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5 **veterinary drugs in human food: genotoxicity testing**
6 **(revision 2)**
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International Cooperation on Harmonisation of Technical Requirements
for Registration of Veterinary Medicinal Products

VICH GL23 (R2) (SAFETY) - GENOTOXICITY

May 2024

Revision at Step 9

For consultation at Step 4

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

Revision at Step 9

Recommended for Consultation at Step 4 of the VICH Process

in May 2024

by the VICH Steering Committee

This Guideline has been developed by the appropriate VICH Expert Working Group and is subject to consultation by the parties, in accordance with the VICH Process. At Step 7 of the Process the final draft will be recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

59 **STUDIES TO EVALUATE THE SAFETY OF RESIDUES OF**
60 **VETERINARY DRUGS IN HUMAN FOOD: GENOTOXICITY TESTING**
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88 1. INTRODUCTION

89 1.1. Objective of the guideline

90
91 In order to establish the safety of veterinary drug residues in human food, a number of toxicological
92 evaluations are recommended, including investigation of possible hazards from genotoxic activity.
93 Many carcinogens and/or genotoxicants have a genotoxic mode of action, and it is prudent to regard
94 genotoxicants as potential carcinogens unless there is convincing evidence that this is not the case.
95 The results of genotoxicity tests will normally not affect the numerical value of an acceptable daily
96 intake (ADI), but they may influence the decision on whether carcinogenicity tests are needed and
97 whether an ADI can be established.

98
99 The objective of this guideline is to ensure international harmonisation of genotoxicity testing of
100 veterinary drug residues.

101 1.2. Background

102
103 This guideline is one of a series of VICH guidelines developed to facilitate the mutual acceptance
104 of safety data necessary for the establishment of ADIs for veterinary drug residues in human food
105 by the relevant regulatory authorities. It should be read in conjunction with the guideline on the
106 overall strategy for the evaluation of veterinary drug residues in human food¹. VICH GL23 was
107 developed after consideration of the International Council for Harmonisation of Technical
108 Requirements for Pharmaceuticals for Human Use (ICH) guidelines for pharmaceuticals for human
109 use: “Genotoxicity: A Standard Battery of Genotoxicity Testing of Pharmaceuticals”² and “Guidance
110 on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals”³.

111
112 For VICH GL23(R2), account is taken of OECD Guidelines for Testing of Chemicals, of the WHO
113 International Programme on Chemical Safety (IPCS) Environmental Health Criteria (EHC) 240⁴, of
114 ICH guideline S2(R1)⁵, of EFSA (2011)⁶, and of national/regional guidelines and the current
115 practices for evaluating the safety of veterinary drug residues in human food in the EU, Japan, the
116 USA, Australia, Canada, New Zealand, and the UK. VICH seeks to minimize animal testing in
117 alignment with the principle of the 3Rs– replacement, refinement, and reduction of animal use in
118 toxicology studies.

119 1.3. Scope of the guideline

120
121 This guideline recommends a Standard Battery of Tests that can be used for the evaluation of the
122 genotoxicity of veterinary drug residues (including parent drug substances and/or metabolites). The
123 Standard Battery of Tests intends to achieve reasonable confidence in the assessment of the
124 genotoxicity potential of veterinary drug residues and to be in harmony with the requirements of ICH
125 for testing human drugs for genotoxicity. This guideline also advises on modifications to the
126 Standard Battery of Tests and on interpretation of test results.

127 2. STANDARD BATTERY OF TESTS

128
129 VICH recommends two options for the Standard Battery of Tests and both options are considered
130 equally suitable for the hazard identification of genotoxicity potential:

- 131
132
- 133 • Option 1 includes a test for gene mutation in bacteria, an *in vitro* test in mammalian cells and
134 an *in vivo* test for chromosomal effects using rodent hematopoietic cells.
 - 135 • Option 2 includes a test for gene mutation in bacteria, an *in vivo* test for chromosomal effects
136 using rodent hematopoietic cells and a second *in vivo* test.
- 137
138

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

143
144 The current versions of OECD test guidelines for genotoxicity should be used to guide the conduct
145 of the tests.

146
147 In most cases, it is the parent drug substance that is tested. In some cases, one or more of the
148 major metabolites that occur as residues in food may also be tested, especially when it is produced
149 in the target species but not produced in the laboratory animal species, and/or it has structural
150 alerts; major metabolites are those comprising ≥ 100 $\mu\text{g}/\text{kg}$ or $\geq 10\%$ of the total residue in a sample
151 collected from the target animal species in the metabolism study⁷. For some regions, testing other,
152 non-major metabolites may also need to be considered, such as when the metabolite has structural
153 alerts that are not present in the molecular structure of the parent drug. Salts, esters, conjugates,
154 and bound residues are usually assumed to have similar genotoxic properties to the parent drug,
155 unless there is evidence to the contrary.

