



American National Standard/American Dental Association
Specification No. 41

Recommended Standard Practices for Biological Evaluation of Dental Materials

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AMERICAN NATIONAL STANDARD/AMERICAN DENTAL ASSOCIATION SPECIFICATION NO. 41 FOR RECOMMENDED STANDARD PRACTICES FOR BIOLOGICAL EVALUATION OF DENTAL MATERIALS

The Council on Scientific Affairs of the American Dental Association has approved American Dental Association Specification No. 41 for Recommended Standard Practices for Biological Evaluation of Dental Materials. This and other specifications for dental materials, instruments and equipment are being formulated by working groups of the ADA Standards Committee on Dental Products (formerly Accredited Standards Committee MD156 for Dental Materials, Instruments and Equipment). The Committee has representation from all interests in the United States in the standardization of materials, instruments and equipment in dentistry. The Council has adopted the specifications, showing professional recognition of their usefulness in dentistry, and has forwarded them to the American National Standards Institute with a recommendation that the specifications be approved as American National Standards. The American National Standards Institute granted approval of ADA Specification No. 41 as an American National Standard on December 22, 2005.

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FOREWORD

(This Foreword does not form a part of ANSI/ADA Specification No. 41 for Recommended Standard Practices for Biological Evaluation of Dental Materials).

This Specification is a revision of ANSI/ADA Specification No. 41, which was approved in 1979, and ANSI/ADA Specification No. 41a, an addendum that was approved in 1982. Both documents were reaffirmed in 2001.

This revision was prepared by the ADA Standards Committee on Dental Products (SCDP) Joint Working Group 1.

The objective of this Specification is to provide detailed guidelines and methodologies for evaluating the biocompatibility of dental materials following International Standard ISO 7405-1997, Dentistry — preclinical evaluation of biocompatibility of medical devices used in dentistry—test methods for dental materials. Therefore, the document is intended to serve as an annex to ISO 7405-1997 for the benefit of United States dental professionals, scientists and manufacturers.

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1 SCOPE

This document covers standard practices for the biological evaluation of the safety of dental materials, including those with pharmacological agents as an integral part of the material.

2 INTRODUCTION

Biological evaluations of medical devices, including dental materials, are performed to determine the potential toxicity resulting from contact of the component materials of a device with the body. The device materials should not (either directly or through the release of their material constituents): (i) produce adverse local or systemic effects; (ii) be carcinogenic; or, (iii) produce adverse reproductive and developmental effects. Therefore, evaluation of any new dental materials intended for human use requires data from systematic testing to ensure that the benefits provided by the final product will exceed any potential risks produced by device materials.

When selecting appropriate tests for the biological evaluation of a dental material, one must consider the chemical characteristics of device materials and the nature, degree, frequency and duration of its exposure to the body. In general, the tests include: acute, sub-chronic and chronic toxicity; irritation to skin and oral mucosal surfaces; sensitization; genotoxicity; carcinogenicity; and usage tests. Depending on characteristics and intended uses of the dental materials, as well as the nature of contact, these general tests may not be sufficient to demonstrate the safety of some specialized dental materials. Additional tests for specific target organ toxicity, such as neurotoxicity and immunotoxicity may be necessary. The specific clinical application and the materials used determine which tests are appropriate.

Some devices are made of materials that have been well characterized chemically and physically in the published literature and have a long history of safe use. For purposes of demonstrating substantial equivalence of such dental materials to other marketed products, it may not be necessary to conduct all tests suggested in the biocompatibility test matrix. Scientific judgment should be used to determine which tests are appropriate.

ISO 10993 Standard, Part 1, uses an approach to test selection that is similar to the Tripartite Guidance, which it replaced. This revision also uses a tabular format (matrix) for laying out test requirements based on the factors discussed above. The matrix consists of two tables: Table 1 - Preclinical Evaluation Tests for Biocompatibility of Dental Materials, and Table 2 - Supplemental Tests Specific for Dental Materials. Information in these tables will assist in determining appropriate toxicology tests for dental materials. Each device will be considered on its own merits, and while not all tests listed are applicable for all dental materials, additional tests not indicated in the tables may be necessary to determine safety. The test matrix is only a framework for selecting tests, not a checklist of every required test. Manufacturers should determine the appropriate tests prior to the initiation of expensive, long-term testing of any new dental materials. A justification for the choice of all methods should be included in the test report. This is of particular importance when methods not included in this International Standard are used. All dental materials should be re-evaluated for biocompatibility when substantial revisions or modifications in formulation, quality, and/or performance specifications are made.

The current documents describing selection of tests for biocompatibility are ISO 10993-1 and the U.S. FDA Blue Book Memorandum, G95-1; tests specific to dental materials are described in ISO 7405). Excerpts from these documents are included to provide a framework for selection of tests needed to determine biocompatibility of dental materials. Should these documents be amended, the most recent version should be consulted. The blue book memorandum G95-1 entitled "Use of International Standard ISO-10993, Biological Evaluation of Medical Devices Part-1: Evaluation and Testing," includes an FDA-modified matrix that designates the type of testing needed for various medical devices

for United States submissions. For biocompatibility evaluation, medical devices are classified by duration and type of contact applicable to dental materials.

It is strongly recommended that laboratory and animal studies be conducted in compliance with Good Laboratory Practice (GLP) regulations and human studies in compliance with Good Clinical Practice (GCP) guidelines.

3 NORMATIVE REFERENCE

- 3.1 U.S. FDA Blue Book Memorandum #G95-1, ISO-10993-1, Biological Evaluation of Medical Devices Part 1: Evaluation and Testing.

(U.S. FDA Blue Book Memorandum #G95-1 is available at: <http://www.fda.gov/cdrh/g951.html>).

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

4 DEFINITIONS

- 4.1 Surface-contacting device: Includes those that contact the surface of intact or breached skin, the surface of intact or breached oral mucosa, and those that contact the external surfaces of dental hard tissue, including enamel, dentin and cementum. Dentin and cementum are considered as surfaces, e.g., after gingival recession.
- 4.2 External communicating device: Includes dental devices that penetrate and are in contact with oral mucosa and dental hard tissues. Dental pulp tissue or bone, or any combination of these, and are exposed to the oral environment.
- 4.3 Implant device: Includes dental devices and implants that are partially or fully embedded within the soft tissue, bone or pulpodentinal system of the tooth, or any combination of these, and are not exposed to the oral environment.
- 4.4 Limited exposure device: Device whose single or multiple use or contact is likely to be up to 24 h.
- 4.5 Prolonged exposure device: Devices whose single, multiple or long-term use or contact is likely to exceed 24 h but not 30 days
- 4.6 Permanent contact device: Device whose single, multiple or long-term use or contact exceeds 30 days.

5 SELECTION OF TESTS

- 5.1 The types of biocompatibility tests to be conducted are divided into three groups. Group I comprises *in vitro* tests of cytotoxicity. General guidance for *in vitro* cytotoxicity tests is provided in ISO 10993-5. Although the use of dentin barrier tests is encouraged for *in vitro* cytotoxicity tests, the order of listing does not indicate any preference for one method over another. Group II tests are *in vivo* tests, including acute systemic toxicity (oral and inhalation), subchronic systemic toxicity (oral and inhalation), skin irritation and intracutaneous reactivity, sensitization, genotoxicity, local effects after implantation. In evaluating materials following implantation, examination of undemineralized sections, in addition to routine demineralized sections, is recommended. Group III comprises dental materials-specific tests not referred to in ISO 10993: pulp and dentin usage test, pulp capping test, and endodontic usage test. Guidance for these tests can be found in ISO 7405:1997(E). Detailed test protocols for methods appropriate to dental materials in all three categories are included in this standard.
- 5.2 When possible, alternative tests are provided to provide options that may be better suited for different types of products. These tests are identified as alternative tests. Some of these tests may not satisfy regulatory requirements of government agencies. Only a test that has been validated shall be considered in the process of test selection.

Table 1. Preclinical evaluation tests for biocompatibility of dental materials

Device Category	Contact duration	Biological Effect									
		Group I	Group II								
			Cytotoxicity ¹	Systemic Toxicity (acute, oral)	Systemic Toxicity (acute, inhalation)	Subchronic Oral Toxicity	Irritation or Intracutaneous Reactivity	Sensitization	Subchronic Inhalation Toxicity	Genotoxicity	Implantation
Surface devices (mucosal membrane)	≤24h	X		X		X		X			
	>24h-30d	X	O	X	X	X		X	X		O
	>30d	X	O	X	X	X		X	X		O
Breached or compromised surfaces	≤24h	X	O	X		X					
	>24h-30d	X	O	X	X	X		X	X		O
	>30d	X	O	X	X	X		X	X		O
External communicating devices	≤24h	X ²	X	X		X					
	>24h-30d	X ²	X	X	X	X		X	X		X
	>30d	X ²	X	X	X	X		X	X		X
Tissue/bone/dentin communicating implant devices ³	≤24h	X	O			X					
	>24h-30d	X	O		X	X		X	O		X
	>30d	X	O		X	X		X	O		X ⁴

X = ISO evaluation tests.

O = Additional tests that may be applicable in the United States.

¹ Extract, direct contact, or diffusion.² Dentin barrier test may also be required.³ Tissue includes tissue fluids and subcutaneous spaces.⁴ Chronic toxicity and/or carcinogenicity tests may also be required.

Table 2. Supplemental tests specific for dental materials

Device Category	Contact duration	Biological Effect		
		Group III		
		Pulp and Dentin Usage	Pulp Capping	Endodontic Usage
Surface devices (mucosal membrane)	≤24h			
	>24h-30d			
	>30d			
	>24h-30d			
	>30d			
External communicating devices	≤24h	X		
	>24h-30d	X		
	>30d	X		
Tissue/bone/dentin communicating implant devices ¹	≤24h		X	X
	>24h-30d		X	X
	>30d		X	X

X = ISO evaluation tests.

¹ Tissue includes tissue fluids and subcutaneous spaces.

6 REPORT OF RESULTS

The test report should include a complete record of all procedures followed, details of the preparation and methods of application of the test material, and all results obtained and any other data necessary for the assessment of results.

A Test substance

- 1 Manufacturer, source, purity, and lot number of test substance
- 2 Physical nature, purity and physicochemical properties, e.g., pH and buffering capacity; volatility; solubility; stability
- 3 If mixture, composition and relative percentages of components

B Vehicle (if applicable)

- 1 Identification, concentration (where appropriate)
- 2 Justification for choice of vehicle

C Test system and experimental conditions

- 1 Cell culture or microbial system
 - (i) Species/strain of cell line or microbial system used
 - (ii) Source of cell line or microbial system

- (iii) Description of culture conditions
 - (iv) Test conditions, including dosage, exposure time and frequency, and evaluation interval and methods
- 2 Animal
 - (i) Species/strain used
 - (ii) Number, age, sex and weight
 - (iii) Source of animals and housing conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of the diet of test animals, etc.
 - (iv) Feed and water consumption data, when applicable
 - (v) Test conditions, including dosage, exposure time and frequency, sampling conditions, and evaluation interval and methods
- 3 Human subject
 - (i) Approval of Institutional Review Board (IRB) and evidence of informed consent of study subjects
 - (ii) Demographic information, including age, gender, and ethnicity
 - (iii) Medical and dental history, when appropriate
 - (iv) Test conditions, such as dosage, exposure time and frequency, sampling conditions, and evaluation interval and methods
- D Results
 - 1 Tabulation of collected data, with mean, standard deviation, and individual values
 - 2 Statistical analysis of data when applicable, including methods, power, significance and p value
 - 3 Interpretation of results
- E Conclusions
- F References

Cited in the body of the report , including references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

7 REFERENCES

1. Meryon, S.D. The model cavity method incorporating dentin. *Int. Endod. J.* 21: pp. 79-84; 1988.
2. Hanks, C.T., Diehl, M.L., Craig, R.G., Makinen, P.L. and Pashley, D.A. Characterization of the "in vitro pulp chamber" using the cytotoxicity of phenol. *J. Oral Pathol.* 18: pp. 97 –107;1989.
3. Schmalz, G. and Schweikl, H. Characterization of an *in vitro* barrier test using a standard toxicant. *J. Endod.*, 20: pp. 592-594;1994.
4. Bruce, R.O. *Fundam. Appl. Toxicol.* 5: pp. 151-157;1985.

5. Christen, O., Read, C., Neroni, M., Thoenen S. and Holz, J. Modele substitutif d'essai biologique *in vitro*. I. Methode normalisée a l'aide de cellules pulpaies humaines. *J. Biol. Buccale* 17: pp. 275-284;1989.
6. Trantor, I.R., Messer, H.H., Birner, R. The effects of neuropeptides (calcitonin gene-related peptide and substance P) on cultured human pulp cells. *J. Dent. Res.*, 74: pp. 1066-1071; 1995.
7. Schmalz, G., Garhammer, P. and Schweikl, H. A commercially available cell culture device modified for dentin barrier tests. *J. Endod.*, 22: pp. 249-252;1996.
8. U.S. Pharmacopoeia-National Formulary.

