International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

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STUDIES TO EVALUATE THE SAFETY OF RESIDUES OF VETERINARY DRUGS IN HUMAN FOOD: GENOTOXICITY TESTING (REVISION 2)

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TABLE OF CONTENTS

1. INTRODUCTION

1.1. Objective of the guideline

In order to establish the safety of veterinary drug residues in human food, a number of toxicological evaluations are recommended, including investigation of possible hazards from genotoxic activity. Many carcinogens and/or genotoxicants have a genotoxic mode of action, and it is prudent to regard genotoxicants as potential carcinogens unless there is convincing evidence that this is not the case. The results of genotoxicity tests will normally not affect the numerical value of an acceptable daily intake (ADI), but they may influence the decision on whether carcinogenicity tests are needed and whether an ADI can be established.

The objective of this guideline is to ensure international harmonisation of genotoxicity testing of veterinary drug residues.

1.2. Background

This guideline is one of a series of VICH guidelines developed to facilitate the mutual acceptance of safety data necessary for the establishment of ADIs for veterinary drug residues in human food by the relevant regulatory authorities. It should be read in conjunction with the guideline on the overall strategy for the evaluation of veterinary drug residues in human food[1]. VICH GL23 was developed after consideration of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines for pharmaceuticals for human use: "Genotoxicity: A Standard Battery of Genotoxicity Testing of Pharmaceuticals"[2] and "Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals"[3].

For VICH GL23(R2), account is taken of OECD Guidelines for Testing of Chemicals, of the WHO International Programme on Chemical Safety (IPCS) Environmental Health Criteria (EHC) 240[4], of ICH guideline S2(R1)[5], of EFSA (2011)[6], and of national/regional guidelines and the current practices for evaluating the safety of veterinary drug residues in human food in the EU, Japan, the USA, Australia, Canada, New Zealand, and the UK. VICH seeks to minimize animal testing in alignment with the principle of the 3Rs– replacement, refinement, and reduction of animal use in toxicology studies.

1.3. Scope of the guideline

This guideline recommends a Standard Battery of Tests that can be used for the evaluation of the genotoxicity of veterinary drug residues (including parent drug substances and/or metabolites). The Standard Battery of Tests intends to achieve reasonable confidence in the assessment of the genotoxicity potential of veterinary drug residues and to be in harmony with the requirements of ICH for testing human drugs for genotoxicity. This guideline also advises on modifications to the Standard Battery of Tests and on interpretation of test results.

2. STANDARD BATTERY OF TESTS

VICH recommends two options for the Standard Battery of Tests and both options are considered equally suitable for the hazard identification of genotoxicity potential:

- Option 1 includes a test for gene mutation in bacteria, an *in vitro* test in mammalian cells and an *in vivo* test for chromosomal effects using rodent hematopoietic cells.

- Option 2 includes a test for gene mutation in bacteria, an *in vivo* test for chromosomal effects using rodent hematopoietic cells and a second *in vivo* test.

In some jurisdictions, legislation requires implementation of the 3Rs wherever possible. Option 1 is therefore recommended unless there is scientific justification for using Option 2, or the second *in vivo* test can be integrated into repeat dose tests without requiring the use of an increased number of animals.

The current versions of OECD test guidelines for genotoxicity should be used to guide the conduct of the tests.

In most cases, it is the parent drug substance that is tested. In some cases, one or more of the major metabolites that occur as residues in food may also be tested, especially when it is produced in the target species but not produced in the laboratory animal species, and/or it has structural alerts; major metabolites are those comprising ≥100 µg/kg or ≥10% of the total residue in a sample collected from the target animal species in the metabolism study[7]. For some regions, testing other, non-major metabolites may also need to be considered, such as when the metabolite has structural alerts that are not present in the molecular structure of the parent drug. Salts, esters, conjugates, and bound residues are usually assumed to have similar genotoxic properties to the parent drug, unless there is evidence to the contrary.

