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Issues Pertaining to the Interpretation of In Vitro Reactivity

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Introduction

Medical devices sold worldwide are evaluated by cytotoxicity testing. Over the last 15 years cytotoxicity testing has evolved from voluntary standards (*1–4*) to national and international standards (*5–7*). The *USP 23–NF 18*, for example, gives users a choice of in vitro or in vivo methods for the evaluation of elastomeric closures, ophthalmic containers, and transfusion and infusion assemblies. The *USP Elution Test* contained in the general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩ offers pass/fail criteria based on cell damage evaluation after 48 hours incubation and is intended to detect xenobiotics that can be extracted from a polymeric material in an aqueous medium. Xenobiotics can be detected using either saline or tissue culture media as the extraction solvent. Cytotoxicity is graded according to a semiquantitative grading system (scores = 0, 1, 2, 3, or 4). Reactivity at a single dose often preempts further investigation. Although the extract may be diluted and then retested for reactivity, this option is not always employed because of widespread confusion regarding the interpretation of the data.

An additional concern is that the same material may either pass or fail the test as a function of the solvent conditions employed because there are many official conditions that are not equivalent to each other or to the alternative official in vivo reactivity test conditions.

As will be described later, cytotoxicity tests are frequently reactive ("positive") even when all in vivo and human tests are nonreactive ("negative"), and it is the sensitivity of these in vitro tests that appears to be their biggest drawback. There are several factors that contribute to in vitro reactivity:

(1) intrinsic cytotoxicity of the test material,
(2) choice of extraction time,
(3) choice of extraction temperature, and
(4) choice of extraction solvent.

In an attempt to illustrate these factors, we have completed two sets of experiments as follows:

(1) tests in which we compared in vitro and in vivo reactivity against the same test material, and
(2) tests wherein the influence of extraction variables (i.e., solvent, serum, time, and temperature) are compared.

From the experimental data we summarize our results in terms of general toxicological principles; furthermore, new criteria for the interpretation of cell culture data are proposed. The data and ideas presented herein may place into perspective the relationship of cytotoxicity and safety testing in vivo and in humans. It is hoped that the present policy of determining product safety will be reexamined.

Methods

For these studies, we employed the *USP Elution Test* contained in the general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩ and the *Biological Reactivity Tests, In Vivo* ⟨88⟩. These tests, among others, have been recommended over the years by the U.S. Food and Drug Administration (FDA) (*6*). Materials tested in vitro were extracted (4 gram/20 mL) in RPMI 1640 tissue culture fluid with 5% calf serum (complete media) for 24 hours at 37°C (or as specified in the tables), while extracts of the same material for in vivo injection were made at 50°C for 72 hours in both saline and cottonseed oil (CSO) as per the *USP* and International Standards Organization (ISO) methods. Although we recognize that the extracting media were different in the in vitro and in vivo tests (tissue culture fluid versus saline/CSO), the comparison was conducted so as to conform exactly to the parameters of the *USP* or ISO.

Results

Tables 1 and 2 summarize the variety of official conditions available to conduct *USP* in vitro and in vivo reactivity tests. The effects of extraction solvent, extraction time, and extraction temperature on in vitro reactivity for seven elastomeric materials are shown in Figure 1 and Table 3.

One hundred twenty-six elastomeric materials were tested with the *USP Elution Test* for cytotoxicity, and 48 were reactive (38%, Table 4). Seventy-six of these materials (including the 48 referred to above) were also tested for in vivo reactivity. The in vivo reactivity rates were 3% and 0% (intracutaneous test and implantation test, respectively). Three categories of response were observed. The two *Class I* plastic test samples (the *USP* general test chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩ describes six *USP Plastic Classes* in *Table 1. Classification of Plastics*) were noncytotoxic but were biologically reactive in vivo by the intracutaneous (cottonseed oil) route, but negative in the intramuscular route. The single *Class II* plastic sample tested was both cytotoxic and biologically reactive intracutaneously. The 33 *Class III* plastic samples tested were cytotoxic but not otherwise biologically reactive. Twenty-three of the 33 samples that were cytotoxic were further characterized in order to determine the noncytotoxic concentration (Table 4).

