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Biological evaluation of medical devices —

Part 33: Guidance on tests to evaluate genotoxicity — Supplement to ISO 10993-3

Évaluation biologique des dispositifs médicaux —
Partie 33: Directives sur les essais pour évaluer la génotoxicité — Supplément à l'ISO 10993-3

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Contents

Page

Contents

Page

Contents

Page

Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT), see the following URL: Foreword — Supplementary information.

The committee responsible for this document is ISO/TC 194, *Biological and clinical evaluation of medical devices*.

ISO 10993 consists of the following parts, under the general title *Biological evaluation of medical devices*:

— *Part 1: Evaluation and testing within a risk management process*

— *Part 2: Animal welfare requirements*

— *Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity*

— *Part 4: Selection of tests for interactions with blood*

— *Part 5: Tests for in vitro cytotoxicity*

— *Part 6: Tests for local effects after implantation*

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 9: Framework for identification and quantification of potential degradation products*

— *Part 10: Tests for irritation and delayed-type hypersensitivity*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

— *Part 13: Identification and quantification of degradation products from polymeric medical devices*

— *Part 14: Identification and quantification of degradation products from ceramics*

— *Part 15: Identification and quantification of degradation products from metals and alloys*

— *Part 16: Toxicokinetic study design for degradation products and leachables*

— *Part 17: Establishment of allowable limits for leachable substances*

— *Part 18: Chemical characterization of materials*

— *Part 19: Physico-chemical, morphological and topographical characterization of materials (Technical specification)*

— *Part 20: Principles and methods for immunotoxicology testing of medical devices (Technical specification)*

— *Part 33: Guidance on tests to evaluate genotoxicity - Supplement to ISO 10993-3 (Technical Report)*

Introduction

Genotoxicity tests are designed to detect compounds which induce genetic damage directly or indirectly by various mechanisms. These tests should enable hazard identification with respect to genetic damages. Expression of gene mutations, large scale chromosomal damage, recombination, and numerical changes are generally considered to be essential for heritable effects and the multi-step carcinogenesis. A positive genotoxicity test provides an indication that further testing can be warranted to determine the carcinogenic potential of the compound. Because the relationship between exposure to particular chemicals and carcinogenesis is established for man, while a similar relationship has been difficult to prove for heritable diseases, genotoxicity tests have been used mainly for the prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with human disease, the suspicion that a compound can induce heritable effects is considered to be just as serious as the suspicion that a compound can induce cancer. In addition, the outcome of such tests can be valuable for the interpretation of carcinogenicity studies.

Biological evaluation of medical devices —

Part 33:
Guidance on tests to evaluate genotoxicity — Supplement to ISO 10993-3

1 Scope

There are differences between the views of regulatory bodies on the subject of genotoxicity testing. The purpose of this Technical Report is to provide background information to facilitate the selection of tests and guidance on the performance of tests.

2 Selection of tests

Since chemicals can induce genetic damage by different mechanisms, a battery of tests sensitive to different types of genetic damage are thought to provide the best assurance for detecting genotoxic hazard. The tests selected usually include tests to detect point mutations and tests to detect chromosomal aberrations. Both bacterial cells and cultured mammalian cells are used to detect genotoxic agents. *in vivo* tests are sometimes incorporated into these test batteries. These tests are sometimes included in the initial test battery or are used to clarify results from *in vitro* tests, see Reference [13].

3 Recommended tests

Although there are some variations in details, the same genotoxicity tests are commonly recommended by most regulatory agencies. The following are commonly recommended tests:

— bacterial reverse mutation test (see OECD 471[1] and Clause 6);

— *in vitro* mammalian chromosome aberration test (see OECD 473[2] and Clause 7);

— *in vitro mammalian micronucleus test* (see OECD 487[6] and Clause 8);

— *in vitro* mammalian cell gene mutation test using mouse lymphoma (L5178Y) cells (see OECD 475[4] and Clause 9);

— *in vivo* mammalian erythrocyte micronucleus test (see OECD 474[3] and Clause 10);

— *in vivo* chromosome aberration test (see OECD 475[5] and Clause 11).

