxicity due to a chemical in solution is different than toxicity due to micelles in solution. Cell membrane damage from micelles is due to the solubilization of cell membrane components, which is a separate issue than a soluble chemical toxicity[15]. Using 0.025% Tween-20 with 1% ACN in PBS increased the solubility of DEHP to 25 μg/ml from 5 μg/ml in PBS alone. However, at the 0.025% Tween-20 concentration, the cells died. At the tolerable tween-20 level of 0.00625%, there was no significant increase in DEHP solubility.

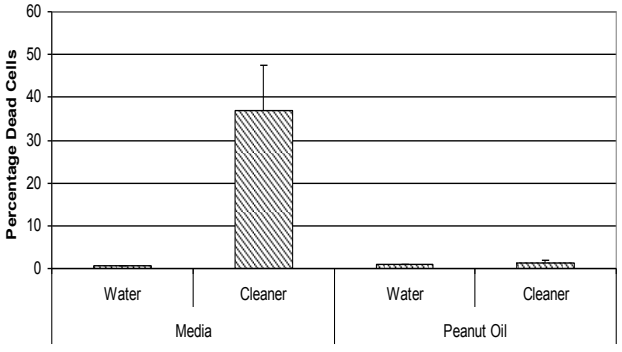

Figure 4: The toxicity of a commercially available enzymatic cleaning agent on cells in test system.

The HPLC analysis of DEHP from peanut oil into dialysis tubing with media alone showed that no detectable DEHP had diffused into the dialysis tubing with media. Atrazine is slightly more water soluble than DEHP and was also examined in diffusing from peanut oil or media into the dialysis tubing with media alone. 15.5 μg/ml of atrazine was present in the dialysis tubing that was placed in media with an initial concentration of 25 μg/ml of atrazine; 0.8 μg/ml of atrazine was present in the dialysis tubing that was placed in peanut oil. The hydrophobic/hydrophilic interaction appears to prevent diffusion across the dialysis from the oil across the dialysis tubing to the cells. The higher concentration of atrazine in the media and the greater toxicity of the cleaning agent in media (Figure 4), would support this hypothesis.

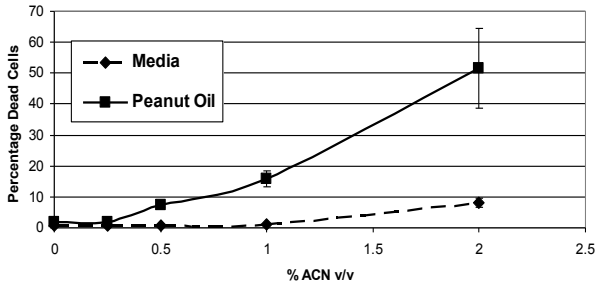

Figure 5: The toxicity of acetonitrile on cells in test system. Significant increase in the toxicity of oil compared to media was seen at and above the 0.05% v/v level ($p<0.005$).

Conclusion

Medical device standards recommend using both a polar and non-polar solvent to extract materials prior to *in vitro* testing. To attempt to address this problem, a system of indirect exposure of cells to oil was developed. By placing cells in dialysis tubing then putting these cells directly in oil, it was expected that exposure to lipophilic compounds would be increased; however, this was not the case. Atrazine, a lipophilic pesticide with limited solubility in water, actually diffused 5 times more from the test system with media than the test system with peanut oil. It is possible other semi-permeable membranes may yield different results. Attempts to increase exposure of the cells to hydrophobic leachates using 2 detergents were examined for their ability to increase the diffusion of lipophilic leachates into the cell culture test system. The use of hydrotropes to increase solubility has been applied to drugs[8] and its application to device leachate testing using this *in vitro* system did not work at concentrations that were non-toxic to cells. This study does illustrate the importance of identifying toxicity due to micelle formation versus that of a soluble chemical(s)

Acknowledgments The authors wish to thank Gail Matson for her editorial expertise, Ann Koustenis for laboratory support, and Dr. Megan Shoff for the technical review. FDA Chief Scientist Challenge Grant partially supported this work.