USE OF ANIMALS, USE OF HUMAN SUBJECTS, AND PERSONAL PROTECTION

1 USE OF ANIMALS

Similar to the importance of the protection of human subjects in scientific research, it is imperative that animal welfare be ensured to minimize the number and exposure of laboratory animals. Studies involving animals must follow pertinent guidelines, e.g., ISO 10993-2: Animal Welfare Requirements and federal laws, e.g., Animal Welfare Act. In addition, such studies should be conducted following Good Laboratory Practice (GLP) regulations. The following list provides general principles to ensure minimum requirements for the use of vertebrate animals in biological testing of dental materials.

- Animal experiments should not be performed until appropriate *in vitro* tests have been conducted (if available). Animal tests should not be performed if *in vitro* tests clearly show that a material or device is toxic or harmful.
- The design of animal experiments should be appropriate to meet the desired objectives.
- Efforts should be made to prevent unnecessary repetition of similar animal tests.
- All persons involved in performing animal experiments should be adequately trained in the humane care of the animal species being used, pertinent guidelines and legislations, and the scientific aspects of the research being conducted.
- Care and handling of the animals should conform to accepted animal husbandry guidelines.
- All experimental procedures, including surgeries and pre- and post-surgical care should follow established veterinary medical and nursing practices.
- Pilot experiments should be performed to allow planning of a minimum number of experiments to provide the required result. If in a standard test the minimum number of animals required is given, that number takes precedence.
- Use of the minimal number of animals possible in the experiment is strongly encouraged; however, this should not preclude use of an adequate sample size in order to yield meaningful data.
- Experimental design should consider using the least invasive test methods in an animal and/or reducing animal experiments by using less invasive methods in the same animal.
- In general an animal should not be used for more than one experiment in a series. The need to avoid undue suffering in the animals used should take precedence over the need to reduce the number of animals used.
- Methods of euthanasia used in animal experiments should produce rapid unconsciousness and subsequent death without evidence of pain or distress.

2 USE OF HUMAN SUBJECTS

All studies that involve human subjects should follow the principles of Good Clinical Practice (GCP). Such studies require that human subjects be adequately informed about the nature of a clinical trial in which they participate and the study be approved by an Institutional Review Board (IRB) or Independent Ethics Committee (IEC) prior to enrolling subjects. The ICH Harmonised Tripartite Guideline For Good Clinical Practice (ICH E6) should be followed when conducting human studies. The principles of the ICH GCP are as follows.

- Clinical trials should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, the Belmont Report, and that are consistent with GCP and the applicable regulatory requirement(s).

- Before a trial is initiated, foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial subject and society. A trial should be initiated and continued only if the anticipated benefits justify the risks.
- The rights, safety, and well-being of clinical trial subjects are the most important considerations and should prevail over interests of science and society.
- All available nonclinical and clinical information on an investigational product should be adequate to support the proposed clinical trial.
- Clinical trials should be scientifically sound, and described in a clear, detailed protocol.
- A trial should be conducted in compliance with a protocol that has received prior IRB or IEC approval or favorable opinion.
- The medical care given to, and medical decisions made on behalf of, subjects should always be the responsibility of a qualified health professional.
- Each individual involved in conducting a trial should be qualified by education, training, and experience to perform his or her respective task(s).
- Informed consent should be obtained from every subject prior to the clinical trial.
- All clinical trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification.
- The confidentiality of records that could identify subjects should be protected by respecting the privacy and confidentiality rules in accordance with regulatory requirement(s).
- Investigational products should be manufactured, handled, and stored in accordance with applicable good manufacturing practice (GMP). They should be used in accordance with the approved protocol.
- Procedures that assure the quality of every aspect of the trial should be implemented.
- The IRB/IEC should safeguard the rights, safety, and well-being of all trial subjects. Special attention should be paid to trials that may include vulnerable subjects.
- Adverse events should be reported according to severity. All serious adverse events (SAEs) should be reported immediately to the sponsor except for those SAEs previously identified as not needing immediate reporting. Immediate reports should be followed promptly by detailed, written reports. Immediate and follow-up reports should identify subjects by unique code numbers assigned to the trial subjects rather than by the subjects' names, personal identification numbers, and/or addresses. The investigator should comply with regulatory requirements related to reporting unexpected serious adverse drug reactions to regulatory authority(ies) and the IRB/IEC.
- An Investigator's Brochure should provide a description of possible risks and adverse drug reactions anticipated on the basis of prior experiences with the product under investigation and with related products. A description should also be provided of precautions or special monitoring to be done as part of the investigational use of the product(s).

3

PERSONAL PROTECTION

Persons engaged in the conduct of clinical and nonclinical studies should take necessary personal sanitation and health precautions to avoid contamination of themselves, human subjects, test and control articles, and test systems. Clothing should be worn that is appropriate for the duties they perform, and such clothing should be changed as often

as necessary to prevent biological, radiological, or chemical contamination. Any individual found to have an illness that may adversely affect the quality and integrity of a study should be excluded from direct contact with any operation or function of a study that may adversely affect the study until the condition is corrected. All personnel should report any health or medical conditions that may reasonably be considered to have an adverse effect on a study. Examples of personal protection include safety eyewear, impermeable gloves, facemasks, and protective outer garments. Specific items required for use depend on the nature of the study being conducted and the associated hazards.

4

REFERENCES

1. Animal Welfare Act as Amended (7 USC, 2131-2156).
2. Code of Federal Regulations Title 9, Volume 1, Parts 1 to 199 Revised as of January 1, 1999, Animals and Animal Products.
3. Public Health Service Policy on Humane Care and Use of Laboratory Animals, Office of Laboratory Animal Welfare, Revised September, 1986, Reprinted October, 2000.
4. Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996 by the National Academy of Sciences).
5. Report of the AVMA Panel on Euthanasia 2000 Report of the AVMA Panel on Euthanasia JAVMA, 218(5) 669-696, 2001.
6. 21CFR Part 58, Good Laboratory Practice For Nonclinical Laboratory Studies.
7. ICH E6, Harmonised Tripartite Guideline For Good Clinical Practice.
8. 21CFR Part 50, Protection of Human Subjects.
9. 21CFR Part 56, Institutional Review Boards.
10. 21 CFR Part 812, Investigational Device Exemptions.
11. 21 CFR Part 820, Good Manufacturing Practices.
12. 45 CFR Part 46, Protection of Human Subjects.
13. Declaration of Helsinki.
14. Health Insurance Portability and Accountability Act (HIPAA) 1996.

PREPARATION OF SAMPLES OF DENTAL MATERIALS

1 GUIDELINES FOR SAMPLE PREPARATION

1.1 General considerations

Procedures for preparing dental material samples are individually described elsewhere in this document for specific testing protocols. This section will provide general guidance for test sample preparation. Preparing samples for biological evaluations presents many challenges. A test sample must be capable of revealing the toxic potential of a dental material under conditions likely to be found in short or long term clinical use. Test materials must be prepared in a way that minimizes bias in the assessment of toxicity. For example, care should be taken to ensure that a testing methodology does not unexpectedly accelerate corrosion or leaching of a test material beyond what would normally be found under clinical conditions. Direct contact with a cell culture medium might be unusually reactive with a metallic test material, causing test cells to be killed by an unrealistically high level of metal ions. This may lead to the conclusion that a dental material is unsuitable for clinical use. On the other hand, an extract of this same material in artificial saliva or buffer might have provided a more clinically relevant concentration of metal ions with the result that this material now may be viewed as suitable for clinical use. Therefore, the investigator is challenged with preparing a test sample in a way that maximizes the clinical toxic predictive value of a testing methodology.

1.2 Applicability of Testing

This document does not apply to dental materials marketed and found to be safe for use prior to the date of this document unless a change in concentration of more than 5% in any component or other substantive change in manufacturing procedures is made.

1.3 Samples

Samples used for inspection and testing should be taken from normal commercial production, if available. Samples must not be past the manufacturer's expiration date at the time of initiation of any test procedure. Specimens for testing should be prepared in accordance with the manufacturer's instructions. The test should be performed on either an extract of the material and/or the material itself. For both methods the test material should be representative of either the final product or a component of the final product that is to be tested.

2 NORMATIVE REFERENCES

- 2.1 The following standards contain provisions that are pertinent to this part of ANSI/ADA Specification 41. All standards are subject to revision, and parties to agreements based on this part of ANSI/ADA Specification 41 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below.
 - 2.2.1 ISO 10993-12, 1996, Biological evaluation of medical materials – Part 12: Sample preparation and reference materials.
 - 2.2.2 ISO 10993-13, Biological evaluation of medical devices – Part 13: Identification and quantification of degradation products from polymeric medical devices.
 - 2.2.3 ISO 10993-14, Biological evaluation of medical devices – Part 14: Identification and quantification of degradation products from ceramics.
 - 2.2.4 ISO 10993-15, Biological evaluation of medical devices – Part 15: Identification and quantification of degradation products from metals and alloys.
 - 2.2.5 ISO 10993-16, Biological evaluation of medical devices – Part 16: Toxicokinetic study design for degradation products and leachables.

- 2.2.6 ISO 10993-17, Biological evaluation of medical devices – Part 17: Establishment of allowable limits for leachable substances.

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

3 DEFINITIONS

- 3.1 For the purposes of this document the following definitions apply.

3.1.1 Material/test material: product, product portion or component thereof that is sampled for biological testing.

3.1.2 Test sample: extract or portion of the test material that is subjected to biological testing.

3.1.3 Extract: liquid resulting from contact with the test material for a specified time and temperature.

4 MATERIAL ACCEPTABILITY

4.1 Materials in current use

Dental materials currently marketed legally at the time of publication of these guidelines should be assumed acceptable unless there is evidence indicating a reasonable doubt.

4.2 New Materials

Newly developed dental materials or components should be subjected to the appropriate tests given in Tables 1 and 2 (Selection of Tests).

4.3 Substantive change in a material

A previously accepted material in which a substantive change in concentration of any component has been made should be subjected to the appropriate tests given in Tables 1 and 2 (Selection of Tests). A substantive change is defined as an increase in concentration of more than 5% in any component.

4.4 Change in application or use of a material

Previously accepted dental materials, in which the manufacturer recommends changes in clinical application or new uses for the material, should be re-evaluated by the appropriate tests if the new application or use could be reasonably expected to increase the toxicity of the product.

4.5 National acceptance

Acceptance regarding the test methods given in these guidelines does not imply compliance with appropriate national regulations.

5 TEST MATERIALS

5.1 New materials

Dental materials not yet marketed should be tested using the product prototype. The manufacturer's instructions and claims for intended use should be presented with the material to be tested. The batch number should be stated and form part of the test report.

5.2 Previously accepted materials

Samples of dental materials for testing should be taken from normal production. At the time of initiation of any test procedure, materials must not be used past their labeled expiration date. The batch number should be stated and should form part of the test report.

6 TEST MATERIAL PREPARATION

6.1 General

The dental material used in the biological assay should be representative of the composition, processes and surface characteristics of the final product. Residues from the processes of manufacturing, fabrication, cleaning, sterilization should be considered integral to the material, material portion or component.

6.2 Materials should be prepared in accordance with the manufacturer's instructions.

6.3 Storage of materials must follow the manufacturer's recommendations.

6.4 If a material is not yet marketed, it should be prepared according to its intended use.

6.5 The amount and surface area of a material should be appropriate to the biological and physical constraints of the test system. Use of a standard sample size for a specific assay is recommended.

6.6 Many dental materials are used in an 'unset' state. Although this state can be transient, it usually represents the most toxic and chemically irritating state of the materials. Where a material exhibits the two states during its clinical use, information should be obtained on the biological response elicited by the material in both states.

6.7 If a material cannot be tested as a whole and contains several materials, each individual material that can come into contact with body tissues in the final product should be evaluated.

6.8 When test samples need to be cut into pieces, the influence of previously unexposed surfaces, e.g., lumens or cut surfaces, should be considered.

6.9 When different materials are present in a single material, the potential for synergies or interactions should be considered in the choice of the test sample.

6.10 Cleanliness

All items used in material preparation should be clean and free from contamination by oil, dust, soap, grease, or previously tested materials. Test samples should be handled to prevent contamination.

6.11 Sterilization

When a sterile material or solution is required, autoclave the sample at 121°C, 15 psi (sea level) or equivalent for 20 min, unless the material is supplied in a sterile and suitable form. When a material is not suitable for autoclaving an alternative means of sterilization may be submitted. Where sterility is required to be checked, the microbial content should be measured in accordance with the United States Pharmacopoeia microbial limits tests.