For medical devices, a battery of tests is commonly used for genotoxicity evaluations. The general strategy identified in ISO 10993-3 is as follows:

a) test for gene mutations in bacteria. Bacterial Reverse Mutation Assay, OECD 471[1] technically modified for medical devices to allow, for example, testing with extracts from devices (see Clause 6);

and either

b) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells, Chromosome aberration test, OECD 473[2] technically modified for medical devices (see Clause 7), or

c) an *in vitro* mouse lymphoma tk assay, OECD 476[5] technically modified for medical devices (see Clause 8) including detection of small (slow growing) and large colonies, or

d) an *in vitro* mammalian cell micronucleus test for chromosomal damage and aneugenicity, OECD 487 technically modified for medical devices, (see Clause 8).

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) recommends a three-test battery described in the ICH S2(R1) Genotoxicity, which can be required for medical devices by some regulatory authorities.

4 Use of *in vitro* tests to detect genotoxicity

In vitro tests are commonly used for identifying the potential of chemicals to induce genotoxicity. Multiple tests are used because no single test detects all known genotoxins. Genotoxins often lead to different effects (e. g. large scale or chromosomal damage vs. small scale damage or point mutations or different DNA sequence specificity). Also, the resulting genetic damage has differing susceptibility to DNA repair. The "ICH test battery" was developed to cast a wider net for detecting genotoxins. Although *in vitro* genotoxicity tests can be considered overly sensitive, these tests detect most rodent genotoxic carcinogens. Comparisons of the "scorecards" of genotoxicity assay with those of rodent carcinogenicity assays have found that the *in vitro* mammalian assays generated a number of "false positives" (i.e. agents testing positive that were not rodent carcinogens). However, it is not clear that the rodent carcinogenicity assay is the appropriate standard, rather than detection of genotoxicity per se.

Later work identified two new classes of pharmaceuticals causing DNA damage by interference with topoisomerases. These are responsible for substantial numbers of the *in vitro* false positives, see Reference [29]. Later work indicated much lower percentages of unexplained *in vitro* positive results with pharmaceuticals, see Reference [16]. Unfortunately, all of the information on the ability of genotoxicity to predict carcinogenicity and germ cell mutagenicity was developed from the analysis of industrial chemicals and pharmaceuticals. Medical device testing usually includes the use of extracts, which often contain complex mixtures of chemicals. Although future effects are unknown, device extracts have generated limited number of positives with unknown constituents to date.

5 Use of *in vivo* tests to detect genotoxicity

The *in vivo* genotoxicity tests are an integral part of the ICH test battery and are used in a weight of evidence approach in the evaluation of pharmaceuticals. For these tests, a demonstration that the chemical or its metabolite has reached the target organ is required. For medical devices, the latter requirement is often difficult to fulfil since complex mixtures are usually tested and the dose of agent(s) in extracts can be below the detection level of the system.

The *in vivo* test for chromosomal damage using rodent haematopoietic cells is included in the test battery to provide additional relevant factors (absorption, distribution, metabolism, excretion) that can influence the genetic activity of chemicals, see Reference [14]. There are also a small number of genotoxic carcinogens that are reliably detected by the *in vivo* bone marrow tests for chromosomal damage that have yielded negative/weak/conflicting results in the pairs of *in vitro* tests outlined in the standard battery options (e.g. bacterial reverse mutation plus one of a selection of possible tests with cytogenetic evaluation of chromosomal damage or bacterial mutation plus the mouse lymphoma tk assay). A few industrial chemical carcinogens such as urethane and benzene fall into this category, see Reference [31].