156
157 In addition to the Standard Battery of Tests, other available information (such as *in silico* data and
158 published literature) may provide additional evidence as part of the weight of evidence assessment
159 for genotoxicity potential of veterinary drug residues. When performing the *in silico* (quantitative)
160 structure-activity relationship ((Q)SAR) assessment, two complementary (Q)SAR methods, i.e.,
161 expert rule-based and statistical-based, should be used⁸. Current (Q)SAR models are effective only
162 for predicting bacterial mutagenicity⁸.

163 **2.1. A test for gene mutation in bacteria**

164
165 The gene mutation test in bacteria is the first test in Options 1 and 2 of the Standard Battery of
166 Tests. An extensive database has been built up for bacterial reverse mutation tests for gene
167 mutation in strains of *Salmonella typhimurium* and *Escherichia coli*. However, the bacterial gene
168 mutation test, whilst being an efficient test for detecting substances with inherent potential for
169 inducing gene mutations, does not detect all substances with mutagenic potential.

170 **2.2. An *in vitro* test in mammalian cells**

171
172 The second test in Option 1 evaluates the potential of a substance to produce chromosomal effects.
173 This can be evaluated using one of the following three tests: (1) an *in vitro* mammalian cell
174 micronucleus test, which detects both clastogenicity and aneugenicity; (2) an *in vitro* chromosomal
175 aberrations test using metaphase analysis, which detects clastogenicity; or (3) an *in vitro* gene
176 mutation test in mammalian cells, which can detect both gene mutation and chromosomal damage.

177 **2.3. An *in vivo* test for chromosomal effects using rodent haematopoietic cells**

178
179 The third test in Option 1 and the second test in Option 2 is an *in vivo* test to ensure the detection
180 of all potential genotoxicants. This could be either a micronucleus test or a chromosomal aberration
181 test.

182 **2.4. A second *in vivo* genotoxicity test**

183
184 The third test in Option 2 could be either the *in vivo* mammalian alkaline comet assay, or the *in vivo*
185 transgenic mouse/rat mutation assay. Other validated *in vivo* tests, such as the *Pig-a* assay, may
186 also be acceptable.

187

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

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

224 **4.1. A test for gene mutation in bacteria**

225
226 A bacterial reverse mutation test should be performed according to OECD Test Guideline 471⁹. This
227 test uses at least five amino acid-requiring strains of *S. typhimurium* and *E. coli* to detect point
228 mutations by base substitutions or frameshifts. It detects mutations which revert 'lack of function'
229 mutations present in the test strains, and restore the functional capability of the bacteria to
230 synthesize an essential amino acids and to allow bacterial growth without supplementation of the
231 amino acid.

232 **4.2. *In vitro* tests for chromosomal effects in mammalian cells**

233
234 An *in vitro* mammalian cell micronucleus test should be performed according to OECD Test
235 Guideline 487¹⁰. This test is a genotoxicity test for the detection of micronuclei in the cytoplasm of
236 interphase cells. Micronuclei may originate from acentric chromosome fragments (i.e., lacking a
237 centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase
238 stage of cell division. The assay detects the activity of clastogenic and aneugenic test substances
239 in cells that have undergone cell division during or after exposure to the test substance. This test
240 would be recommended for the detection of aneuploidy and, thus, as preferred test for
241 clastogenicity.

242
243 An *in vitro* chromosome aberration test should be performed according to OECD Test Guideline
244 473¹¹. This test identifies substances that cause structural chromosomal aberrations from
245 clastogenic events in cultured mammalian cells. Structural aberrations may be of two types: at
246 chromosome level, or at chromatid level. Polyploidy (including endoreduplication) could arise in
247 chromosome aberration assays *in vitro*. While aneugens can induce polyploidy, polyploidy alone
248 does not indicate aneugenic potential and can simply indicate cell cycle perturbation or cytotoxicity.
249 This test is not designed to measure aneuploidy.

250 **4.3. *In vitro* tests for gene mutation in mammalian cells**

251
252 A mammalian cell gene mutation test using *Hprt* and *xprt* genes should be performed according to
253 OECD Test Guideline 476¹². This test can be used to detect gene mutations. In this test, the genetic
254 endpoints used measure mutation at hypoxanthine-guanine phosphoribosyl transferase (HPRT),
255 and at a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The HPRT and XPRT
256 mutation tests detect different spectra of genetic events.

257 A mammalian cell gene mutation test using thymidine kinase (TK) gene should be performed
258 according to OECD Test Guideline 490¹³. This test can be used to detect gene mutations. The Test
259 Guideline includes two alternative *in vitro* mammalian gene mutation assays requiring two specific
260 TK heterozygous cells lines: L5178Y *TK*⁺-3.7.2C cells for the mouse lymphoma assay (MLA) and
261 TK6 *TK*⁺ cells for the TK6 assay. Genetic events detected using the *tk* locus include both gene
262 mutations and chromosomal events.

