# METHODS FOR DETERMINING SUB-ACUTE, SUB-CHRONIC, AND CHRONIC TOXICITY OF CHEMICAL COMPOUNDS

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**Abstract:** The safety of pharmacological, chemical agents, and dietary supplements gains a high level of public interest. The creation and introduction of new drugs go through two stages – preclinical and clinical. Pharmaceutical products must undergo a range of general toxicology studies during the preclinical stage to provide information on the safety of a potential new drug in at least two different animal species. General toxicology studies begin with an acute toxicity study of the compound and progress to longer exposure durations, a minimum of six months. The preclinical toxicity studies can be divided into 1) according to the duration of the survey and its purposes: acute, sub-acute, sub-chronic, and chronic toxicity studies; 2) according to the number of doses administered: single-dose toxicity studies (acute toxicity study) and repeated-dose toxicity studies (sub-acute, sub-chronic, and chronic toxicity studies); 3) according to the location of drug exposure: systemic toxicity studies (acute, sub-acute, sub-chronic, and chronic toxicity studies) and local toxicity studies (dermal toxicity, ophthalmic toxicity studies). This review will discuss the main methods for determining the sub-acute, sub-chronic, and chronic toxicity of chemical compounds. Sub-acute toxicity studies aim to determine the doses to be tested for sub-chronic toxicity. Long-term treatment with the substance under investigation allows the registration of a toxic effect, manifested after a certain latent period. Sub-chronic and chronic toxicity are investigated during the fourth phase of preclinical trials. During the trial, changes in body weight and water and food consumption, changes affecting the skin, fur, mucous membranes, respiratory and cardiovascular systems, behavior and motor activity, and blood and urine tests are monitored daily. Specific toxicological studies are conducted – a study of allergenicity, local tolerance, and toxicity, a study of immunotoxicity, a study of reproductive toxicity and teratogenicity, peri- and postnatal toxicity, mutagenicity, carcinogenicity, etc. After completion of the experiment and euthanasia of the animals, histological examinations are performed to establish potential adverse drug reactions. In this review, the most used tests in the study of the above-mentioned different types of chronic toxicity are discussed. The safety of potential new drugs must be adequately assessed in preclinical studies with various methods for determining acute, sub-acute, sub-chronic, and chronic toxicity. If the test compound has shown acceptable efficacy and safety during preclinical testing, it moves on to testing in human clinical trials.
**Keywords:** preclinical studies, sub-acute toxicity, sub-chronic toxicity, chronic toxicity, methods

## 1. INTRODUCTION

The safety of pharmacological, chemical agents, and dietary supplements gains a high level of public interest. The creation and introduction of new drugs go through two stages - preclinical and clinical. Pharmaceutical products must undergo a range of general toxicology studies during the preclinical stage to provide information on the safety of a potential new drug in at least two different animal species. General toxicology studies begin with an acute toxicity study of the compound and progress to longer exposure durations, a minimum of 6 months.

The preclinical toxicity studies can be divided into:

- according to the duration of the survey and its purposes: acute, sub-acute, sub-chronic, and chronic toxicity studies (Table 1);
- according to the number of doses administered: single-dose toxicity studies (acute toxicity study) and repeated-dose toxicity studies (sub-acute, sub-chronic, and chronic toxicity studies);
- according to the location of drug exposure: systemic toxicity studies (acute, sub-acute, sub-chronic, and chronic toxicity studies) and local toxicity studies (dermal toxicity, ophthalmic toxicity studies).

Sub-acute, sub-chronic, and chronic toxicity studies are performed to determine the risk of long-term exposure to substances used in daily life or to establish potential toxic effects with repeated administration of the test substances, for example drugs.

***Table 1. Toxicity determined in vivo and application of the data obtained.***

| Type of toxicity | Duration of study | Application |
|---|---|---|
| Acute | Up to 24 hours | Risk assessment after single exposure to high doses |
| Sub-acute | 2-4 weeks | Risk assessment for frequent exposure to the test substance |
| Sub-chronic | Minimum of 3 months | Risk assessment for frequent exposure to the test substance |
| Chronic | Minimum of 6 months | Determination of a potential carcinogenic effect |

This review will discuss the main methods for determining the sub-acute, sub-chronic, and chronic toxicity of chemical compounds.

**2. SUB-ACUTE TOXICITY STUDY**

Sub-acute toxicity studies aim to determine the doses to be tested for sub-chronic toxicity. Long-term treatment with the substance under investigation allows the registration of a toxic effect, manifested after a certain latent period. Usually, the animals studied are of one biological species (rats or dogs). They are divided into groups of 10, and three doses are tested.