The value of including *in vivo* tests as part of the initial genotoxicity assessment is controversial. The limited sensitivity of *in vivo* tests to detect a significant number of carcinogens (see Reference [10] and Reference [27]) can argue against their use. However, the concern that a small group of biologically active compounds that are known or suspected human carcinogens cannot be easily detected by *in vitro* tests (see Reference [26]) argues for their use in circumstances where the extent of exposure to biologically active constituents of a medical device indicates the need for greater reassurance.

6 Bacterial reverse mutation assay

6.1 General

The following procedure for the bacterial reverse mutation assay was adapted for medical devices from OECD 471.[1] For evaluation of genotoxic potential of medical devices, medical device material, extracts or extracted and evaporated residues can be applied to test systems.

When two extracts are used, genetic potential of each extract should be evaluated in accordance with this Clause.

Suspensions of bacterial cells are exposed to the test sample in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after 48 h or 72 h of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

6.2 Preparations

6.2.1 Bacteria

Cultures of bacteria in late exponential growth or early stationary phase of growth (approximately 10^9 cells/ml) should be used.

The recommended culture temperature is 37 °C.

The recommended combination of strains is

— *S. typhimurium* TA1535, and

— *S. typhimurium* TA1537 or TA97 or TA97a, and

— *S. typhimurium* TA98, and

— *S. typhimurium* TA100, and

— *E. coli* WP2 *uvrA*, or *E. coli* WP2 *uvrA* (pKM101), or *S. typhimurium* TA102.

Established procedures for stock culture preparation, marker verification, and storage should be used.

The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains and tryptophan for *E. coli* strains).

The following phenotypic characteristics should be checked:

a) presence or absence of R-factor plasmids, where appropriate:

   1) ampicillin resistance in strains TA98, TA100, and TA97a or TA97 and WP2 *uvrA* (pKM101);

   2) ampicillin + tetracycline resistance in strain TA102;

b) the presence of characteristic mutations:

   1) rfa mutation in *S. typhimurium* through sensitivity to crystal violet;

   2) *uvrA* mutation in *E. coli* or *uvrB* mutation in *S. typhimurium*, through sensitivity to ultraviolet light.

The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

6.2.2 Medium

An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin or tryptophan, to allow for a few cell divisions, is used.

6.2.3 Metabolic activation

Bacteria should be exposed to the test sample both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction S9 prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and ß-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range from 5 % volume fraction to 10 % volume fraction in the S9 mix.

The supplier and the S9 quality control information (e.g. preparation method, rodent strain, concentration of P450 inducer, etc.) should be recorded. If the S9 is an in-house source, then source and method of preparation should be documented. Regardless, the S9 activity should be verified using two reference promutagens in a defined strain (e.g. *S. typhimurium* TA100) and compared to the historical control.

The concentration of S9 homogenate should be expressed as activity units per plate since different suppliers can prepare S9 differently, e.g. use different co-factors in S9 mix, different ratio of tissue to homogenizing fluid during S9 preparation.

The buffer and component concentrations should be defined.

Simple omission of the S9 mix component in the top agar is not recommended in the absence of metabolic activation system, as the differing volumes of the agar overlay will alter the perceived dose of compound (at least initially, depending on solubility and/or diffusion into the basal agar). The S9 mix should be replaced with an appropriate buffer.

6.2.4 Test sample preparation

The selection of a sample preparation procedure for any medical device should consider the chemical composition and physicochemical properties of the material(s) used in the medical device. ISO 10993-12 should be consulted for sample preparation guidance. Additional information is provided in ISO 10993-3, Annex A.

— Medical devices or materials that can be dissolved or suspended in a solvent can be dosed directly to the assay (see ISO 10993-3, Annex A, Method A).

— Medical devices or materials that are not soluble in a solvent can be dosed using extracts as test samples. The choice of extraction methods depends on the percentage of extractables obtained from the test sample (see ISO 10993-3, Annex A, Method B and Method C).