In the case of an aortic balloon (latex) material that was tested as per biocompatibility requirements of the FDA, in vitro reactivity was not diminished by exhaustive wash-

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ing techniques, yet the material conformed to the *USP Class VI* plastics criteria, extended to include a seven-day implant (Table 5).

Discussion

The data in Tables 1 and 2 illustrate the variety of *USP* options for extraction and preparation of test materials. The *USP* does not give definitive guidance on the use of these extraction options: the user selects the conditions. The data in Table 3 and Figure 1 demonstrate with elastomeric materials how the variety of extraction conditions (i.e., extraction time and temperature, and serum and solvents used in extraction) can lead to significantly different in vitro reactivity. In our experience, the optimal extraction temperature for performing cytotoxicity testing of medical devices is 50°C for 72 hours (1) because the extraction is exhaustive, and (2) the temperature facilitates leaching of xenobiotics without damaging heat-labile xenobiotics. The data in Table 4 show, again with elastomeric materials, the lack of correlation between in vitro and in vivo reactivity. The data in Table 5 show that latex, used as part of a marketed, safe, cardiac balloon catheter, is also highly reactive in vitro but nonreactive in vivo. These data illustrate the potential confusion associated with *USP* interpretation criteria. This FDA-accepted invasive material does not conform to the requirements for *USP* ophthalmic containers.

The *USP* criteria need to recognize that materials may vary in their relative in vitro reactivity. Some materials that are safe are reactive in vitro but not in vivo. In the results presented here (Table 4), 33 elastomeric samples were reactive in vitro but only one was reactive in vivo. In our experience we have never observed an elastomeric material to be both reactive in vitro and reactive by the systemic route, in vivo. Furthermore, of the three samples that were reactive in vivo, two were not reactive in vitro (a 66% rate of error). We suggest that the in vitro test must be interpreted in terms of an acceptable threshold dose and related to an in vivo system so as to provide meaningful safety data.

A course of action for situations such as described above is to determine the level (X) of cytotoxicity and the extent, if any, of any corresponding in vivo reactivity. When there is no in vivo reactivity detected, the in vitro test may be used for routine monitoring of reactivity with the understanding that the expected level of in vitro reactivity should not be more than X. A batch or lot exceeding the limit of X would suggest an increased potential for toxicity and, perhaps, a manufacturing process drifting away from validated conditions. An accept/reject system such as this can be put in place for every category of material evaluated by the *USP* general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩ methodology. In this way, the in vivo data are used to establish the in vitro criteria, thus eliminating the need for subsequent routine in vivo testing.

The concept of the "degree" of cytotoxicity as opposed to "presence" of cytotoxicity, as shown in these experiments, is an extension of the suggestions made in previous studies with parenteral drugs (*8*, *9*) and topical formulations (*10*). In those studies it was demonstrated that intramuscular irritation testing and ocular irritation testing were useful calibrators of the pass/fail criteria that could be subsequently assigned to routine dose-response cell culture assays, and that for Valium® and hydrocortisone acetate formulations, the presence of in vitro cytotoxicity could not be viewed as an indicator of in vivo toxicity because the formulations had an established record of clinical safety.

In vitro reactivity was inversely related to extraction temperature in the materials examined (Fig. 1). Significantly more reactivity was noted when tissue culture medium containing 5% calf serum was used for extraction at 37°C as compared to when saline was used at 50°C, 70°C, or 121°C. Test materials that were extracted in tissue culture medium without calf serum were significantly more reactive at 50°C and 70°C than when extracted in physiological saline (Table 3). Since it was not clear whether the observed in vitro reactivity represented a positive or negative finding, a series of comparative tests were performed utilizing both in vitro and in vivo systems (Table 4). All the test materials were elastomeric. The *USP Intracutaneous Test* contained in the general test chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩ using cottonseed oil (CSO) was chosen as the in vivo reference assay because we have found it to be the most frequently positive component of the Class VI test for plastics. Reactivity was substantially less in vivo than in vitro when grading was performed as per the *USP*. In the in vivo experiments, reactivity was only observed when cottonseed oil was the solvent.