**3. SUB-CHRONIC AND CHRONIC TOXICITY STUDY**

Sub-chronic and chronic toxicity are investigated during the fourth phase of preclinical trials. During the trial, changes in body weight and water and food consumption, changes affecting the skin, fur, mucous membranes, respiratory and cardiovascular systems, behavior and motor activity, and blood and urine tests are monitored daily. Specific toxicological studies are conducted – a study of allergenicity, local tolerance, and toxicity, a study of reproductive toxicity and teratogenicity, a study of mutagenicity, etc. Very often, the study of chronic toxicity is combined with a study of the carcinogenic potential of the substance. After completion of the experiment and euthanasia of the animals, histological examinations are performed to establish potential adverse drug reactions.

**4. OPHTHALMOTOXICITY**

*In vivo* testing is performed by instilling 0.1 ml of liquid substance or introducing 100 mg of solid substance into one eye of a rabbit. Changes 1, 24, 48, 72 hours, and 7 days after application are monitored. *In vitro* tests have been proposed to replace *in vivo* studies, but there is still no *in vitro* method that can predict with sufficient accuracy the ophthalmic toxicity of a test substance.

**5. ALLERGENICITY**

The frequency of skin allergic reactions has been continuously increasing in recent years. Irritant dermatitis is usually caused by direct skin contact with the chemical, while allergic contact dermatitis is the result of a systemic immune response that leads to skin changes. In allergic reactions, after the first exposure of the organism to the given chemical substance, there follows a period during which hypersensitivity to the chemical agent develops. Repeated exposure of the organism to the relevant substance leads to clinical symptoms of allergy (usually 12 to 48 hours after repeated exposure).

Skin sensitization studies are conducted on experimental animals (guinea pigs). The test substance is applied for 2 to 4 weeks on the skin or intradermally. Low, non-irritating doses are then administered for 48 hours, and the treated skin surface is observed for possible allergic manifestations (edema, redness). The changes are compared with those obtained after the initial 2 to 4-week treatment (direct toxic effect).

**6. PHOTOTOXICITY AND PHOTOSENSITIZATION**

Xenobiotics providing high concentrations in the skin can undergo bioactivation under the action of UVB rays and cause phototoxicity and/or photosensitization (photoallergy). These reactions are relatively rare and their testing is analogous to skin sensitization testing.

**7. REPRODUCTIVE TOXICITY AND TERATOGENICITY**

The first reproductive toxicity test of the test substance should be conducted after the 90-day three-dose sub-chronic toxicity test. Dose selection is related to data of sub-acute and sub-chronic toxicity tests. Doses tested for reproductive toxicity should not evoke general systemic toxicity in the parent organism.

Several xenobiotics can damage the developing organism and lead to severe and irreversible structural malformations. Embryotoxicity and teratogenicity studies aim to establish possible adverse effects of the test substance during the period of organogenesis. These studies are usually performed with two doses and in two animal species.

***In vitro* reproductive toxicity tests.** Several alternative methods to animal experiments have been developed, but due to the complexity of the development process, none of them can replace *in vivo* studies. *In vitro* tests are primarily used for screening to prevent experiments with substances with marked reproductive toxicity, embryotoxicity, and teratogenicity. Preliminary research can be done on cell cultures, organ cultures, embryos, etc.

**8. DETERMINATION OF PERI- AND POSTNATAL TOXICITY**

This type of research follows the effects of the test substance on the survival and development of the offspring in the period after birth. These are usually performed on rats, but the specific characteristics of this animal species make it difficult to extrapolate the obtained results to humans.

**9. CARCINOGENICITY AND GENOTOXICITY**

Despite the existence of numerous short-term *in vivo* and *in vitro* tests, long-term follow-up (lifetime, 24-month treatment in rats) remains the most reliable method for determining the carcinogenic effects of a given substance. Many factors can influence the analysis and interpretation of results obtained in experimental animals. When determining the absolute carcinogenic risk for a given xenobiotic, it should be taken into account that in some cases the development of a neoplastic process is not related to the application of the substance under investigation. The method of lifelong treatment of rats is reliable since practically any substance with carcinogenic properties for humans causes tumor formation in rodents as well. The disadvantages of the method are its long duration and high cost.

There are *in vivo* carcinogenicity tests of limited duration. They are based on treating experimental animals with the test substance for a shorter period (e.g., 52 weeks or less) than lifelong treatment. However, this type of research is not accepted by regulatory agencies as an alternative to conventional research.

In recent years, numerous tests of limited duration have been developed to predict the carcinogenic properties of chemical substances. Most of them are based on the possible genetic material damage (genotoxicity) by the xenobiotic or its metabolites.

***10. IN VIVO* AND *IN VITRO* GENOTOXICITY TESTS**

Mutagenesis is the inducing of a change in the genetic material of cells or an organism, which is subsequently passed on to offspring. Changes can affect a single gene, a set of genes, or entire chromosomes.

Genotoxicity is a broader term that concerns potentially damaging effects on genetic material, but these are not always related to mutagenesis.

There is evidence that many carcinogens are also mutagens, so substances with potential carcinogenic effects are first investigated for mutagenic effects or damaging effects on chromosomes and then tested for carcinogenicity in rodents.