Test extracts should be used within 24 h of preparation. Extracts should, if possible, be used immediately after preparation to prevent adsorption on to the extraction container or other changes in composition. If an extract is stored longer than 24 h, then the stability and homogeneity of the extract under the storage conditions should be verified.

6.3 Test conditions

6.3.1 Solvents

The test solvent should be selected in accordance with ISO 10993-12 or ISO 10993-3, Annex A, and should be compatible with the survival of the bacteria and the S9 activity. Rationale for solvent selection should be documented. If the selected solvent has not been commonly used, evidence/data demonstrating compatibility should be presented. If other than well-known solvents are used, their inclusion should be supported by data indicating their compatibility.

6.3.2 Exposure concentrations

The maximum test concentrations will depend on the solubility and cytotoxicity of the test compound or the cytotoxicity of the test sample extract.

Dose Range Finding Study (DRF study)

A DRF study may be conducted prior to the main study if cytotoxicity of the test sample is expected to be significant, e.g. cytotoxicity or growth inhibition greater than 50 %.

Cytotoxicity can be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a test sample can be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye.

Limit Study

For soluble, non-cytotoxic test compounds (determined in the DRF study), a single test at one dose level of at least 5 mg/plate or 5 µl/plate (see ISO 10993-3, Annex A, Method A or, if feasible, Method B) or 0,1ml of a 100 % (neat) test sample extract (see ISO 10993-3, Annex A, Method C) is acceptable. For test articles prepared following guidance provided in ISO 10993-12, in most situations, a limit study using 100 % test sample extract is acceptable and no further dosing study is necessary.

Main Dosing Study

If the test sample shows visible signs of precipitation or is cytotoxic already below dose level of 5 mg/plate or 5 µl/plate or 100 % of a test sample extract, a full study with at least five different analysable concentrations of the test sample should be used. For cytotoxic test compounds/test sample extracts, the dose levels for revertant frequency should cover a range from the maximum to little or no cytotoxicity. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment. The precipitate should not interfere with scoring.

Criteria to be taken into consideration when determining the highest amount of test sample to be used should include cytotoxicity and solubility in the final treatment mixture. It can be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity might be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a test sample can be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The recommended maximum test concentration will depend on the test sample preparation method selected.

a) For soluble non-cytotoxic test samples (see ISO 10993-3, Annex A, Method A), the recommended maximum test concentration is 5 mg/plate or 5 µl/plate. For non-cytotoxic test samples that are not soluble at 5 mg/plate or 5 µl/plate (in case of liquid chemicals), one or more concentrations tested should be insoluble in the final treatment mixture. Test samples that are cytotoxic already below 5 mg/plate or 5 µl/plate (in case of liquid chemicals) should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

b) For test samples in accordance with ISO 10993-3, Annex A, Method B, the recommended maximum test concentration is 5 mg/plate, if feasible. For non-cytotoxic test samples that are not soluble at 5 mg/plate, one or more concentrations tested should be insoluble in the final treatment mixture. The precipitate should not interfere with the scoring. Test samples that are cytotoxic below 5 mg/plate should be tested up to a cytotoxic concentration.

c) For test samples in accordance with ISO 10993-3, Annex A, Method C, the recommended maximum test concentration is 100 % of the test sample extract.

When a precipitate is observed or the test sample is cytotoxic, at least five different analysable concentrations of the test sample should be used. For preliminary experiments, test concentrations using

half log intervals can be helpful. Smaller intervals can be appropriate when subsequent experiments are performed. If the test sample is soluble and not cytotoxic, a single maximum concentration is acceptable.

6.3.3 Controls

Concurrent positive and negative (vehicle) controls (both with and without metabolic activation) should be included for each strain. Except for the treatment with the test sample preparation, the positive and negative control groups should be processed in an identical manner as the treatment groups.

A viable bacterial count on the stock culture should be demonstrated and recorded as part of each assay.

The induced mutation rates with the reference mutagen should be within the range reported in the literature.