In regard to understanding toxicological mechanisms that may be involved, a comparison of in vivo and in vitro data is problematic because of the differences in sample preparation and the potential for metabolism in vivo to a more or less toxic xenobiotic. Sample preparation achieves separation of the extractives from the materials according to physicochemical properties such as solubility and chemical partitioning. The in vitro assays are essentially concentration assays with a target cell that lacks the enzymes for in vivo metabolism. The data from the two elastomeric materials that were reactive in vivo, but not in vitro, suggest that the extracted xenobiotic is metabolized to a more toxic agent. For these reasons we question why *USP* specifies that transfusion and infusion assemblies may be tested either in vitro or in vivo. The conclusions reached are likely to be different based on whether an in vivo or in vitro result is used.

In view of the data presented here, it appears that more research is necessary. The chemical nature of the extractives from elastomeric and rigid device polymers that produce the true-positive, false-positive, and false-negative cell culture is an important object of future study. Furthermore, the effects of differing extraction conditions (sol-

vent, time, and temperature—see Table 3) are critical determinants of reactivity that must be more fully evaluated. If such studies are undertaken, steps will be required to minimize interlaboratory variation such as standardization of extraction time, extraction temperature, and extraction medium. A collaborative study would facilitate proper decision making related to device biocompatibility.

In vitro data must not be viewed alone. Rejection criteria based on single-dose cytotoxicity as referenced in the *USP* general test chapter *Containers* ⟨661⟩ on plastic containers should be revisited, and guidance on how to interpret the testing of more diluted materials (as allowed in both the *USP* and ISO methods) should be provided. It is appropriate to examine critically the present *USP* specifications for monograph materials. Three different requirements exist as follows:

(1) The *USP* general test chapter on *Elastomeric Closures for Injection* ⟨381⟩ specifies that elastomeric closures for injection meet the requirements if they comply with the standards specified under the *USP* general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩, or if they do not meet the in vitro specifications, they must be further tested systemically according to the requirements of the *USP* general test chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩.

(2) The *USP* general test chapter *Transfusion and Infusion Assemblies and Similar Medical Devices* ⟨161⟩ specifies that transfusion and infusion assemblies meet the requirements if they comply with the standards under the *USP* general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩ or the standards under *USP* general test chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩.

(3) The *Containers for Ophthalmics—Plastics* section of the *USP* general test chapter *Containers* ⟨661⟩ specifies through cross-reference that containers for ophthalmics meet the requirements if they comply with the standards under the *USP* general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩ and cannot be released based on the standards under *USP* general test chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩.

Recommendations

- Perform a collaborative study.
- Define which extraction system is most appropriate for materials covered in the *USP*, and eliminate unnecessary options.
- Standardize interpretation criteria for materials covered in the *USP*.
- Modify the *USP* general test chapter *Biological Reactivity Tests, In Vivo* ⟨88⟩ to allow ophthalmic containers to be tested and released based on the *USP XXII Eye Irritation Test* adopted in the *Fifth Supplement* and deleted in the *Ninth Supplement*.
- Provide an information chapter on the development of dose response data and correlation of in vitro reactivity to in vivo reactivity.

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APPENDIX I

A list of terms and their definitions as used in this work.

Biocompatibility test: A series of in vitro and in vivo tests designed to determine the safety of an article and usually restricted to devices and their components; identical to the terms "toxicology" and "safety testing" which are used in the chemical and pharmaceutical industries.

Systemic effect: A toxic effect on internal body organs induced by injection or absorption through the skin or lungs.

Tripartite: A document on biocompatibility testing subscribed to by the United States, Canada, and the United Kingdom promulgated in 1987 (available from the US FDA, Division Small Manufacturers' Assistance Center for Devices and Radiological Health, Rockville, MD).

Xenobiotic: A substance obtained from an extraction of a medical device which is responsible for biological reactivity.