6.12 If sterilized test samples are prepared for the test procedure, the effect of sterilization should be considered.

7 PREPARATION OF MATERIAL FOR DIRECT CONTACT TESTS

7.1 The preferred sample of a solid specimen should have at least one flat surface.

Adjustments should be made for other shapes and physical states.

7.2 Test materials that are normally supplied non-sterile but are sterilized before use should be sterilized by the method recommended by the manufacturer and handled aseptically throughout the extraction and test procedure.

7.3 The effect of sterilization methods or agents on the material should be considered in defining the preparation of a test material prior to use in a test system.

7.4 Test materials not required to be sterile in use should be used as supplied and handled aseptically throughout the extraction and test procedure.

7.5 Liquids tested on solid media should be applied directly onto the media or deposited onto a biologically inert absorbent material, such as filter discs.

7.6 If an insoluble material is to be used for oral dosing in animals, a test material should be prepared by:

- A Grinding an appropriate quantity of the material into a powder using a freezer mill to a sieve size of 200 μm or less; or,
- B Lathe turning 200 μm cuttings using a clean tungsten carbide tipped cutter with distilled water as a coolant, if necessary.

Collect the turnings in a clean stainless steel tray.

7.6.1 Paste or rubbery materials may be blended for 30 seconds in a high-speed homogenizer, dry or in a suspending medium as needed.

8 PREPARATION OF EXTRACTS OF SOLID TEST MATERIALS

8.1 General

8.1.1 If extracts of a dental material are required for a test protocol, the extraction vehicle and conditions of extraction used should be appropriate to the nature and use of the final product. Extraction conditions that simulate clinical conditions are preferred. Extraction conditions must also reflect predictability of the tests.

8.1.2 Extraction is a complex process influenced by time, temperature, surface area-to-volume ratio, extraction medium and phase equilibrium of the material. The effects of higher temperatures or other conditions on extraction kinetics and the identity of leachate(s) should be considered carefully if accelerated or exaggerated extraction is used.

8.1.3 If necessary, repeated extraction followed by concentration to obtain sufficient extractable substance(s) for analysis may be used to increase the sensitivity of the test. Situations may arise necessitating a need to exaggerate clinical use conditions using extraction methods that yield high levels of leachate. Results derived from the use of this extract need to be viewed in light of these exaggerations. Judgment needs to be used in interpreting the results as to their appropriateness to actual use conditions and potential material toxicity.

8.1.4 If particulates are generated during extraction, their effect on the test used should be considered.

8.1.5 Extraction should be performed in sterile, chemically inert closed containers using aseptic techniques. Extracts should, when possible, be used immediately after preparation to prevent adsorption onto the extraction container or other changes in composition. If an extract is stored longer than 24 h, the stability of the extract under conditions of storage should be verified.

8.2 Temperature

8.2.1 Extraction time and temperature are dependent on physicochemical characteristics of the material and extraction vehicle. Recommended conditions are:

- A Not less than 24 h at $37 \pm 1^\circ\text{C}$;
- B 72 h at $50 \pm 2^\circ\text{C}$, ± 2 h;
- C 24 h at $70 \pm 2^\circ\text{C}$, ± 2 h;
- D 1 h at $121 \pm 2^\circ\text{C}$, ± 0.2 h.

8.2.2 Extraction conditions usually should simulate as closely as possible the conditions under which the material will normally be used.

8.2.3 Extraction temperature must not cause material degradation.

8.2.4 Extraction procedures using culture medium with serum can only be used at 37 °C for at least 24 hours.

8.3 Surface Area

8.3.1 The ratio of surface area of the material to the volume of extraction vehicle should be sufficient to cover the material in the solvent volume.

8.3.2 The ratio between the surface area of the material and the volume of extraction vehicle should be no more than 6 cm²/ml and no less than 0.5 cm²/ml. The surface area should be calculated on the basis of overall sample dimensions, not taking into account surface irregularity and porosity. However, actual surface characteristics should be considered in the interpretation of test results. Extraction ratios ($\pm 10\%$) for test material thickness of 0.5 mm: 6 cm²/ml; $>0.5 \leq 1.0$ mm: 3 cm²/ml; >1.0 mm: 1.25 cm²/ml. If the surface area is indeterminate, then 0.1 to 0.2 g/ml should be used.

8.3.3 Mass/volume or surface area/volume extraction ratios related to evaluation of powders or porous surfaces may be used provided that they simulate conditions during clinical use or result in a relevant measure of hazard potential.

8.3.4 Hydrocolloids may be tested by first determining the amount of extractant absorbed by the material. The test sample should be 2 g of material, and the extract volume should be 20 ml more than the extractant absorbed.

8.3.5 Materials such as elastomers, composites, amalgam, coated or surface-treated materials should be tested intact whenever possible. Other materials may also be cut into small pieces before extraction to enhance submersion in the extract liquid.

8.4 Extraction media

8.4.1 Solvents selected as extractants should be suitable for use in specific biological test systems, and should simulate the extraction that occurs during clinical use of the material and maximize the amount of leachate. Example solvents include:

- Polar liquid: water; physiological saline; liquid culture media without serum.
- Nonpolar liquid: freshly refined vegetable oil, e.g., sesame or cottonseed oil.
- Additional extraction liquids: 5% ethanol in water or physiological saline (v/v); polyethylene glycol 400 (diluted to a physiological osmotic pressure); dimethylsulfoxide (DMSO is cytotoxic in some assay systems at concentrations greater than 0.5%); liquid culture media with serum; artificial saliva.
- Other liquids appropriate to the nature and use of the material, if their effects are known.

8.4.2 Extracts should not routinely be processed by filtration, centrifugation or other methods to remove suspended particulates. However, if such processing is necessary, the rationale should be presented.

8.4.3 Artificial saliva solutions should be considered for materials intended for use in the oral cavity. For example, restorative alloys, casting alloys, orthodontic alloys, impression or composite materials should be extracted in an artificial saliva solution such as, but not limited to, the Fusayama (1963) solution formulation as modified by Holland (1991). Other artificial saliva formulations are listed in Leung and Darvell (1997). Artificial saliva preparations for the relief of xerostomia are not acceptable as extraction media, since these formulations do not sufficiently simulate human saliva. Extract should be used immediately and sterilized by filtration through a 0.22 μ m hydrophilic polysulfone membrane immediately before use.

Modified Fusayama Solution:

NaCl	0.400 g
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KCl	0.400 g
NaH ₂ PO ₄ · 2H ₂ O	0.780 g
Na ₂ S · 9H ₂ O	0.005 g
Urea	1.000 g
CaCl ₂ · 2H ₂ O	0.795 g
Distilled H ₂ O	1000 ml

pH adjusted to 6.0 (a precipitate may form above pH 6.0)

Calcium chloride should be dissolved in about 50 ml of distilled water and added last before pH adjustment. All chemicals should be of analytical grade, and pH adjusted with 1.0 M HCl or 1.0 M NaOH as needed immediately before final volume adjustment. The solution be prepared fresh prior to an experiment.

9 TEST REPORT

9.1 The test report should include the following documentation of sample preparation:

- Source of material, material portion or component;
- Lot or batch number;
- Description of processing, cleaning or sterilization treatments;
- Extraction techniques, including documentation of the conditions for extraction.

10 REFERENCES

1. Fusayama, T; Katayori, T; Nomoto, S. Corrosion of gold and amalgam placed in contact with each other. *J. Dent. Res.* 42 (5): 1183-1197;1963.
2. Holland, R.I. Use of potentiodynamic polarization technique for corrosion testing of dental alloys. *Scandinavian J. Dent. Res.* 1975-85; 1991.
3. Leung, V. W.-H. and Darvell, B.W. Artificial salivas for *in vitro* studies of dental materials, *J. Dentistry* 25 (6): 475-484.

CYTOTOXICITY

AGAR DIFFUSION TEST

(Recommended Test)

1 OBJECTIVE

The test is designed to demonstrate nonspecific cytotoxicity of dental materials after diffusion through agar or agarose.

2 NORMATIVE REFERENCES

ISO 10993-5, ISO 7405

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

3 SAMPLE PREPARATION

3.1 For the preparation of test dental materials, the manufacturer's recommended instructions should be followed. The test should be performed on either an extract of the material or the material itself, according to the guidance in ISO 10993-5, clause 4.

3.2 For solid materials, prepare circular test specimens of approximately 5 mm diameter, with a flat surface to ensure adequate contact with the agar overlay.

3.3 For setting materials, insert the freshly mixed material into glass or polytetrafluoroethylene rings of internal diameter 5 mm and height 5 mm. When testing materials in the freshly mixed state, place the rings on the agar prior to inserting the material. When testing after various setting periods, fill the rings so that the material is flush with the rim and allow it to set at $37\pm 2^{\circ}\text{C}$ and a relative humidity of $90\pm 10\%$ until ready for testing.

3.4 For fluid specimens or extracts, place 0.1 ml of the fluid on a borosilicate microglass filter disc of 5 mm diameter, placed on the agar.

NOTE — Suitable discs can be prepared from prefilters. Millipore prefilter AP2505500 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

4 CONTROL SPECIMENS

Use control specimens as defined in the section "Sample Preparations" in this Document.

5 CELL LINE

5.1 American Type Culture Collection CCL 1 fibroblasts (NCTC clone 929) free of mycoplasma should be used; other cell lines may be used if reproducibility and accuracy of the response can be demonstrated.

6 CULTURE MEDIUM, REAGENTS AND EQUIPMENT

6.1 Eagle's Basal Medium containing 2.2 g/l sodium bicarbonate, 3.0 g/l HEPES and 50 ml/l newborn calf serum.

6.2 Methods used for cell dissociation should not interfere with cell growth.

7 TEST PROCEDURE

7.1 Prepare a double concentration of Eagle's Basal Medium omitting HEPES and reducing sodium bicarbonate to 1 g/l.

7.2 Prepare either 3% agar or 3% agarose in distilled water. Sterilize agar by autoclaving and medium by filtration.

- 7.3 Prepare the vital stain by diluting a stock solution of 1% aqueous neutral red solution 1/100 with 0.01 ml/l phosphate-buffered saline solutions immediately before use. Store neutral red solutions protected from light. Use culture dishes of diameter 50 mm to 100 mm suitable for tissue culture.
- 7.4 Culture cells until they reach the end of log growth phase. Pipette 10 ml of cell suspension (2.5×10^5 cells/ml) into a sufficient number of culture dishes and incubate at $37 \pm 2^\circ \text{C}$ in a water-saturated atmosphere with 5% carbon dioxide for 24 h. Heat the sterile agar to 100°C in a water bath and allow it to cool to 48°C . Mix 1 part of agar with 1 part of double-concentration, freshly prepared nutrient medium and heat to 48°C . Aspirate the liquid nutrient medium from each culture dish and replace with 10 ml of freshly prepared agar/nutrient medium mixture.
- 7.5 Allow the agar nutrient medium to solidify at room temperature (approximately 30 min). Add 10 ml neutral red solution and keep dark for 15-20 min. Aspirate excess neutral red.
- 7.6 Protect the culture from light in the presence of neutral red, as the cells can be damaged.
- 7.7 Apply to each dish two samples of test material, one positive control, one negative control and one extraction medium control if the last was used. Keep the specimens as far as possible from each other and from the wall of the culture dish. Incubate at $37 \pm 2^\circ \text{C}$ in a water-saturated atmosphere with 5% carbon dioxide for 24 h. Examine each test material at least in quadruplicate, i.e., two dishes per test material.

8 PARAMETERS OF ASSESSMENT

- 8.1 A decolorization zone around test materials and controls should be assessed using an inverted microscope with a calibrated screen, and a Decolorization Index (8.2) and a Lysis Index (8.3) determined for each specimen in accordance with the following criteria:

8.2 Decolorization Index

Index	Description
0	No decolorization detectable
1	Decolorization only under the test substance
2	Decolorization zone not greater than 5.0 mm from the test substance
3	Decolorization zone not greater than 10.0 mm from the test substance
4	Decolorization zone greater than 10.0 mm from the test substance
5	The total culture is decolorized

8.3 Lysis Index

Index	Description
0	No cell lysis detectable
1	Less than 20% cell lysis
2	20% to 40% cell lysis
3	>40% to <60% cell lysis
4	60% to 80% cell lysis
5	More than 80% cell lysis

- 8.4 Calculate the median Decolorization Index and Lysis Index for each test material and present the cell response as follows:

Cell response = Decolorization Index/Lysis Index

If the index values for four replicates of the test substance differ by more than 2 units in the range 0 to 3, repeat the test. With indices of 4 and 5, no repetition is necessary. When extracts are tested, subtract the median index of the extraction medium alone from the median index of the extraction medium containing test substance to obtain the index for the test substance alone. If the median index for the extraction medium serving as a control is greater than 1, repeat the test using a different extraction medium.