In addition to the Standard Battery of Tests, other available information (such as *in silico* data and published literature) may provide additional evidence as part of the weight of evidence assessment for genotoxicity potential of veterinary drug residues. When performing the *in silico* (quantitative) structure-activity relationship ((Q)SAR) assessment, two complementary (Q)SAR methods, i.e., expert rule-based and statistical-based, should be used[8]. Current (Q)SAR models are effective only for predicting bacterial mutagenicity[8].

2.1. A test for gene mutation in bacteria

The gene mutation test in bacteria is the first test in Options 1 and 2 of the Standard Battery of Tests. An extensive database has been built up for bacterial reverse mutation tests for gene mutation in strains of *Salmonella typhimurium* and *Escherichia coli*. However, the bacterial gene mutation test, whilst being an efficient test for detecting substances with inherent potential for inducing gene mutations, does not detect all substances with mutagenic potential.

2.2. An *in vitro* test in mammalian cells

The second test in Option 1 evaluates the potential of a substance to produce chromosomal effects. This can be evaluated using one of the following three tests: (1) an *in vitro* mammalian cell micronucleus test, which detects both clastogenicity and aneugenicity; (2) an *in vitro* chromosomal aberrations test using metaphase analysis, which detects clastogenicity; or (3) an *in vitro* gene mutation test in mammalian cells, which can detect both gene mutation and chromosomal damage.

2.3. An *in vivo* test for chromosomal effects using rodent haematopoietic cells

The third test in Option 1 and the second test in Option 2 is an *in vivo* test to ensure the detection of all potential genotoxicants. This could be either a micronucleus test or a chromosomal aberration test.

2.4. A second *in vivo* genotoxicity test

The third test in Option 2 could be either the *in vivo* mammalian alkaline comet assay, or the *in vivo* transgenic mouse/rat mutation assay. Other validated *in vivo* tests, such as the *Pig-a* assay, may also be acceptable.

3. MODIFICATIONS TO THE STANDARD BATTERY

For most substances, the Standard Battery of Tests should be sufficient for genotoxicity testing. In some instances, there is a need for modifications to the choice of tests or to the protocols of the individual tests. A scientific justification should be given for not using the Standard Battery of Tests.

The physicochemical properties of a substance (e.g., pH, solubility, stability, and volatility) can sometimes make standard test conditions inappropriate. It is essential that due consideration is given before tests are conducted. Modified protocols should be used where it is evident that standard conditions will likely give a false negative or false positive result. The OECD Guidelines for Testing of Chemicals for the genotoxicity tests provide advice on the susceptibility of the individual tests to the physical characteristics of the test substance as well as advice on compensatory measures that may be taken.

Alternative genotoxicity tests (e.g., other validated genotoxicity studies, including new approach methods) can be considered on a case-by-case basis; however, their use should be justified.

3.1. Antimicrobials

Bacteria may be susceptible to inhibition by antimicrobial substances. For such substances, it would be appropriate to perform a gene mutation test in bacteria using concentrations up to the limit of cytotoxicity in accordance with the respective OECD guidelines, and to supplement the bacterial test with an *in vitro* test for gene mutation in mammalian cells.

3.2. Metabolic activation

The *in vitro* tests should be performed in the presence and absence of a metabolic activation system. Metabolic activation systems other than the standard S9 mix from induced livers of rats may be used, such as human microsomal preparations or S9 mix from induced livers of hamsters. A scientific rationale should be given to justify the choice of an alternative metabolic activation system.

4. OVERVIEW OF RECOMMENDED TESTS

The tests of the Standard Battery in Options 1 and 2 are listed in the table below, with their respective OECD guidelines.