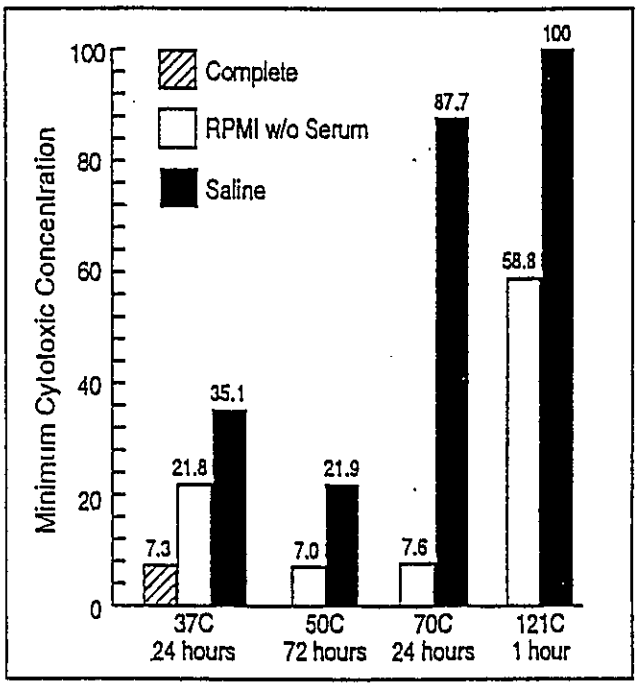

Fig. 1. Comparative cytotoxic titers versus extraction conditions average of seven reactive elastomeric materials in three extract systems.

NOTE—Saline at 121 °C was not reactive (the smaller the number the greater the reactivity).
The highest titers were obtained from extracts made from tissue culture fluid media, with or without serum.

Table 1. *USP* Biological Reactivity Test Parameters.

Extraction Media	Extraction Temperature	Extraction Time (hr)	Route of Administration	Remarks
1. Saline	50°C	72	iv,* ic†	
2. Saline	70°C	24	iv, ic	
3. Saline	121°C	1	iv, ic	High temperature will destroy heat-labile toxicants or volatile toxicants.
4. Saline	Same as 1 to 3 above	Same as 1 to 3 above	In vitro	The extract is diluted 1 to 4 in 1X tissue culture medium containing 5% serum of 1:2 in 2X tissue culture medium containing 5% serum after extraction.
5. Tissue Culture Medium	Same as 1 to 3 above	Same as 1 to 3 above	In vitro	The extract is not diluted as in number 4 above.
6. Tissue Culture Medium containing 5% serum	37	24	In vitro	No extraction dilution as in number 4 above.
7. 5% Ethanol in Saline	Same as 1 to 3 above	Same as 1 to 3 above	iv, ic	
8. Vegetable oil and PEG 400‡	Same as 1 to 3 above	Same as 1 to 3 above	ip,‡ ic‡	PEG§ is reactive with certain polyvinyl chloride additives (phthalates) upon intracutaneous injection.

* intravenous (iv)
† intracutaneous (ic)
‡ = PEG 400 extract is diluted 4.1 to 1 with saline prior to intraperitoneal (ip) injection, and 7.4 to 1 with saline prior to intracutaneous (ic) injection.
§ Data on file at Gibraltar Laboratories, Inc.

Table 2. Test Material Quantities per 20-mL Solvent.

1. Plastic Sheets	$\leq$ 0.5 mm thick, 120 cm^2
2. Plastic Sheets	$>$ 0.5 mm thick, 60 cm^2
3. Elastomeric closure	25 cm^2
4. Elastomeric closure when surface area cannot be determined	2 g
5. Plastics when surface area cannot be determined	4 g
6. Transfusion and Infusion Assemblies	4 g/20 mL
7. Ophthalmic Devices	4 g/20 mL

Table 3. Minimum Concentration (in Percent) of Elastomeric Material Extracts That Was Not Cytotoxic (Grade Is ≤2).

Sample	USP 37°C 24 Hr	37°C 24 Hr	37°C 24 Hr	37°C 72 Hr	37°C 72 Hr	37°C 120 Hr	37°C 120 Hr	USP 50°C 72 Hr	USP 50°C 72 Hr	USP 70°C 24 Hr	USP 70°C 24 Hr	USP 121°C 1 Hr	USP 121°C 1 Hr
	Complete medium	RPMI	Saline	RPMI	Saline	RPMI	Saline	RPMI	Saline	RPMI	Saline	RPMI	Saline
1. 65636	6.25	>25	25	12.5	12.5	12.5	6.25	6.25	12.5	6.25	>25	>25	>25
2. 66900	3.12	6.25	6.25	NT	NT	6.25	NT	3.12	6.25	3.12	>25	>25	>25
3. 66911	6.25	>25	>25	>25	>25	12.5	>25	12.5	>25	12.5	>25	>25	>25
4. 66910	3.12	6.25	12.5	>25	>25	12.5	6.25	6.25	12.5	12.5	>25	>25	>25
5. 68453	6.25	>25	>25	6.25	6.25	NT	NT	3.12	6.25	1.56	>25	>12.5	>25
6. 77786	6.25	6.25	>25	25	12.5	NT	NT	6.25	>25	12.5	>25	>25	>25
7. 68896	1.56	6.25	6.25	6.25	6.25	NT	NT	1.56	6.25	3.12	6.25	6.25	>25