NOTE — For a valid test, an intact cell layer should be found under the negative control.

9 ASSESSMENT OF RESULTS

- 9.1 All information gathered in the test should be taken into account in assessing test results, particularly any differences in results between experimental and control groups. The cell response is based on the median Decolorization Index and Lysis Index of at least four replicate tests. The cell response is therefore not necessarily an integer and the scale used for interpreting the cell response should be continuous. A useful way to grade test materials is presented below.

Scale	Cell response	Interpretation
0	0/0	Noncytotoxic
1	1/1	Mildly cytotoxic
2	2/2 to 3/3	Moderately cytotoxic
3	4/4 to 5/5	Severely cytotoxic

- 9.2 Results of the assessment should be included in the test report.

CYTOTOXICITY**FILTER DIFFUSION TEST****(Alternative Test)****1 OBJECTIVE**

The test is designed to demonstrate nonspecific cytotoxicity of dental materials after diffusion through a cellulose acetate filter.

2 NORMATIVE REFERENCES

ISO 10993-5, ISO 7405

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

3 SAMPLE PREPARATION

3.1 To prepare test dental materials, the manufacturer's recommended instructions should be followed. The test should be performed on either an extract of the material or the material itself, according to the guidance in ISO 10993-5, clause 4.

3.2 For solid materials, prepare circular test specimens of approximately 5 mm diameter, with a flat surface to ensure adequate contact with the agar overlay. The mass of the test specimens should not exceed 3.5 g.

3.3 For setting materials, insert the freshly mixed material into glass or polytetrafluoroethylene rings of internal diameter 5 mm and height 5 mm. When testing materials in the freshly mixed state, place the rings on the agar prior to inserting the material. When testing after various setting periods, fill the rings so that the material is flush with the rim and allow it to set at $37 \pm 2^\circ\text{C}$ and a relative humidity of $90 \pm 10\%$ until ready for testing.

3.4 For fluid specimens or extracts, imbibe 0.1 ml of the fluid on a borosilicate microglass filter disc of 5 mm diameter, placed on the agar.

NOTE — Suitable discs can be prepared from prefilters. Millipore prefilter AP2505500 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

4 CONTROL SPECIMENS

Use control specimens as defined in the section "Sample Preparations" in this Document.

5 CELL LINE

5.1 American Type Culture Collection CCL 1 fibroblasts (NCTC clone 929) free of mycoplasma should be used; other cell lines may be used if reproducibility and accuracy of the response can be demonstrated.

6 CULTURE MEDIUM, REAGENTS AND EQUIPMENT

6.1 Eagle's Basal Medium containing 2.2 g/l sodium bicarbonate, 3.0 g/l HEPES and 50 ml/l new-born calf serum.

6.2 Methods used for cell dissociation should not interfere with cell growth.

6.3 Use cellulose acetate filters, 47 mm diameter, 0.45 μm pore size. Millipore HAWD 04753, HA IF 047 SO is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement of this product.

7 TEST PROCEDURE

- 7.1 Prepare a double concentration of Eagle's Basal Medium omitting HEPES and reducing sodium bicarbonate to 1 g/l.
- 7.2 Prepare either 3% agar or 3% agarose in distilled water. Sterilize agar by autoclaving and medium by filtration.
- 7.3 Prepare solutions either for succinate dehydrogenase staining or for nonspecific hydrolase staining.
- 7.4 For succinate dehydrogenase staining, prepare the following stock solutions:
 - 7.4.1 Succinate solution: 13.6 g sodium succinate in 100 ml phosphate buffer, pH 7.6
 - 7.4.2 Nitro blue tetrazolium chloride solution: 100 mg nitro blue tetrazolium chloride in 100 ml phosphate buffer, pH 7.6
 - 7.4.3 Phenazine methosulfate solution: 4 mg phenazine methosulfate in 10 ml distilled water, fresh
 - 7.4.4 Prepare a staining solution of 1 ml succinate solution, 9 ml nitro blue tetrazolium chloride solution and 1 ml phenazine methosulfate solution
- 7.5 For nonspecific hydrolase staining, prepare a stock solution of fluorescein diacetate consisting of 5 mg fluorescein diacetate in 1 ml acetone. For use, add 20 µl of stock solution to 100 ml phosphate-buffered saline. Use Petri dishes of 50 mm to 100 mm diameter, suitable for tissue culture.
- 7.6 Culture the cells until they reach the end of the log growth phase. Place cellulose acetate filters in the bottom of a sufficient number of Petri dishes and pipette 6 ml of cell suspension (2.5×10^5 cells/ml) into each. Incubate at $37 \pm 2^\circ \text{C}$ in a water-saturated atmosphere with 5% carbon dioxide for 24 h.
- 7.7 Pipette 5 ml of freshly prepared agar nutrient medium mixture kept at 48°C into a sufficient number of Petri dishes and allow it to solidify at room temperature.
- 7.8 Aspirate excess nutrient medium from the dishes containing cellulose acetate filters, wash the filters with phosphate-buffered saline at $37 \pm 2^\circ \text{C}$ and place them on top of the agar, cell side down.
- 7.9 Apply three to five test specimens on top of the filter in each dish and incubate for a further 2 h at $37 \pm 2^\circ \text{C}$ in a water-saturated atmosphere with 5% carbon dioxide. Ensure that the specimens are in close contact with the surface of the filter.
- 7.10 If the test material does not show toxicity after 2-h incubation, repeat the test with 24-h incubation. In each dish include one positive control and one negative control.
- 7.11 Controls of a filter with a cell monolayer but without test specimens, and of a filter without cells but with test specimens should be used. When extracts are tested, a control using the extraction medium alone should also be used.
- 7.12 Each test specimen should be examined at least in quadruplicate.
- 7.13 After incubation, remove the test specimens and gently loosen the filter from the agar. Assess cytochemically the area of reduced cell enzyme activity by method A or method B.
- 7.14 Method A: Demonstrate succinate dehydrogenase (enzyme classification 1.3.99.1).¹ The incubation period is 3 h at $37 \pm 2^\circ \text{C}$. Wash the filter in distilled water and air-dry prior to measurement.
- 7.15 Method B: Demonstrate nonspecific hydrolase by incubation with fluorescein diacetate solution for 30 min at 4°C . Examine the filter under ultraviolet light.
- 8 ASSESSMENT OF CELL DAMAGE
 - 8.1 Cell damage should be assessed as follows:
 - 8.1.1 Measuring the area of decolorization, e.g., by means of an image analysis system, or

8.1.2 Using the following scale:

Scale	Grading assessment	Area of decolorization
0	No difference in staining intensity across the filter	None
1	A zone of reduced staining intensity, or an unstained zone with a diameter less than that (5 mm) of the test specimen	<20 mm ²
2	An unstained zone 5 mm to 7 mm in diameter of the test specimen	20 mm ² to 40 mm ²
3	An unstained zone greater than 7 mm in diameter of the test specimen	>40 mm ²

8.2 The filters beneath negative control specimens and control filters should be uniformly stained dark blue (if succinate dehydrogenase is used) or light green (if nonspecific hydrolase is used). Control filters without cells allow determination of a possible effect of the test specimen on the filter.

9 ASSESSMENT OF RESULTS

9.1 Information gathered in the test should be taken into account in assessing the test results, particularly any differences in results between the experimental and control groups. A useful way to grade test materials is presented below:

Scale	Interpretation
0	Noncytotoxic
1	Mildly cytotoxic
2	Moderately cytotoxic
3	Severely cytotoxic

9.2 Results of the assessment should be included in the test report.

10 REFERENCE

1. ISO 10993-5, 1999, Biological Evaluation of Medical Devices - Part 5: Tests for *in vitro* Cytotoxicity, Second Edition.

CYTOTOXICITY

DIRECT CONTACT TEST

(Recommended for Solid Materials)

1 OBJECTIVE

The test is designed to demonstrate nonspecific cytotoxicity of dental materials from direct contact.

2 NORMATIVE REFERENCES

ISO 10993-5, ISO 7405

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

3 SAMPLE PREPARATION

3.1 Dental materials that have various shapes, sizes or physical states, i.e., liquid or solid, may be tested without modification in cytotoxicity assays. The preferred sample of a solid specimen should have at least one flat surface. Adjustments should be made for other shapes and physical states.

3.2 To prepare test materials, the manufacturer's recommended instructions should be followed. The test should be performed either on an extract of the material and/or on the material itself, according to the guidance in ISO 10993-5, clause 4.

3.3 For solid materials, prepare circular test specimens of approximately 5 mm diameter, with a flat surface to ensure adequate contact with the cell layer surface. The mass of the test specimens should not exceed 3.5 g.

3.4 For setting materials, insert the freshly mixed material into glass or polytetrafluoroethylene rings of internal diameter 5 mm and height 5 mm. When testing materials in a freshly mixed state, place the rings on the agar prior to inserting the material. When testing after various setting periods, fill the rings so that the material is flush with the rim and allow it to set at $37 \pm 2^\circ \text{C}$ and a relative humidity of $90 \pm 10\%$ until ready for testing.

4 CONTROL SPECIMENS

4.1 Use an appropriate noncytotoxic control specimen for comparison. High-density polyethylene polymers and aluminum oxide ceramic rods for dental material have been used as negative controls.

4.2 An organo-tin stabilized poly(vinylchloride) has been used as a positive control.

5 CELL LINE

5.1 American Type Culture Collection CCL 1 fibroblasts (NCTC clone 929) free of mycoplasma should be used; other cell lines may be used if reproducibility and accuracy of the response can be demonstrated.

6 CULTURE MEDIUM, REAGENTS AND EQUIPMENT

6.1 Eagle's Basal Medium containing 2.2 g/l sodium bicarbonate, 3.0 g/l HEPES and 50 ml/l newborn calf serum is recommended.

6.2 Methods used for cell dissociation should not interfere with cell growth.

7 TEST PROCEDURES

7.1 Culture the cells until they reach the end of the log growth phase. Pipette an appropriate number of cells into each well or flask, and incubate at $37 \pm 2^\circ \text{C}$ in a humidified atmosphere with 5% carbon dioxide for 24 h.

- 7.2 A minimum of three replicates should be used for test samples and controls.
- 7.3 Examine the culture with a microscope.
- 7.4 Remove and discard the culture medium, then add fresh culture medium to each vessel.
- 7.5 Carefully place individual specimens of a test sample on the cell layer in the center of each replicate vessel. Ensure that the specimen covers approximately one-tenth of the cell layer surface. Exercise care to prevent unnecessary movement of the specimens as this could cause physical trauma to the cells that is evidenced by patches of dislodged cells.
- 7.6 For testing extracts of test material, an aliquot should be added to each vessel with fresh culture medium. If DMSO is used as the solvent, make sure its final content is not greater than 0.5%.
- 7.7 When appropriate, specimens can be placed in culture vessels prior to addition of cells.
- 7.8 Prepare replicate vessels for both the negative control and the positive control material.
- 7.9 Incubate the vessels under the same conditions as in 7.1 for 24, 48, and 72h.

8 ASSESSMENT OF CELL DAMAGE

- 8.1 Qualitative evaluation: examine the cells microscopically to assess changes, e.g., general morphology, vacuolization, detachment, cell lysis and membrane. The change from normal morphology should be recorded as indicated below.

Scale	Reactivity	Grading assessment
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under the specimen
2	Mild	Zone limited to area under the specimen
3	Moderate	Zone extends 0.5-1.0 cm beyond specimen
4	Severe	Zone extends >1.0 cm beyond specimen

- 8.2 Cell damage can also be assessed quantitatively: measure cell death, inhibition of cell growth, cell proliferation or colony formation. The number of cells, amount of protein, release of enzymes, release of vital dye, reduction of vital dye or other measurable parameter may be quantified by objective means. The objective measure and response shall be recorded in the test report.
- 8.2.1 For some methods of determining cytotoxicity, a zero time or baseline cell culture control may be necessary.
- 8.3 Ensure that care is taken in the choice of evaluation methods as the test results may be invalid if a test specimen releases substances that interfere with the test system or measurement.
- 8.4 If differences in the test result for replicate culture vessels are evident, the test is either inappropriate or invalid.
- 8.5 If negative, positive and any other controls (reference, medium, blank, reagent, etc.) do not have the expected response in the test system, the assay should be repeated.

9 ASSESSMENT OF RESULTS

- 9.1 All information gathered in a test should be taken into account in assessing the test results, particularly any differences

in results between the experimental and control groups. A useful way to grade test materials is presented below.

Scale	Interpretation
0	Noncytotoxic
1	Slightly cytotoxic
2	Mildly cytotoxic
3	Moderately cytotoxic
4	Severely cytotoxic

9.2 Results of the assessment should be included in the test report.

ACUTE SYSTEMIC TOXICITY

SINGLE DOSE ACUTE TOXICITY TEST

(Alternative Test)

1 OBJECTIVE

This test is an alternative method to the classic LD₅₀ procedure for determination of acute systemic toxicity of dental materials using a single dose via the oral route.