Table 1. Tests of the Standard Battery in Options 1 and 2

<table>
<tr><th>Type</th><th>Test</th><th>Section number</th><th>Option 1</th><th>Option 2</th><th>OECD TG number</th></tr>
<tr><td rowspan="5">In vitro</td><td>Bacterial reverse mutation test</td><td>4.1</td><td>First test</td><td>First test</td><td>471</td></tr>
<tr><td>Mammalian cell micronucleus test</td><td>4.2</td><td rowspan="4">Second test (one of these tests)</td><td rowspan="4"></td><td>487</td></tr>
<tr><td>Chromosome aberration test in mammalian cells</td><td>4.2</td><td>473</td></tr>
<tr><td>Mammalian cell gene mutation test using Hprt and xprt genes</td><td>4.3</td><td>476</td></tr>
<tr><td>Mammalian cell gene mutation test using thymidine kinase gene</td><td>4.3</td><td>490</td></tr>
<tr><td rowspan="4">In vivo</td><td>Mammalian erythrocyte micronucleus test</td><td>4.4</td><td rowspan="2">Third test (one of these tests)</td><td rowspan="2">Second test (one of these tests)</td><td>474</td></tr>
<tr><td>Mammalian bone marrow chromosome aberration test</td><td>4.4</td><td>475</td></tr>
<tr><td>Mammalian alkaline comet assay</td><td>4.5</td><td rowspan="2"></td><td rowspan="2">Third test (one of these tests)</td><td>489</td></tr>
<tr><td>Transgenic rodent somatic and germ cell mutation assay</td><td>4.5</td><td>488</td></tr>
</table>

4.1. A test for gene mutation in bacteria

A bacterial reverse mutation test should be performed according to OECD Test Guideline 471[9]. This test uses at least five amino acid-requiring strains of *S. typhimurium* and *E. coli* to detect point mutations by base substitutions or frameshifts. It detects mutations which revert 'lack of function' mutations present in the test strains, and restore the functional capability of the bacteria to synthesize an essential amino acids and to allow bacterial growth without supplementation of the amino acid.

4.2. *In vitro* tests for chromosomal effects in mammalian cells

An *in vitro* mammalian cell micronucleus test should be performed according to OECD Test Guideline 487[10]. This test is a genotoxicity test for the detection of micronuclei in the cytoplasm of interphase cells. Micronuclei may originate from acentric chromosome fragments (i.e., lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The assay detects the activity of clastogenic and aneugenic test substances in cells that have undergone cell division during or after exposure to the test substance. This test would be recommended for the detection of aneuploidy and, thus, as preferred test for clastogenicity.

An *in vitro* chromosome aberration test should be performed according to OECD Test Guideline 473[11]. This test identifies substances that cause structural chromosomal aberrations from clastogenic events in cultured mammalian cells. Structural aberrations may be of two types: at chromosome level, or at chromatid level. Polyploidy (including endoreduplication) could arise in chromosome aberration assays *in vitro*. While aneugens can induce polyploidy, polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation or cytotoxicity. This test is not designed to measure aneuploidy.

4.3. *In vitro* tests for gene mutation in mammalian cells

A mammalian cell gene mutation test using *Hprt* and *xprt* genes should be performed according to OECD Test Guideline 476[12]. This test can be used to detect gene mutations. In this test, the genetic endpoints used measure mutation at hypoxanthine-guanine phosphoribosyl transferase (HPRT), and at a transgene of xanthineguanine phosphoribosyl transferase (XPRT). The HPRT and XPRT mutation tests detect different spectra of genetic events.

A mammalian cell gene mutation test using thymidine kinase (TK) gene should be performed according to OECD Test Guideline 490[13]. This test can be used to detect gene mutations. The Test Guideline includes two alternative *in vitro* mammalian gene mutation assays requiring two specific TK heterozygous cells lines: L5178Y $TK^{+/-}$-3.7.2C cells for the mouse lymphoma assay (MLA) and TK6 $TK^{+/-}$ cells for the TK6 assay. Genetic events detected using the *tk* locus include both gene mutations and chromosomal events.

4.4. *In vivo* tests for chromosomal effects

The mammalian *in vivo* micronucleus test as described in OECD Test Guideline 474[14] is used for the detection of damage to the chromosomes or the mitotic apparatus of erythroblasts, by analysis of erythrocytes, which are sampled in bone marrow and/or peripheral blood cells of the test animals, usually rodents (mice or rats). This test identifies substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. This test can be integrated into repeat-dose toxicity studies.