263 **4.4. *In vivo* tests for chromosomal effects**

264
265 The mammalian *in vivo* micronucleus test as described in OECD Test Guideline 474¹⁴ is used for
266 the detection of damage to the chromosomes or the mitotic apparatus of erythroblasts, by analysis
267 of erythrocytes, which are sampled in bone marrow and/or peripheral blood cells of the test animals,
268 usually rodents (mice or rats). This test identifies substances that cause cytogenetic damage which
269 results in the formation of micronuclei containing lagging chromosome fragments or whole
270 chromosomes. This test can be integrated into repeat-dose toxicity studies.

271 The mammalian *in vivo* chromosome aberration test, as described in OECD Test Guideline 475¹⁵,
272 detects structural chromosome aberrations induced by test substances in bone marrow cells of the
273 test animals, usually rodents (mice or rats).

274 **4.5. Second *in vivo* test for chromosomal effects**

275
276 The *in vivo* mammalian alkaline Comet assay (also called *in vivo* alkaline single cell gel
277 electrophoresis assay), as described in OECD Test Guideline 489¹⁶, identifies substances that
278 cause DNA damage. Under alkaline conditions, this assay can detect single and double stranded
279 breaks. This test can be integrated into repeat-dose toxicity studies.

280
281 The transgenic rodent somatic and germ cell mutation assay, as described in OECD Test Guideline
282 488¹⁷, detects gene mutations in both somatic and germ cells. In this assay, transgenic rats or mice
283 that contain multiple copies of chromosomally integrated plasmid or phage shuttle vectors are used
284 as the test system. The transgenes contain reporter genes for the detection of various types of
285 mutations induced by test substances during a 28-day treatment period.

286 **4.6. Integration of *in vivo* genotoxicity testing in repeat-dose toxicity studies**

287
288 VICH recommends combining the *in vivo* tests described above with repeat-dose toxicity studies,
289 whenever possible. Further guidance can be found in ICH S2(R1)⁵, IPCS⁴ and OECD Test Guideline
290 474¹⁴.

291 **5. EVALUATION OF TEST RESULTS**

292
293 The evaluation of the genotoxic potential of a substance should take into account the totality of the
294 findings and acknowledge the intrinsic values and limitations of both *in vitro* and *in vivo* tests. Other
295 available information (such as *in silico* data and published literature) may provide additional
296 evidence as part of the weight of evidence assessment for genotoxicity potential of veterinary drug
297 residues⁴.

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

300 If a substance gives a clearly positive result for mutagenicity in the bacteria gene mutation test,
301 additional *in vivo* testing including carcinogenicity tests may be needed. In some jurisdictions, the
302 consequences of positive findings in genotoxicity tests are regulated in legislation¹⁸.

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

308 If a clear conclusion cannot be reached with the Standard Battery of Tests, follow-up considerations
309 and strategies can be found in ICH⁵, IPCS⁴ and OECD¹⁹.

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360 **7. GLOSSARY**

361

362 The following definitions apply for purposes of this guideline:

- 363 Aneugenicity: The ability to cause aneuploidy.
- 364 Aneuploidy: Numerical deviation of the modal number of chromosomes in a cell or organism,
365 other than an extra or reduced number of complete sets of chromosomes.
- 366 Clastogen: An agent that produces structural changes of chromosomes, usually detectable
367 by light microscopy.
- 368 Clastogenicity: The ability to cause structural changes of chromosomes (chromosomal
369 aberrations).
- 370 Cytogenetics: Chromosome analysis of cells, normally performed on dividing cells when
371 chromosomes are condensed and visible with a light microscope after staining.
- 372 Gene mutation: A detectable permanent change within a single gene or its regulating
373 sequences. The change may be a point mutation, insertion, deletion, etc.
- 374 Genotoxicity: A broad term that refers to any deleterious change in the genetic material
375 regardless of the mechanism by which the change is induced.
- 376 Micronucleus: Particle in a cell that contains microscopically detectable nuclear DNA; it might
377 contain a whole chromosome(s) or a broken centric or acentric part(s) of
378 chromosome(s). The size of a micronucleus is usually defined as less than 1/5
379 but more than 1/20 of the main nucleus.
- 380 Mutagenicity: The capacity to cause a permanent change in the amount or structure of the
381 genetic material in an organism or cell that may result in change in the
382 characteristics of the organism or cell. The alteration may involve changes to
383 the sequence of bases in the nucleic acid (gene mutation).
- 384 Polyploidy: An extra or reduced number of complete sets of chromosomes.
385