2 NORMATIVE REFERENCES

ISO 10993-5

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

US FDA Federal Register August 26, 1996 (61 FR 43934)

(FDA Federal Register documents are available from FDA, 5630 Fishers Lane - Room 1061- HFA-305, Rockville, MD, 20852; or at: <http://www.accessdata.fda.gov/scripts/oc/ohrms/index.cfm>).

3 TERMS AND DEFINITIONS

3.1 Acute toxicity: Those adverse effects occurring following administration of a single dose of a substance or multiple doses given within 24 hours.

3.2 Dose: The amount of test substance administered. Dose is expressed as weight of test substance per unit weight of test animal, e.g., mg/kg.

3.3 LD₅₀ (median lethal dose): A statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals. The LD₅₀ value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

4 GENERAL CONSIDERATION

4.1 Acute toxicity studies in animals are usually necessary for any product intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans.

4.2 Studies should be designed so that the maximum amount of information is obtained from the smallest number of animals. Calculating lethal parameters, e.g., LD₅₀, using large numbers of animals, as was done previously, is not recommended (Federal Register, October 11, 1988, 53 FR 39650). To avoid causing excessive pain or tissue damage in animals, products with irritant or corrosive characteristics should not be administered in concentrations that produce severe toxicity solely from local effects.

4.3 All available information on the test substance should be considered by the testing laboratory prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physicochemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; toxicological data on structurally related substances; and the anticipated use(s) of the substance. This information is necessary to satisfy all concerned that the test is relevant for the protection of human health, and will help in the selection of an appropriate starting dose.

5 TEST ANIMALS

- 5.1 Studies should be conducted in at least two mammalian species, including a nonrodent species when reasonable. The preferred rodent species is the rat, although other rodent species may be used.
- 5.2 Where nonrodent species are appropriate for investigation, use of fewer animals may be considered. Any data providing information on acute effects in nonrodent species, including preliminary dose-range finding data for repeat-dose toxicity studies, may be acceptable.
- 5.3 Normally females are used. This is because literature surveys of conventional LD₅₀ tests show that usually there is little difference in sensitivity between the sexes, but in those cases where differences are observed, females are generally slightly more sensitive. However, if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive then this sex should be used. When the test is conducted in males, adequate justification should be provided.
- 5.4 Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant.

6 ANIMAL HOUSING AND FEEDING CONDITIONS

- 6.1 The temperature of the experimental animal room should be 22±3° C. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark.
- 6.2 For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

7 PREPARATION OF ANIMALS AND DOSES

- 7.1 Animals should be randomly selected, marked to permit individual identification, and kept in cages for at least 5 days prior to dosing to allow acclimatization to laboratory conditions.
- 7.2 In general, test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation.
- 7.3 Where a liquid end product or mixture is to be tested however, the use of undiluted test substance at a constant concentration may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities.
- 7.4 In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1ml/100g of body weight. In the case of aqueous solutions, however, 2 ml/100g body weight can be considered.
- 7.5 With respect to formulation of dosing preparations, use of an aqueous solution/suspension/emulsion is recommended when possible, followed in order of preference by a solution/ suspension/emulsion in oil, e.g., corn oil, or other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known.
- 7.6 Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

8 EXPERIMENTAL PROCEDURES

- 8.1 Acute toxicity studies in animals should ordinarily be conducted using two routes of administration:
- 8.1.1 The route intended for human administration

- 8.1.2 Intravenous administration, if feasible
- 8.2 When intravenous dosing is proposed in humans, use of this route alone in animal testing is sufficient.
- 8.3 Oral administration of doses
 - 8.3.1 Animals should be fasted prior to dosing, e.g., with the rat, food but not water should be withheld overnight; with the mouse, food but not water should be withheld for 3-4 hours. Following the period of fasting, animals should be weighed and the test substance administered.
 - 8.3.2 The test substance should be administered in a single dose by gavage using a stomach tube or suitable intubation canula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.
 - 8.3.3 After a substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice. When a dose is administered in fractions over a period of time, it may be necessary to provide animals with food and water, depending on the length of time.
- 8.4 Selection of doses should be based on available information on the test substance and the route of administration.
- 8.5 Use of vehicle control groups should be considered.
- 8.6 For compounds with low toxicity, the maximum feasible dose should be administered.
- 9 OBSERVATION
 - 9.1 Animals should be observed for 14 days after administration.
 - 9.2 All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity should be recorded.
 - 9.3 Gross necropsies should be performed on all animals, including those sacrificed moribund, found dead, or terminated at 14 days.
 - 9.4 If acute toxicity studies in animals are to provide the primary safety data supporting single dose safety/kinetic studies in humans, e.g., a study screening multiple analogs to aid in the selection of a lead compound for clinical development, the toxicity studies should be designed to assess dose-response relationships and pharmacokinetics.
 - 9.5 Clinical pathology and histopathology should be monitored at an early time and at termination, i.e., ideally, for maximum effect and recovery.

ACUTE SYSTEMIC TOXICITY

ORAL – FIXED DOSE PROCEDURE

(Recommended Test)

1 OBJECTIVE

The test is an alternative method to the classic LD₅₀ procedure for the determination of acute systemic toxicity of dental materials using fixed doses via oral route.

2 NORMATIVE REFERENCES

ISO 10993-5

(ISO standards are available from the American National Standards Institute, 25 West 43rd St., New York, NY 10036. ISO dental standards are available from the American Dental Association, 211 E. Chicago Ave., Chicago, IL 60611).

OECD Guideline 420

(OECD documents are available from OECD, 2, rue André Pascal, F-75775 Paris Cedex 16, France and at: www.oecd.org)

3 TERMS AND DEFINITIONS

3.1 Acute oral toxicity: Those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours.

3.2 Dose: The amount of test substance administered. Dose is expressed as weight of test substance per unit weight of test animal, e.g., mg/kg.

3.3 Evident toxicity: A general term describing clear signs of toxicity following the administration of test substance, such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status, or probable mortality in most animals can be expected.

3.4 Impending death: The moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbency or tremor.

3.5 Moribund status: A state of dying or inability to survive, even if treated.

3.6 Predictable death: The presence of clinical signs indicative of death at a known time in the future before the planned end of an experiment, for example, inability to reach water or food.

3.7 Oral LD₅₀ (median lethal dose): A statistically derived single dose of a substance that can be expected to cause death in 50 percent of animals when administered by the oral route. The LD₅₀ value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg). It is also expressed as the time until death, i.e., days.

4 GENERAL CONSIDERATION

4.1 It is a principle of the method that in the main study only moderately toxic doses are used, and that administration of doses that are expected to be lethal should be avoided. Also, doses that are known to cause marked pain and distress, due to corrosive or severely irritant actions, need not be administered. Moribund animals, or animals in obvious pain or showing signs of severe and enduring distress should be humanely killed, and are considered in interpretation of test results in the same way as animals that died on test.

4.2 All available information on the test substance should be considered by the testing laboratory prior to conducting a study. Such information will include the identity and chemical structure of the substance; its physicochemical

properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; toxicological data on structurally related substances; and the anticipated use(s) of the substance. This information is necessary to satisfy all concerned that the test is relevant for the protection of human health, and will help in the selection of an appropriate starting dose.

5 PRINCIPLE OF TEST

- 5.1 Groups of animals of a single sex (see section 6.2) are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2,000 mg/kg (where an exception is warranted, an additional fixed dose of 5,000 mg/kg may be considered).
- 5.2 An initial dose level is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality.
- 5.3 Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the dose causing evident toxicity or no more than one death is identified, when no effects are seen at the highest dose, or when deaths occur at the lowest dose.

6 SELECTION OF ANIMAL SPECIES

- 6.1 The preferred rodent species is the rat, although other rodent species may be used.
- 6.2 Normally females are used. This is because surveys of conventional LD₅₀ tests show that there is usually little difference in sensitivity between sexes, but in cases where differences are observed, females are generally slightly more sensitive. However, if knowledge of the toxicologic or toxicokinetic properties of structurally-related chemicals indicates that males are likely to be more sensitive, then this sex should be used. When the test is conducted in males, adequate justification should be provided.
- 6.3 Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal at the commencement of its dosing should be between 8 and 12 weeks old and its weight should fall in an interval within $\pm 20\%$ of the mean weight of any previously dosed animals.

7 ANIMAL HOUSING AND FEEDING CONDITIONS

- 7.1 The temperature of the experimental animal room should be $22 \pm 3^\circ \text{C}$. Although relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark.
- 7.2 For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

8 PREPARATION OF ANIMALS AND DOSES

- 8.1 Animals should be randomly selected, marked to permit individual identification, and kept in cages for at least 5 days prior to the start of dosing to allow acclimatization to laboratory conditions.
- 8.2 In general, test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation.
- 8.3 Where a liquid end product or mixture is to be tested however, use of an undiluted test substance at a constant concentration may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities.
- 8.4 In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1 ml/100g of body weight; however in the case of aqueous solutions, 2 ml/100g body weight can be considered.

- 8.5 Regarding formulation of dosing preparations, an aqueous solution/suspension/emulsion is recommended when possible, followed in order of preference by a solution/suspension/emulsion in oil, e.g., corn oil, and then solution in other vehicles. For vehicles other than water, toxicological characteristics of the vehicle should be known.
- 8.6 Doses must be prepared shortly prior to administration unless stability of the preparation over the period during which it will be used is known and shown to be acceptable.

9 EXPERIMENTAL PROCEDURES

9.1 Administration of doses

- 9.1.1 Animals should be fasted prior to dosing, e.g., with the rat, food but not water should be withheld over-night; with the mouse, food but not water should be withheld for 3-4 hours. After fasting, animals should be weighed and the test substance administered.
- 9.1.2 The test substance should be administered in a single dose by gavage using a stomach tube or suitable intubation canula. If a single dose is not possible, the dose may be given in smaller fractions over a period not to exceed 24 hours.
- 9.1.3 After the substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice. When a dose is administered in fractions over a period of time, it may be necessary to provide animals with food and water depending on the length of time between doses.

9.2 Sighting study

- 9.2.1 The purpose of a sighting study is to allow selection of an appropriate starting dose for the main study. The test substance is administered to single animals in a sequential manner following the flow charts in Figure 1. The sighting study is completed when a decision on the starting dose for the main study can be made (or if a death is seen at the lowest fixed dose).
- 9.2.2 The starting dose for a sighting study is selected from fixed dose levels of 5, 50, 300 and 2,000 mg/kg as a dose expected to produce evident toxicity based, when possible, on evidence from *in vivo* and *in vitro* data from the same chemical and from structurally related chemicals. In the absence of such information, the starting dose will be 300 mg/kg.
- 9.2.3 A period of at least 24 hours will be allowed between the dosing of each animal. All animals should be observed for at least 14 days after initial dose administration.
- 9.2.4 As an exception, and only when justified by specific regulatory needs, the use of an additional upper fixed dose level of 5,000 mg/kg may be considered. To protect animal welfare, testing at 5,000 mg/kg is discouraged and should only be considered when there is a strong likelihood that the results of such a test would have a direct relevance for protecting animal or human health.
- 9.2.5 When an upper fixed dose of 5,000 mg/kg is considered, the following procedure should be followed.
- 9.2.5.1 Decision rules governing the sequential procedure presented in Figure 1 are extended to include a 5,000 mg/kg dose level. Thus, when a sighting study starting dose of 5,000 mg/kg is used outcome A (death) will require a second animal to be tested at 2,000 mg/kg; outcomes B and C (evident toxicity or no toxicity) will allow selection of 5,000 mg/kg as the main study starting dose.
- 9.2.5.2 Similarly, if a starting dose other than 5,000 mg/kg is used, then testing will progress to 5,000 mg/kg in the event of outcomes B or C at 2,000 mg/kg; a subsequent 5,000 mg/kg outcome A will dictate a main study starting dose of 2,000 mg/kg, and outcomes B and C will dictate a main study starting dose of 5,000 mg/kg.