For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used. The following chemicals are examples of suitable positive controls for assays with metabolic activation:

— 9,10-Dimethylanthracene [CAS no. 781-43-1];

— 7,12-Dimethylbenzanthracene [CAS no. 57-97-6];

— Congo Red [CAS no. 573-58-0] (for the reductive metabolic activation method);

— Benzo(a)pyrene [CAS no. 50-32-8];

— Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)];

— 2-Aminoanthracene [CAS no. 613-13-8].

2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9 mix. If 2-Aminoanthracene is used, each batch of S9 should also be characterized with a mutagen that requires metabolic activation by microsomal enzymes, e.g. benzo(a)pyrene, dimethylbenzanthracene.

For assays performed without metabolic activation system, examples of strain-specific positive controls are given in Table 1.

Table 1 — Examples of strain-specific positive controls

Chemical	CAS No.	Strain
Sodium azide	26628-22-8	TA1535 and TA100
2-Nitrofluorene	607-57-8	TA98
9-Aminoacridine or ICR191	17070-45-0 90-45-9	TA1537, TA97, and TA97a
Cumene hydroperoxide Or Methyl methanesulfonate	80-15-9 66-27-3	TA102
Mitomycin C	50-07-7	WP2 *uvrA* and TA102
N-Ethyl-*N*'-nitro-*N*-nitrosoguanididine or 4-Nitroquinoline 1-oxide or *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine	4245-77-6 56-57-5 70-25-7	WP2, WP2 *uvrA*, and WP2 *uvrA* (pKM101)
NOTE Other appropriate positive control reference substances may be used.		

Table 1 *(continued)*

Chemical	CAS No.	Strain
Furylfuramide (AF-2)	3688-53-7	WP2 *uvrA* plasmid-containing strains
NOTE Other appropriate positive control reference substances may be used.		

6.4 Procedure

6.4.1 Treatment with test sample

For the plate incorporation method, without metabolic activation, usually 0,1 ml of the test sample, 0,1 ml of fresh bacterial culture (containing at least 10^8 viable cells) and 0,5 ml of sterile buffer are mixed with 2,0 ml of overlay agar. For the assay with metabolic activation, usually 0,5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 % to 10 % volume fraction in the metabolic activation mixture) are mixed with the overlay agar (2,0 ml), together with the bacteria and test sample/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

For the preincubation method, the test sample/test solution is preincubated with the test strain (containing at least 10^8 viable cells) and sterile buffer or the metabolic activation system (0,5 ml) usually for 20 min or more at 37 °C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0,1 ml of test sample or extract, 0,1 ml of bacteria, and 0,5 ml of S9 mix or sterile buffer, are mixed with 2,0 ml of overlay agar. Tubes should be aerated during pre-incubation by using a shaker.

For an adequate estimate of variation, triplicate plating should be used at each dose level.

The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels.

If quantitative comparisons are to be made between experiments carried out in the presence and absence of S9 mix, the experiments should be performed on the same day.

For *in vitro* assays with built-in confirmatory elements such as multiple treatment lengths or tests with and without metabolic activation, further confirmatory testing in the case of clearly negative or positive test results is not usually needed. Equivocal results can require repeating tests, possibly with a modified protocol such as appropriate spacing of the test concentrations.

6.4.2 Incubation

All plates in a given assay should be incubated at 37 °C for 48 h to 72 h. After the incubation period, the number of revertant colonies per plate is counted.

6.4.3 Data collection

Automated colony counters should be calibrated against a series of authentic hand-counted plates encompassing a range of mutant colonies, from very low to very high counts and colonies of varying sizes.

The condition of the bacterial background for evidence of test article extract toxicity should be evaluated by using a dissecting microscope or darkfield counter. The precipitate should be evaluated by visual examination without magnification. Toxicity and degree of precipitation should be scored relative to the corresponding extraction blank using the standardized method (e.g. table at the end of document).