NOTES—

- For 6 of the 7 samples tested, the greatest extent of reactivity was observed with complete medium at 37°C for 24 hours, and in all eight of the samples the least reactivity occurred with saline at 121°C for 1 hour. The results illustrated that even the most reactive material (#7) can appear nonreactive at 121°C in saline, which is a difference of reactivity of at least 16 times or 400%. Boldface indicates the condition with the greatest degree of reactivity.
- NT = not tested.

Sample results

Sample 1: The official *USP* conditions give substantially different results. A heat labile xenobiotic shows no reactivity in saline at 70°C or 121°C. Reactivity in saline and RPMI increases with extraction time and temperature; in RPMI, reactivity is not observed at 37°C (24 hours) and at 121°C.

Sample 2: The official *USP* conditions give substantialy different results. Reactivity in RPMI is greater at 50°C and 70°C than at 37°C. Reactivity in saline is the same at 37°C for 24 hours as at 50°C for 72 hours. Reactivity in saline is not seen at 70°C nor with either saline or RPMI at 121°C.

Sample 3: No reactivity was observed with saline. The same reactivity was observed at 50°C or 70°C with RPMI.

Sample 4: Reactivity with RPMI or saline is constant or is diminished as the temperature of extraction increases from 37°C to 121°C.

Sample 5: Reactivity is greatest with RPMI at 70°C for 24 hours and is reduced by eightfold at 121°C.

Sample 6: Reactivity is greatest at 37°C and 50°C and is diminished at 70°C and is absent at 121°C.

Sample 7: Reactivity is greatest in complete medium at 37°C for 24 hours and in RPMI at 50°C for 72 hours and is diminished by at least fourfold at 121°C.

Table 4. Reactivity of Elastomeric Materials.

1. In vitro complete medium with 5% calf serum (24 hours at 37°C)

number tested	number nonreactive	number reactive
126	78 (62%)	48 (38%)

2. Endpoint analysis: determination of the nonreactive in vitro extract concentration

	concentration of extract				
number tested	50%	25%	12.5%	6.25%	3.12%
23	7	7	1	6	2

Remark: If the equivalent of the saline extraction conditions had been used here the extract would likely contain 75% less xenobiotics and an additional 14 samples would be graded as nonreactive (i.e., ≤2).

3. In vitro and intracutaneous test

	in vitro		in vivo (CSO)	
number tested	number nonreactive	number reactive	number nonreactive	number reactive
86	53 (62%)	33 (38%)	83 (97%)	3* (3%)

Remark: All 86 materials were nonreactive when tested in vivo with saline.

* One sample of the three was also reactive in vitro.

Table 5. Absence of an In Vitro Threshold Dose: Intra-aortic Balloon.

Concentration of Extract Tested	In Vitro Cytotoxicity Results: Time of Hot Water Wash (minutes)				In Vivo *Class VI* Biocompatibility Results: Irritation and Systemic Effects With Undiluted Extract			
	45	60	120	180		Test	Control	Difference
neat	4*	4*	4*	4*	Saline	0.21	0.13	0.08
12.5%	4*	4*	4*	4*	EtOH/ saline	0.04	0.33	0.29
6.25%	4*	3*	4*	3*	PEG 400	0.04	0.00	0.04
3.125%	2*	1*	2*	2*	CSO	0.88	0.50	0.38
1.5%	0*	0*	1*	1*				

* Numerical grades as defined in the general test chapter *Biological Reactivity Tests, In Vitro* ⟨87⟩ in *Table 2. Reactivity Grades for Elution Test.*

NOTES—
- 0 = No cytotoxicity noted in L-929 cell culture in vitro assay; no in vivo local or systemic toxicity noted (intracutaneous, intramuscular, intravenous, and intraperitoneal assays in mice and rabbits).
- Dilutions were made with sterile RPMI 1640 containing 5% calf serum.

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