- 9.2.6 In cases where an animal tested at the lowest fixed dose level (5 mg/kg) in a sighting study dies, the normal procedure is to terminate the study and assign the substance to Globally Harmonized System (GHS) Class 1 (Figure 1). However, if further confirmation of the classification is required, an optional supplementary procedure may be conducted, as follows.
- 9.2.6.1 A second animal is dosed at 5,000 mg/kg.
- 9.2.6.2 If this second animal dies, then GHS Class 1 is confirmed, and the study is terminated.
- 9.2.6.3 If a second animal survives, then a maximum of three additional animals will be dosed at 5,000 mg/kg. Because there will be a high risk of mortality, these animals should be dosed in a sequential manner to protect animal welfare. The time interval between dosing each animal should be sufficient to determine that the previous animal is likely to survive.
- 9.2.6.4 If a second death occurs, the dosing sequence should be terminated, and no further animals will be dosed. The classification will be as shown in Figure 2 at the 5,000 mg/kg fixed dose: Class 1 if there are 2 or more deaths (outcome A) at 5,000 mg/kg, or Class 2 if there is 1 death (outcome B) at 5,000 mg/kg.
- 9.3 **Main study**
- 9.4 **Numbers of animals and dose levels**
- 9.4.1 Action to be taken following testing at the starting dose level is indicated by the flow charts in Figure 2. One of three actions will be required; either stop testing and assign an appropriate hazard classification class, test at a higher fixed dose or test at a lower fixed dose. However, a dose level that caused death in a sighting study will not be revisited in the main study, to protect animal welfare. Experience has shown that the most likely outcome at the starting dose level will be that the substance can be classified and no further testing will be necessary.
- 9.4.2 A total of five animals of one sex will normally be used for each dose level investigated. The five animals will be made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals (except in the unusual circumstance that a dose level used on the main study was not included in the sighting study).
- 9.4.3 The time interval between dosing at each level is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose should be delayed until one is confident of survival of the previously dosed animals. A period of 3 or 4 days between dosing at each dose level is recommended to allow an observation of delayed toxicity. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response.
- 9.4.4 When use of an upper fixed dose of 5,000 mg/kg is considered, the following procedure should be followed.
- 9.4.4.1 Decision rules governing the sequential procedure presented in Figure 3 are extended to include a 5,000 mg/kg dose level. Thus, when a main study starting dose of 5,000 mg/kg is used, outcome A (≥ 2 deaths) will require testing of a second group at 2,000 mg/kg; outcome B (evident toxicity and/or ≤ 1 death) or C (no toxicity) will result in the substance being unclassified according to GHS.
- 9.4.4.2 Similarly, if a starting dose other than 5,000 mg/kg is used, then testing will progress to 5,000 mg/kg in the event of outcome C at 2,000 mg/kg; a subsequent 5,000 mg/kg outcome A will result in the substance being assigned to GHS class 5, and outcomes B or C will lead to the substance being unclassified.
- 9.5 **Limit Test**
- 9.5.1 Using the normal procedure, a sighting study with a starting dose of 2,000 mg/kg (or 5,000 mg/kg as an exception) followed by dosing of a further four animals at this level serves as an acceptable limit test.
- 9.6 **Observations**

- 9.6.1 Animals should be observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study for animal welfare reasons or are found dead.
- 9.6.2 The duration of observation should not be fixed rigidly. It should be determined by toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed.
- 9.6.3 All observations should be systematically recorded, with individual records maintained for each animal.
- 9.6.4 Additional observations will be necessary if animals continue to display signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma.
- 9.6.5 Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.
- 9.7 **Body weight**
- 9.7.1 Individual weights of animals should be determined shortly before a test substance is administered and at least weekly thereafter.
- 9.7.2 Weight changes should be calculated and recorded.
- 9.8 **Pathology**
- 9.8.1 All test animals (including those that die during a test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy.
- 9.8.2 All gross pathological changes should be recorded for each animal.
- 9.8.3 Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after initial dosing may also be considered because it may yield useful information.
- 10 **CRITERIA FOR CLASSIFICATION OF TEST SUBSTANCES WITH EXPECTED LD₅₀ VALUES EXCEEDING 2,000 mg/kg WITHOUT THE NEED FOR TESTING**
- 10.1 Test substances could be classified in the hazard Class classification defined by: 2,000mg/kg <LD₅₀ < 5,000mg/kg (GHS Class 5) in the following cases:
- 10.1.1 If reliable evidence is already available that indicates an LD₅₀ to be in the range of Class 5 values; or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.
- 10.1.2 Through extrapolation, estimation or measurement of data if assignment to a more hazardous class is not warranted and reliable information is available indicating significant toxic effects in humans, or any mortality is observed when tested up to class 4 values by the oral route, or where expert judgment confirms significant clinical signs of toxicity, when tested up to Class 4 values, except for diarrhea, piloerection or an ungroomed appearance, or where expert judgment confirms reliable information indicating the potential for significant acute effects from other animal studies.
- 11 **REFERENCES**
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Figure 1. Flow Chart for The Sighting Study

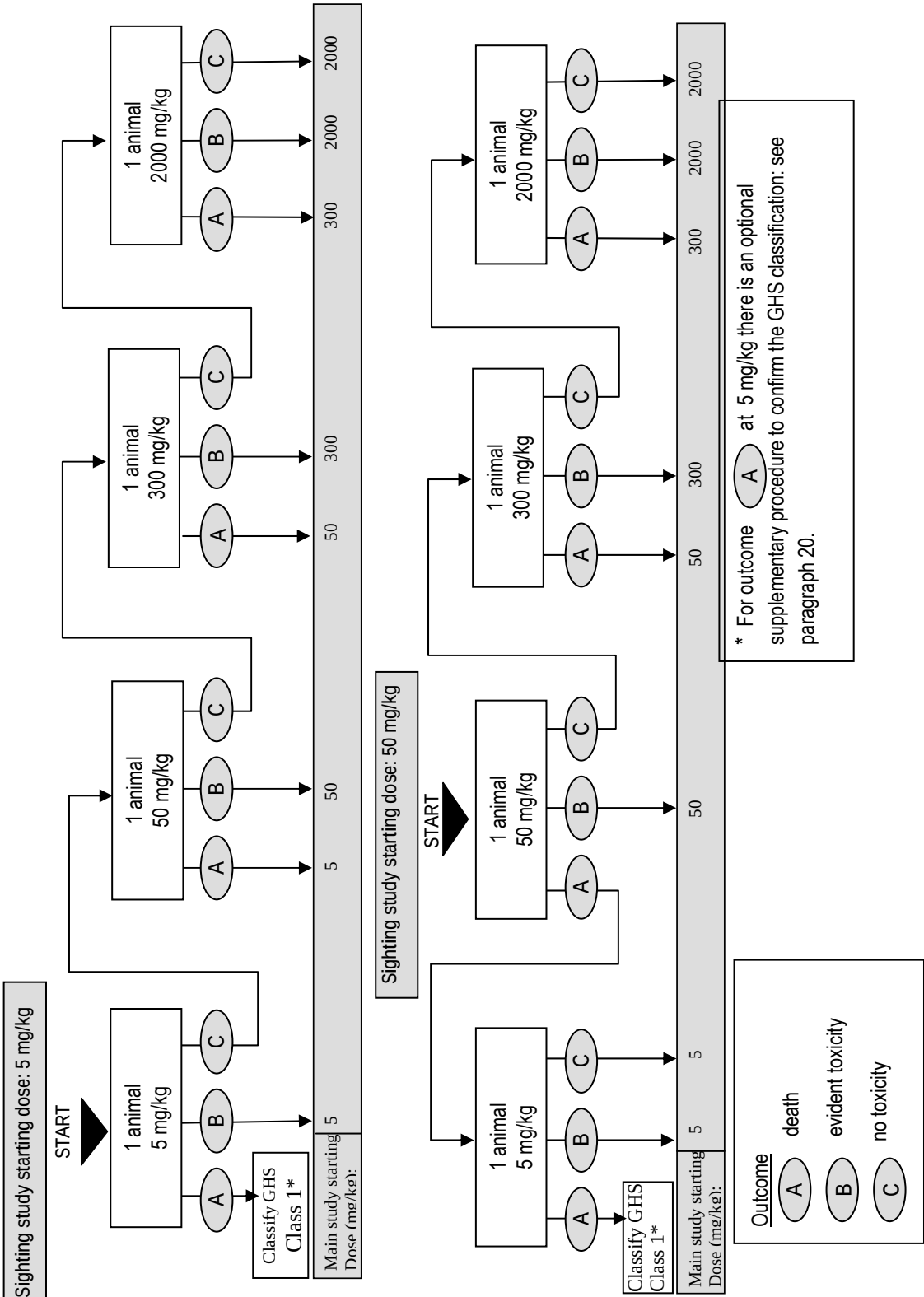


Figure 1. Flow Chart for The Sighting Study (Continued)