Short-term tests fall into two groups: 1) tests that identify gene mutations and 2) tests that identify changes in the structure and/or number of chromosomes.

One of the most commonly used tests for mutagenicity is the Ames test. The test uses a mutant strain of *Salmonella* that cannot synthesize the amino acid histidine, due to one or more mutations in the group of genes encoding the synthesis of this amino acid. Bacteria carrying the mutation cannot grow and form colonies in a medium that does not contain histidine. These bacteria can revert to the original type (capable of synthesizing histidine) spontaneously (a rare phenomenon) or upon exposure to a genotoxic substance. An advantage of the test is that it not only allows the identification of potentially genotoxic substances but also provides information on the type of DNA damage. The use of certain strains allows the identification of mutagens with alkylating properties, and the use of others - the detection of mutations caused by reactive oxygen species and oxidizing agents. Some strains allow the identification of mutagens leading to frameshift mutations (e.g., polycyclic aromatic hydrocarbons). The advantages of the method are 1) high reliability when testing known carcinogens and non-carcinogens, 2) high sensitivity, 3) the possibility to determine the mechanism of occurrence of mutations, and 4) the possibility to study complex mixtures of substances. The method is fast and relatively cheap. Disadvantages of the method are: 1) some carcinogens have a

specific mechanism of action and cannot be identified with this test (asbestos, metals), 2) possibility of qualitative determination of the genotoxic potential, but not the dose at which this effect occurs 3) its implementation requires sterile conditions and 4) it is not suitable for substances of bacterial origin.

There are also tests for mutagenicity of chemical substances using eukaryotic organisms. These are studies on *Drosophila melanogaster*, cultured mammalian cells, and *in vivo* tests on animals. *In vivo* gene mutation detection tests are not included in routine toxicology studies.

**Studies for chromosomal aberrations**

In addition to gene mutations, structural chromosomal aberrations or a change in the number of chromosomes are characteristic of most of the tumor cells studied. Chromosomal aberrations (deletions and translocations) can lead to the activation of proto-oncogenes or inactivation of tumor suppressor genes. There are *in vitro* and *in vivo* tests for the detection of chromosomal aberrations.

*In vitro* cytogenetic analysis is based on the treatment of proliferating cells with the test substance and the detection of chromosomal aberrations after one or more cell cycles.

*In vivo* analysis is most commonly performed in rodents by the micronucleus test. This test involves treating young animals with the test chemical or potential drug substance for 6 to 8 weeks. Substances inducing chromosomal aberrations or breaks in immature erythroblasts in the bone marrow lead to the formation of micronuclei during cell division. The reason for their formation is the inability of the resulting fragments of chromosomes to participate in the separation of chromosomes from the spindle. During the maturation process of erythroblasts, degradation of the cell nucleus occurs, but micronuclei are preserved and can be visualized by appropriate staining.

**Interpretation of data obtained in short-term tests**

A substance is classified as a potential carcinogen if a positive result is reported in one *in vitro* mutagenicity test and one *in vivo* test.

## 11. IMMUNOTOXICITY

Immunotoxicity is the property of a xenobiotic to affect the immune system, resulting in an altered immune response in affected individuals. An immunotoxic response can occur in response to a chemical influence, which can lead to immunosuppression (reduced resistance of the body to infectious agents and neoplasms) or impaired regulation of the functions of the immune system (development of an autoimmune reaction). The parameters characterizing the immune functions are investigated during the sub-acute and sub-chronic toxicity tests (28 and 90-day studies).

Additional studies of effects on the functions of immunocompetent cells are performed for 1) substances that have shown immunotoxicity during sub-acute and sub-chronic toxicity tests and 2) chemicals for which such effects are suspected based on data from previous experiments or relationship structure-activity.

## 12. NEUROTOXICITY

Neurotoxicity is the ability of a chemical substance to cause unwanted effects on the central and peripheral nervous system or sensory organs. Tests are performed during acute toxicity and long-term toxicity studies. The behavior and motor activity of experimental animals, reflexes, and physiological and neuromuscular parameters are monitored.

## 13. EFFECTS ON THE ENDOCRINE SYSTEM

Substances that disrupt the normal functioning of the immune system act by several mechanisms:

- imitation of the action of hormones normally synthesized in the body (estrogens, testosterone) and induction of similar chemical reactions in it;
- blocking hormone receptors in cells and antagonizing the action of natural hormones;
- influencing the biosynthesis, transport, metabolism, and excretion of hormones and changes in their concentrations.

Various tests have been proposed for the detection of these effects in organisms, but there is still no test that allows the detection of all possible effects of the test substance on endocrine functions. Many toxic effects related to the endocrine system are detected during other toxicity tests.

## 14. CONCLUSION

The safety of potential new drugs must be adequately assessed in preclinical studies with different methods for determining acute, sub-acute, sub-chronic, and chronic toxicity. If the test compound has shown acceptable efficacy and safety during preclinical testing, it moves on to testing in human clinical trials.

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