Abreviations

DPBS	Dulbecco's phosphate buffed saline
DEHP	Diethylhexyl-phthtalate
NRL	Natural rubber latex
PEG	Polyethylene glycol
HPLC	High performance liquid chromatography
PPE	Polypropylene
PS	Polystyrene
GI	Gastrointestinal
ACN	Acetonitrile

References

1. FDA Guidance document. Use of International Standard ISO-10993, 'Biological Evaluation of Medical Devices Part 1: Evaluation and Testing'. http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080735.htm. Accessed January 27 2012.
2. ASTM F 748 – 98, 1998. Standard Practice for Selecting Generic Biological Test Methods for Materials and Devices. In: the Annual Book of ASTM Standards, ASTM West Conshohocken, PA, USA. 1998
3. ASTM F 619 - 03, 2003. Standard Practice for Evaluation of Medical Plastics. In: the Annual Book of ASTM Standards, ASTM West Conshohocken PA (USA). 2003
4. International Standards Organization. Biological evaluation of medical devices - Part 12: Sample preparation and reference materials. In AAMI Standards and Recommended Practices Biological Evaluation of Medical Devices Arlington, VA, USA. 2007
5. International Standards Organization. Biological evaluation of medical devices - Part 5: Testing for cytotoxicity: *in vitro* methods. In AAMI Standards and Recommended Practices Biological Evaluation of Medical Devices Arlington, VA, USA. 1999
6. ASTM F 619 - 79 (97), 1997. Standard Practice for Evaluation of Medical Plastics. In: the Annual Book of ASTM Standards, ASTM West Conshohocken, PA, USA. 1997
7. Lucas, A.D., Lappalainen, S.K., Wray-Cahn, D. (2009). Hyperthermia can increase the cytotoxicity of exogenous compounds. Biomedical Instrumentation and Technology. *43*, 73-79.
8. Sharma. A,, Jain, C.P. (2009). Techniques to Enhance Solubility of Poorly Soluble Drugs: A Review. J of Global Pharma Tech. *2(2)*, 18-28.
9. Godar, D.E., Miller, S.A. Thomas DP. (1994). Immediate and Delayed Apoptotic Cell Death Mechanisms: UVA verses UVB and UVC Radiation. Cell Death and Differentiation. *1*, 59-69.
10. Godar, D.E., Lucas, A.D. (1995). Spectral Dependence of UV-Induced Immediate and Delayed Apoptosis: The Role of Membrane and DNA Damage. Photochemistry And Photobiology. *62*,108-113.
11. Nagele, P. (2001). Misuse of standard error of the mean (SEM) when reporting variability of a sample. A critical evaluation of four anaesthesia journals. British Journal of Anesthesiology. *90(4)*, 514-516.
12. Steinheimer, T.R. (1993). HPLC Determination fo Atrazine and Principal Degradates in Agricultural Soils and Associated Surface and Ground Water. J Agric and Food Chem. *41(4)*, 588-595.
13. Kambia, K., Dine, T., Gressier, B., Gerne, A-F., Luyckx, M., Brunet, C., Michaud, L., Gottrand, F. (2001). High-performance liquid chromatographic method for the determination of di(2-ethylhexyl) phthalate in total parenteral nutrition and in plasma. J. Chromatog. B. *755*, 297-303.
14. McClelland. R,, Pinder. A. (1994). Detection of Salmonella Typhimurium in Dairy Products with Flow Cytometry and Monoclonal Antibodies. Applied and Environmental Microbiology. *60(12)*, 4255-4262.
15. Warisnoicharoen, W., Lansley, A.B., Lawrence, M.J. (2003). Toxicological Evaluation of Mixtures of Nonionic Surfactants, Alone and in Combination with Oil. J of Pharmaceutical Sciences. *92(4)*, 859-868.