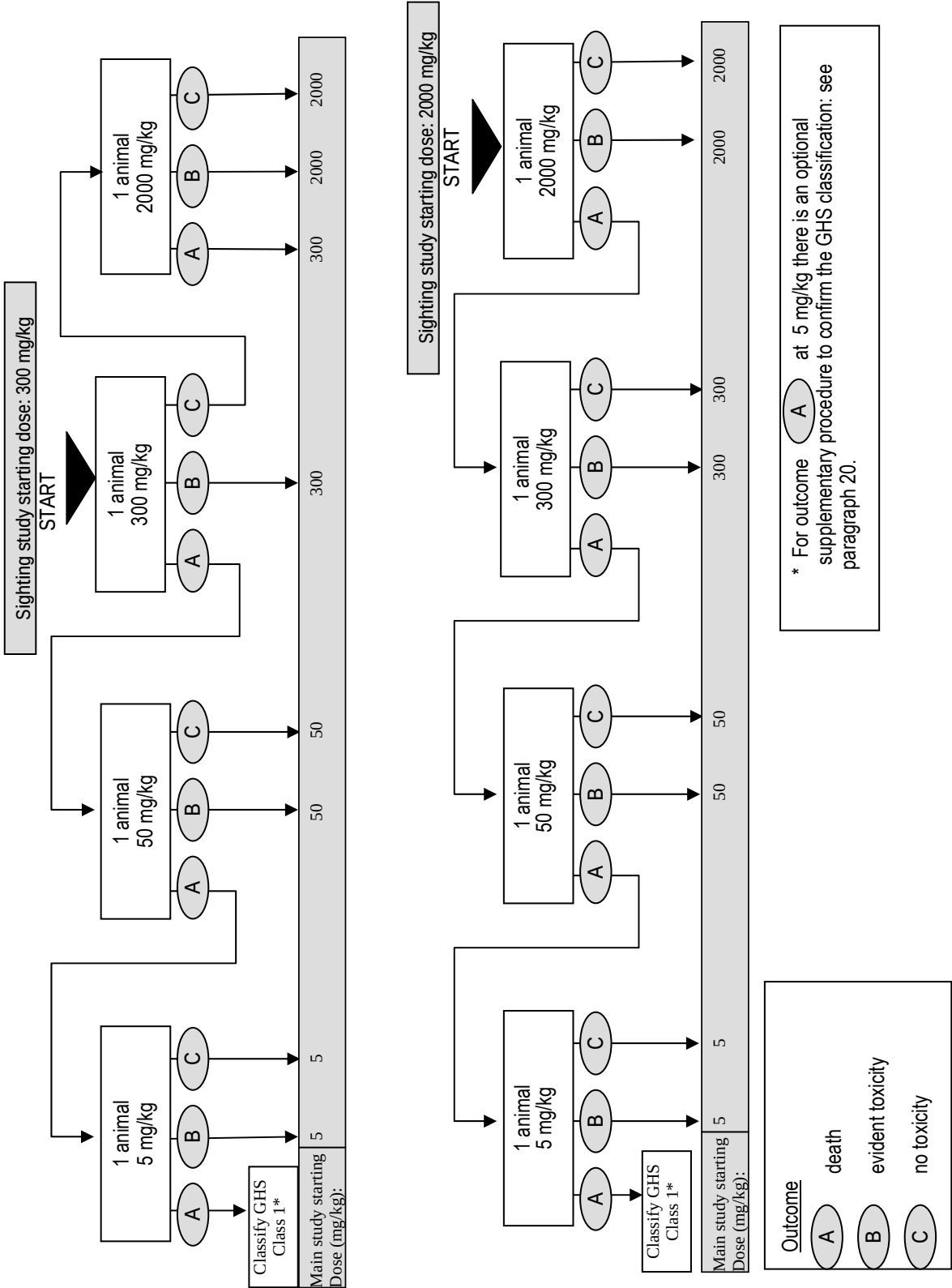


Figure 2. Flow Chart for The Main Study

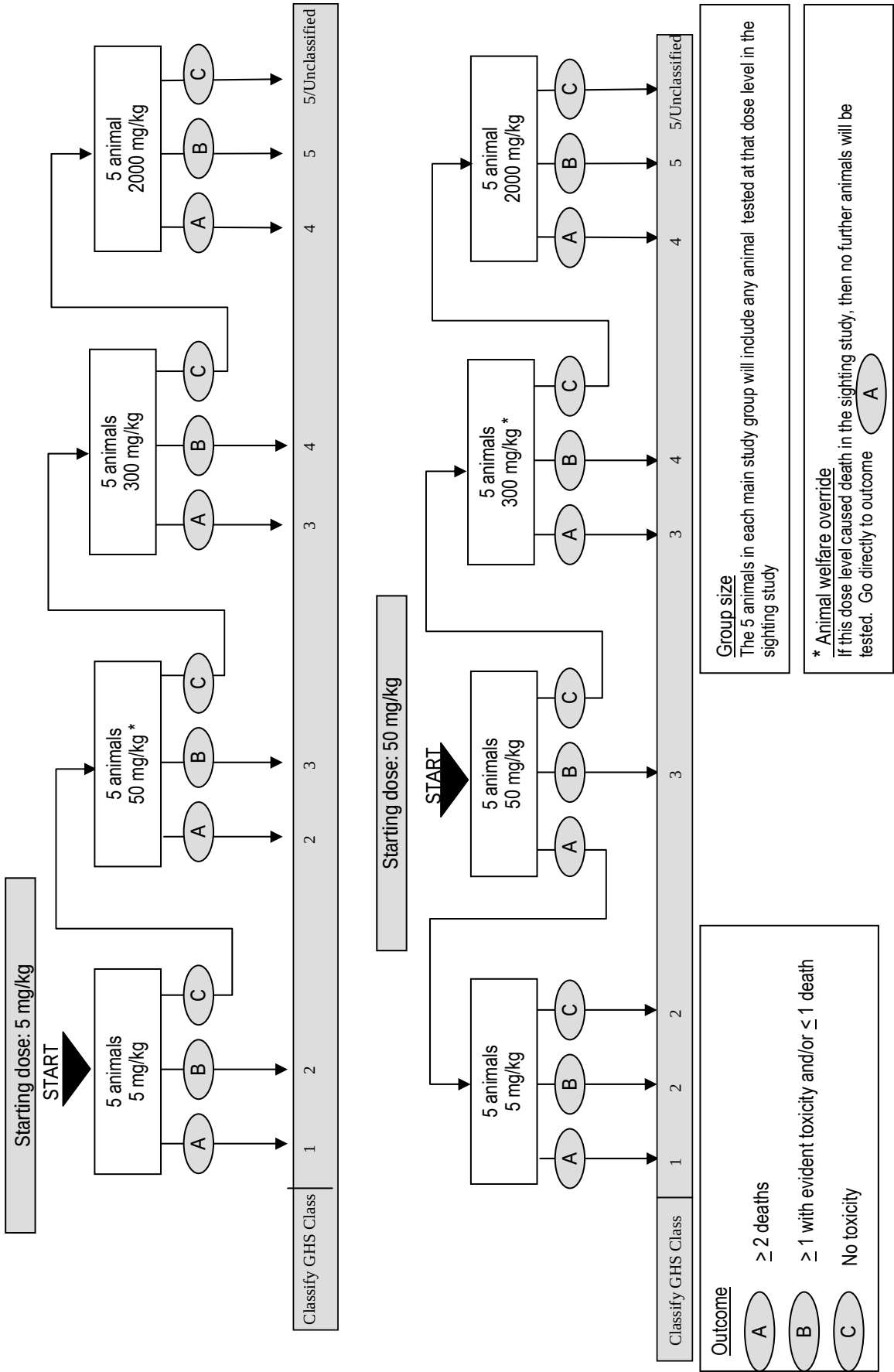
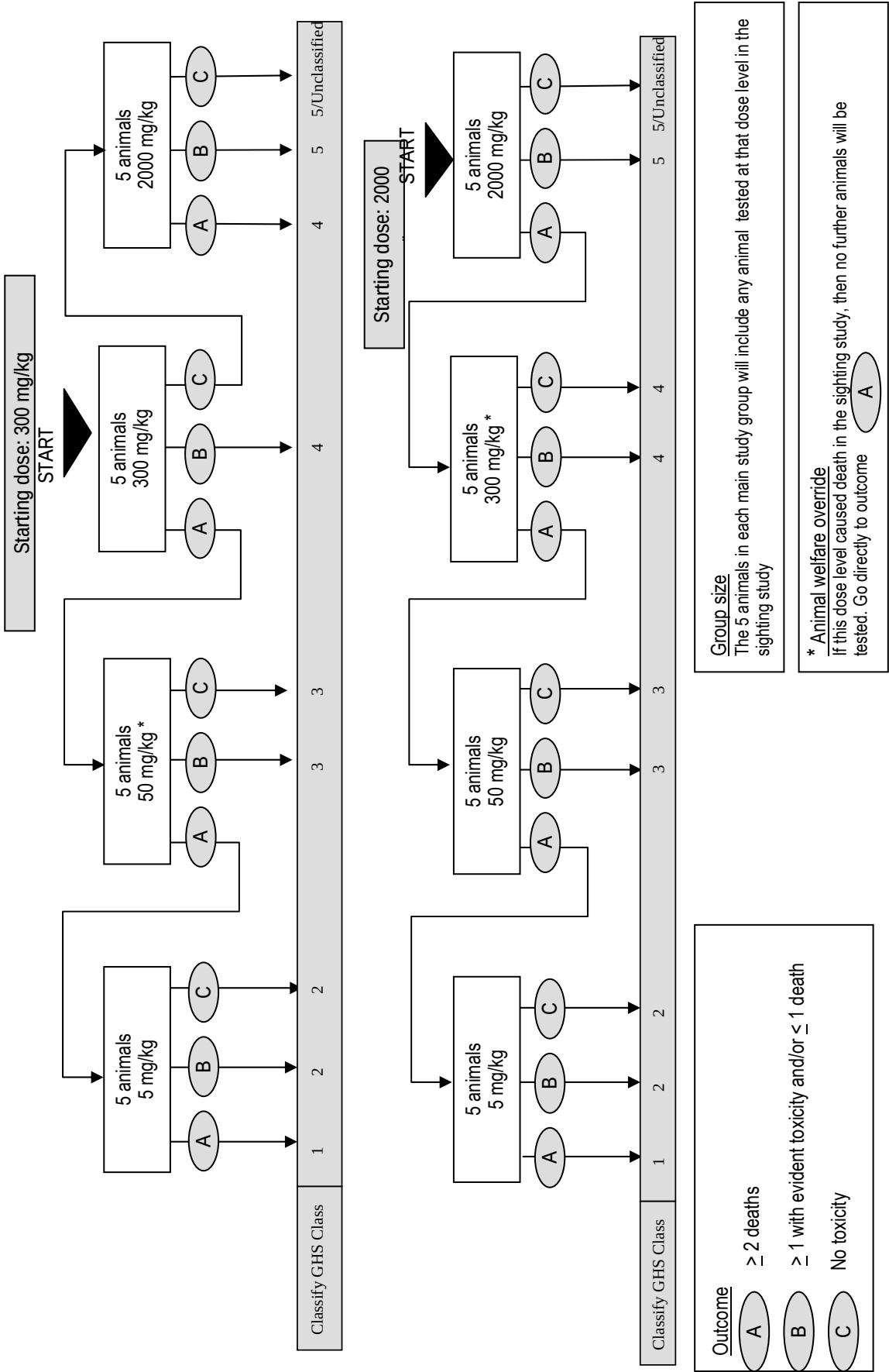


Figure 2. Flow Chart for The Main Study (Continued)



ACUTE SYSTEMIC TOXICITY

ORAL– UP-AND-DOWN PROCEDURE

(Alternative Test)

1 OBJECTIVE

The test is an alternative method to the classic LD₅₀ procedure for the determination of acute systemic toxicity of dental materials using the up and down (UAD) procedure via oral route.

2 NORMATIVE REFERENCES

ISO 10993-5, OECD Guideline 425

3 TERMS AND DEFINITIONS

- 3.1 Acute oral toxicity: The adverse effects following a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.
- 3.2 Delayed death: Means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.
- 3.3 Dose: The amount of test substance administered. Dose is expressed as weight, e.g., g, mg, or as weight of test substance per unit weight of test animal, e.g., mg/kg.
- 3.4 Dose progression factor: Sometimes termed a dose spacing factor, refers to the multiple by which a dose is increased, i.e., the dose progression, when an animal survives or the divisor by which it is decreased when an animal dies.
- 3.5 Oral LD₅₀ (median lethal dose): A statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD₅₀ value is expressed in terms of weight of test substance per unit weight of test animal, i.e., mg/kg.
- 3.6 Limit dose: Refers to a dose at an upper limitation on testing, i.e., 2,000 or 5,000 mg/kg.
- 3.7 Moribund status: Being in a state of dying or inability to survive, even if treated.
- 3.8 Nominal sample size: Refers to the total number of tested animals, reduced by one less than the number of like responses at the beginning of the series, or by the number of tested animals up to but not including the pair that creates the first reversal. For example, for a series where X and O indicate opposite animal outcomes (for instance, X dies within 48 hours and O survives) in a pattern as follows: OOOXXOXO, we have the total number of tested animals (or sample size in the conventional sense) as 8 and the nominal sample size as 6. This particular example shows 4 animals following a reversal. It is important to note whether a count in a particular part of the guideline refers to nominal sample size or to total number tested. For example, the maximum actual number tested is 15. When testing is stopped based on that basis, the nominal sample size will be less than or equal to 15. Members of the nominal sample start with the (r-1st) animal (the animal before the second in the reversal pair – see reversal below).
- 3.9 Probit: An abbreviation for the term “probability integral transformation” and a probit dose-response model permits a standard normal distribution of expected responses, i.e., one centered to its mean and scaled to its standard deviation, *sigma*; to doses (typically in a logarithmic scale to be analyzed as if it were a straight line with slope the reciprocal of *sigma*). A standard normal lethality distribution is symmetric; hence, its mean is also its true LD₅₀ or median response.
- 3.10 Reversal: A situation where nonresponse is observed at some dose, and a response is observed at the next dose tested, or vice versa, i.e., response followed by nonresponse. Thus, a reversal is created by a pair of responses. The first such pair occurs at animals numbered r-1 and r.

- 3.11 *Sigma*: The standard deviation of a lognormal curve describing the range of tolerances of test subjects to a chemical (where a subject is expected to be capable of responding if the chemical dose exceeds the subject's tolerance). The estimated *sigma* provides an estimate of the variation among test animals in response to a full range of doses. See slope and probit.
- 3.12 Slope (of the dose-response curve): A value related to the angle at which a dose response curve rises from the dose axis. In the case of probit analysis, when responses are analyzed on a probit scale against dose on a log scale this curve will be a straight line and the slope is the reciprocal of *sigma*, the standard deviation of the underlying test subject tolerances, which are assumed to be normally distributed. See probit and *sigma*.
- 3.13 Stopping rule: Used in this guideline synonymously with 1) a specific stopping criterion and 2) the collection of all criteria determining when a testing sequence terminates. In particular, for the main test, the stopping rule is used in section 5 as a shorthand for the criterion that relies on comparison of ratios to a critical value.

4 BACKGROUND

- 4.1 The concept of an up-and-down testing approach was first described by Dixon and Mood.¹⁴ In 1985, Bruce proposed to use an up-and-down procedure (UAD) for the determination of acute toxicity of chemicals. There exist several variations of the up-and-down experimental design for estimating an LD₅₀. This guideline is based on the procedure of Bruce adopted by ASTM in 1987 and revised in 1990. A study comparing the results obtained with the UAD, the conventional LD₅₀ test and the Fixed Dose Procedure was published in 1995. Since the early papers of Dixon and Mood, investigations continued to examine the best conditions for use of this approach.
- 4.2 The UAD test procedure described below is of value in minimizing the number of animals required to estimate the acute oral toxicity of a chemical. In addition to an estimation of LD₅₀ and confidence intervals, the test allows observation of signs of toxicity.

5 GENERAL CONSIDERATION

- 5.1 All available information on a test substance should be considered by the testing laboratory prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physical chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; toxicological data on structurally related substances or similar mixtures; and the anticipated use(s) of the substance. This information is useful to determine the relevance of the test for the protection of human health and the environment, and will help in the selection of an appropriate starting dose.
- 5.2 The method permits estimation of an LD₅₀ with a confidence interval and the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemicals that cause acute toxicity.
- 5.3 If no information is available to make a preliminary estimate of the LD₅₀ and the slope of the dose-response curve, results of computer simulations have suggested that starting near 175 mg/kg and using half-log units (corresponding to a dose progression of factor 3.2) between doses will produce the best results. This starting dose should be modified if a substance is likely to be highly toxic. The half-log spacing provides for a more efficient use of animals, and increases accuracy in predicting an LD₅₀ value. Because the method has a bias toward the starting dose, it is essential that initial dosing occur below the estimated LD₅₀. (See sections of Main Test and Dosing Procedures for discussion of dose sequences and starting values.) However, for chemicals with large variability, bias can still be introduced in the lethal estimates and the LD₅₀ will have a large statistical error, similar to other acute toxicity methods. To correct for this, the main test includes a stopping rule keyed to properties of the estimate rather than a fixed number of test observations.
- 5.4 The method is easiest to apply to materials that produce death within one or two days. The method would not be practical to use when considerably delayed death (five days or more) can be expected.