The mammalian *in vivo* chromosome aberration test, as described in OECD Test Guideline 475[15], detects structural chromosome aberrations induced by test substances in bone marrow cells of the test animals, usually rodents (mice or rats).

4.5. Second *in vivo* test for chromosomal effects

The *in vivo* mammalian alkaline Comet assay (also called *in vivo* alkaline single cell gel

electrophoresis assay), as described in OECD Test Guideline 489[16], identifies substances that cause DNA damage. Under alkaline conditions, this assay can detect single and double stranded breaks. This test can be integrated into repeat-dose toxicity studies.

The transgenic rodent somatic and germ cell mutation assay, as described in OECD Test Guideline 488[17], detects gene mutations in both somatic and germ cells. In this assay, transgenic rats or mice that contain multiple copies of chromosomally integrated plasmid or phage shuttle vectors are used as the test system. The transgenes contain reporter genes for the detection of various types of mutations induced by test substances during a 28-day treatment period.

4.6. Integration of *in vivo* genotoxicity testing in repeat-dose toxicity studies

VICH recommends combining the *in vivo* tests described above with repeat-dose toxicity studies, whenever possible. Further guidance can be found in ICH S2(R1)[5], IPCS[4] and OECD Test Guideline 474[14].

5. EVALUATION OF TEST RESULTS

The evaluation of the genotoxic potential of a substance should take into account the totality of the findings and acknowledge the intrinsic values and limitations of both *in vitro* and *in vivo* tests. Other available information (such as *in silico* data and published literature) may provide additional evidence as part of the weight of evidence assessment for genotoxicity potential of veterinary drug residues[4].

Clearly negative results for genotoxicity in a series of tests, including the Standard Battery of Tests, will usually be taken as sufficient evidence of an absence of genotoxicity.

If a substance gives a clearly positive result for mutagenicity in the bacteria gene mutation test, additional *in vivo* testing including carcinogenicity tests may be needed. In some jurisdictions, the consequences of positive findings in genotoxicity tests are regulated in legislation[18].

If a substance gives clearly positive result(s) for *in vitro* genotoxicity tests, but a clearly negative result in the *in vivo* genotoxicity test(s) such as those performed using bone marrow, it will be necessary to confirm whether it is genotoxic with another *in vivo* genotoxicity test using a target tissue other than bone marrow. The most appropriate test should be chosen with justification on a case-by-case basis.

If a clear conclusion cannot be reached with the Standard Battery of Tests, follow-up considerations and strategies can be found in ICH[5], IPCS[4] and OECD[19].

6. REFERENCES

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7. GLOSSARY

The following definitions apply for purposes of this guideline:

Aneugenicity:	The ability to cause aneuploidy.
Aneuploidy:	Numerical deviation of the modal number of chromosomes in a cell or organism, other than an extra or reduced number of complete sets of chromosomes.
Clastogen:	An agent that produces structural changes of chromosomes, usually detectable by light microscopy.
Clastogenicity:	The ability to cause structural changes of chromosomes (chromosomal aberrations).
Cytogenetics:	Chromosome analysis of cells, normally performed on dividing cells when chromosomes are condensed and visible with a light microscope after staining.
Gene mutation:	A detectable permanent change within a single gene or its regulating sequences. The change may be a point mutation, insertion, deletion, etc.
Genotoxicity:	A broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced.
Micronucleus:	Particle in a cell that contains microscopically detectable nuclear DNA; it might contain a whole chromosome(s) or a broken centric or acentric part(s) of chromosome(s). The size of a micronucleus is usually defined as less than 1/5 but more than 1/20 of the main nucleus.
Mutagenicity:	The capacity to cause a permanent change in the amount or structure of the genetic material in an organism or cell that may result in change in the characteristics of the organism or cell. The alteration may involve changes to the sequence of bases in the nucleic acid (gene mutation).
Polyploidy:	An extra or reduced number of complete sets of chromosomes.