5.5 Calculations should be used to facilitate animal-by-animal testing sequences and provide final estimates.

5.6 Moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed, and are considered in interpretation of test results in the same way as animals that died on test.

5.7 A limit test can be used efficiently to identify chemicals that are likely to have low toxicity.

6 PRINCIPLE OF THE LIMIT TEST

6.1 The limit test is a sequential test that uses a maximum of 5 animals. A test dose of up to 2,000, or as an exception 5,000 mg/kg, may be used. The procedures for testing at 2,000 and 5,000 mg/kg are slightly different. The selection of a sequential test plan increases the statistical power and also has been designed to intentionally bias the procedure towards rejection of the limit test for compounds with LD₅₀ near the limit dose; i.e., to err on the side of safety. As with any limit test protocol, the probability of correctly classifying a compound will decrease as the actual LD₅₀ more nearly resembles the limit dose.

6.2 Criteria are provided for classification of test substances with expected LD₅₀ values that exceed 2,000 mg/kg without the need for testing.

6.2.1 Test substances could be classified in the hazard classification defined by: 2,000 mg/kg < LD₅₀ < 5,000 mg/kg (Class 5 in the GHS) in the following cases.

6.2.1.1 If reliable evidence is already available that indicates an LD₅₀ to be in the range of class 5 values; or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.

6.2.1.2 Through extrapolation, estimation or measurement of data if assignment to a more hazardous class is not warranted, and reliable information is available indicating significant toxic effects in humans, or any mortality is observed when tested up to class 4 values by the oral route, or where expert judgment confirms significant clinical signs of toxicity, when tested up to class 4 values, except for diarrhea, piloerection or an ungroomed appearance, or where expert judgment confirms reliable information indicating the potential for significant acute effect from other animal studies. 7777

7 PRINCIPLE OF THE MAIN TEST

7.1 The main test consists of a single ordered dose progression in which animals are dosed, one at a time, at 48-hour intervals. The first animal receives a dose a step below a level of the best estimate of the LD₅₀. If the animal survives, the dose for the next animal is increased to a factor of 3.2 times the original dose; if it dies, the dose for the next animal is decreased by a similar dose progression.

NOTE – 3.2 is the default dose escalation factor; see Main Test for further guidance for choice of dose-spacing factors.

7.2 Each animal should be observed carefully for up to 48 hours before making a decision on whether and how much to dose the next animal. That decision is based on the 48-hour survival pattern of all animals up to that time. A combination of stopping criteria is used to keep the number of animals low while adjusting the dosing pattern to reduce the effect of a poor starting value or low slope. Dosing is stopped when one of these criteria is satisfied, at which time an estimate of the LD₅₀ and a confidence interval are calculated for the test based on the status of all the animals at termination. For most applications, testing will be completed with only 4 animals after initial reversal in animal outcome. An LD₅₀ is calculated using the method of maximum likelihood.

7.3 The results of the main test procedure serve as a starting point for a computational procedure to provide a confidence interval (CI) estimate where feasible. A description of the basis for this CI is outlined in the section of Data Calculation and Reporting.

8 SELECTION OF ANIMALS SPECIES

- 8.1 The preferred rodent species is the rat although other rodent species may be used.
- 8.2 Normally female rats are used because conventional LD₅₀ tests show that usually there is little difference in sensitivity between sexes, but in cases where differences are observed, females are generally slightly more sensitive. However, if knowledge of toxicologic or toxicokinetic properties of structurally-related chemicals indicates that males are likely to be more sensitive, then this sex should be used. When the test is conducted in males, adequate justification should be provided.
- 8.3 Healthy young adult animals of commonly-used laboratory strains should be employed. Females should be nulliparous and non-pregnant. At the commencement of dosing, each animal should be between 8 and 12 weeks old and its weight should fall in an interval within $\pm 20\%$ of the mean initial weight of any previously dosed animals.

9 ANIMAL HOUSING AND FEEDING CONDITIONS

- 9.1 Temperature in the experimental animal room should be $22 \pm 3^\circ \text{C}$. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light and 12 hours dark.
- 9.2 Animals should be housed individually.
- 9.3 For feeding, conventional rodent laboratory diets may be used with an unlimited supply of drinking water.

10 PREPARATION OF ANIMALS AND DOSING

- 10.1 Animals should be randomly selected, marked to permit individual identification, and kept in cages at least 5 days prior to dosing to allow acclimatization to laboratory conditions. As with other sequential test designs, care must be taken to ensure that animals are available in the appropriate size and age range for the entire study.
- 10.2 In general, test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested, however, the use of undiluted test substance, i.e., at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of a test animal. In rodents, this volume should not normally exceed 1 ml/100g of body weight; however with aqueous solutions, 2 ml/100g body weight can be considered.
- 10.3 With respect to formulation of dosing preparations, use of an aqueous solution/suspension/emulsion is recommended when possible, followed in order of preference by a solution/ suspension/emulsion in oil, e.g., corn oil, and then possibly solution in other vehicles.
- 10.4 For vehicles other than water, toxicological characteristics of the vehicle should be known.
- 10.5 Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

11 EXPERIMENTAL PROCEDURES

11.1 Administration of doses

- 11.1.1 The test substance is administered in a single dose by gavage using a stomach tube or suitable intubation cannula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.
- 11.1.2 Animals should be fasted prior to dosing, e.g., with the rat, food but not water should be withheld overnight; with the mouse, food but not water should be withheld for 3-4 hours.

11.1.3 After fasting, animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and dose is calculated according to the fasted body weight.

11.1.4 After a substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice. Where a dose is administered in fractions over a period of time, it may be necessary to provide animals with food and water depending on the time.

11.2 Limit test and main test

11.2.1 The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity below regulatory limit doses.

11.2.2 Information about toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance.

11.2.3 In those situations where there is little or no information about its toxicity, or in which a test material is expected to be toxic, the main test should be performed.

11.3 Limit test

11.3.1 Limit test at 2,000 mg/kg

11.3.1.1 Dose one animal at the test dose. If the animal dies, conduct the main test to determine an LD₅₀. If the animal survives, dose four additional animals, one at a time. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period (see paragraph 30 for initial observation period). Late deaths should be counted the same as other deaths. The results are evaluated as follows (O=survival, X=death).

11.3.1.2 The LD₅₀ is less than the test dose (2,000 mg/kg) when three or more animals die.

OXOXX

OOXXX

OXXOX

OXXXU (U can be O or X)

If a third animal dies, conduct the main test.

11.3.1.3 The LD₅₀ is greater than the test dose (2,000 mg/kg) when three or more animals survive.

OOOOO

OOOXO

OOOOX

OOOXX

OXOXO

OXOOU (U can be O or X)

OOXXO

OOXOU (U can be O or X)

OXXOO

11.3.2 Limit test at 5,000 mg/kg

11.3.2.1 As an exception, and only when justified by specific regulatory needs, use of a dose at 5000 mg/kg may be considered. Recognizing the need to protect animal welfare, testing of animals in Class 5 ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health or the environment.

11.3.2.2 Dose one animal at the test dose. If the animal dies, conduct the main test to determine the LD₅₀. If the animal survives, dose two additional animals. If both animals survive, the LD₅₀ is greater than the limit dose and the test is terminated, i.e., carried to full 14-day observation without dosing further animals.

11.3.2.3 If one or both animals die, then dose an additional two animals, one at a time. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. Late deaths should be counted the same as other deaths. The results are evaluated as follows (O=survival, X=death, and U=Unnecessary).

11.3.2.4 The LD₅₀ is less than the test dose (5,000 mg/kg) when three or more animals die.

OXOXX

OOXXX

OXXOX

OXX XU (U can be O or X, the dosing of the 5th animal is not necessary)

11.3.2.5 The LD₅₀ is greater than the test dose (5,000 mg/kg) when three or more animals survive.

OOOOO

OXOXO

OXOOU (U can be O or X, the dosing of the 5th animal is not necessary)

OOXXO

OOXOU (U can be O or X, the dosing of the 5th animal is not necessary)

OXXOO

11.4 Main test

11.4.1 Single animals are dosed in sequence, usually at 48 h intervals. However, time intervals between dosing are determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. The time interval may be adjusted as appropriate, e.g., in case of an inconclusive response. The test is simpler to implement when a single time interval is used to make sequential dosing decisions. Nevertheless, it is not necessary to recalculate dosing or likelihood-ratios if the time interval changes midtest. To select a starting dose, all available information, including information on structurally-related substances and results of other toxicity tests on the test material, should be used to approximate an LD₅₀ as well as the slope of the dose-response curve.

11.4.2 The first animal is dosed a step below the best estimate of the LD₅₀. If the animal survives, the second animal receives a higher dose. If the first animal dies or appears moribund, a second animal receives a lower dose. The dose progression factor should be chosen to be the antilog of 1/(the estimated slope of the dose-response curve) and should remain constant throughout testing. When there is no information on the slope of the substance to be tested, a dose progression factor of 3.2 is used. Using the default progression factor, doses would be selected from the sequence

- 1.75, 5.5, 17.5, 55, 175, 550, 2,000 (or 1.75, 5.5, 17.5, 55, 175, 550, 1,750, 5,000 for specific regulatory needs). If no estimate of the lethal of a substance is available, dosing should be initiated at 175 mg/kg. In most cases, this dose is sublethal and will reduce the pain and suffering. If animal tolerances to the chemical are expected to be highly variable, i.e., slopes are expected to be ≤ 2.5 , consideration should be given to increasing the dose progression factor beyond the default 0.5 on a log dose scale, i.e., 3.2 progression factor, prior to starting the test. Table 1 includes a table of dose progressions for whole number slopes ranging from 1 to 8 with a starting dose of 175 mg/kg.
- 11.4.3 Dosing continues depending on fixed-time interval, e.g., 48-hour outcomes of all animals up to that time. The testing stops when one of the following criteria are met.
- 11.4.3.1 3 consecutive animals survive at the upper bound;
- 11.4.3.2 5 reversals occur in any 6 consecutive animals tested;
- 11.4.3.3 At least 4 animals have followed the first reversal and the specified likelihood-ratios exceed the critical value.
- NOTE — For a wide variety of combinations of LD₅₀ and slopes, stopping rule 11.4.3.3. will be satisfied with 4 to 6 animals after the test reversal. In some cases for chemicals with steep dose-response curve slopes, additional animals (up to a total of fifteen tested) may be needed.
- 11.4.4 When stopping criteria have been attained, an estimated LD₅₀ should be calculated from animal outcomes at test termination using the method described in the section of Data Compilation and Reporting.
- 11.4.5 Moribund animals killed for humane reasons are considered in the same way as animals that died on test. If an animal unexpectedly dies late in the study and there are other survivors at that dose or above, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. If subsequent survivors also die, and it appears that all dose levels exceed the LD₅₀ it would be most appropriate to start the study again beginning at least two steps below the lowest dose with deaths (and increasing the observation period) since the technique is most accurate when the starting dose is below the LD₅₀. If subsequent animals survive at or above the dose of the animal that dies, it is not necessary to change the dose progression since information from the animal that has now died will be included into the calculations as a death at a lower dose than subsequent survivors, pulling the LD₅₀ down.
- 11.5 Dose sequence for main test
- 11.5.1 Up-and-Down Dosing Procedure: For each run, animals are dosed, one at a time, usually at 48-hour intervals. The first animal receives a dose a step below the level of the best estimate of the LD₅₀. This selection reflects an adjustment for a tendency to bias away from the LD₅₀ in the direction of the initial starting dose in the final estimate. The overall pattern of outcomes is expected to stabilize as dosing is adjusted for each subsequent animal. See 11.5.3 for further guidance on choice of dose spacing factors.
- 11.5.2 Default Dose Progression: Once a starting dose and dose spacing are decided, the investigator should list all possible doses including the upper limit (usually 2,000 or 5,000 mg/kg). Doses that are close to the upper limit should be removed from the progression. The stepped nature of this design provides for the first few doses to function as a self-adjusting sequence. Because of the tendency for positive bias, in the event that nothing is known about the substance, a starting dose of 175 mg/kg is recommended. If the default procedure is to be used for the main test, dosing will be initiated at 175 mg/kg and doses will be spaced by a factor of 0.5 on a log dose scale. The doses to be used include 1.75, 5.5, 17.5, 55, 175, 550, 2,000 or, for specific regulatory needs, 1.75, 5.5, 17.5, 55, 175, 550, 1,750, 5,000. For certain highly toxic substances, the dosing sequence may need to be extended to lower values.
- 11.5.3 In the event a dose progression factor other than the default is deemed suitable, Table 1 provides dose progressions for whole number multiples of slope, from 1 to 8.