B.39. UNSCHEDULED DNA SYNTHESIS (UDS) TEST

WITH MAMMALIAN LIVER CELLS *IN VIVO*

1. METHOD

This method is a replicate of the OECD TG 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo* (1997).

1.1 INTRODUCTION

The purpose of the unscheduled DNA Synthesis (UDS) test with mammalian liver cells i*n vivo* is to identify test substances that induce DNA repair in liver cells of treated animals (see 1,2,3,4).

This *in vivo* test provides a method for investigating genotoxic effects of chemicals in the liver. The end-point measured is indicative of DNA damage and subsequent repair in liver cells. The liver is usually the major site of metabolism of absorbed compounds. It is thus an appropriate site to measure DNA damage *in vivo*.

If there is evidence that the test substance will not reach the target tissue, it is not appropriate to use this test.

The end-point of unscheduled DNA synthesis (UDS) is measured by determining the uptake of labelled nucleosides in cells that are not undergoing scheduled (S-phase) DNA synthesis. The most widely used technique is the determination of the uptake of tritium-labelled thymidine (^{3}H-TdR) by autoradiography. Rat livers are preferably used for *in vivo* UDS tests. Tissues other than the livers may be used, but are not the subject of this method.

The detection of a UDS response is dependent on the number of DNA bases excised and replaced at the site of the damage. Therefore, the UDS test is particularly valuable to detect substance-induced “longpatch repair” (20-30 bases). In contrast, “shortpatch repair” (1-3 bases) is detected with much lower sensitivity. Furthermore, mutagenic events may result because of non-repair, misrepair or misreplication of DNA lesions. The extent of the UDS response gives no indication of the fidelity of the repair process. In addition, it is possible that a mutagen reacts with DNA but the DNA damage is not repaired via an excision repair process. The lack of specific information on mutagenic activity provided by the UDS test is compensated for by the potential sensitivity of this endpoint because it is measured in the whole genome.

See also General Introduction Part B.

1.2 DEFINITIONS

Cells in repair: a net nuclear grain (NNG) higher than a preset value, to be justified at the laboratory conducting the test.

Net nuclear grains (NNG): quantitative measure for UDS activity of cells in autoradiographic UDS tests, calculated by subtracting the average number of cytoplasmic grains in nucleus-equivalent cytoplasmic areas (CG) from the number of nuclear grains (NG): NNG = NG - CG. NNG counts are calculated for individual cells and then pooled for cells in a culture, in parallel cultures, etc.

Unscheduled DNA Synthesis (UDS): DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents.

1.3 PRINCIPLE OF THE TEST METHOD

The UDS test with mammalian liver cells *in vivo* indicates DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents. The test is usually based on the incorporation of ^{3}H-TdR into the DNA of liver cells which have a low frequency of cells in the S-phase of the cell cycle. The uptake of ^{3}H-TdR is usually determined by autoradiography, since this technique is not as susceptible to interference from S-phrase cells as, for example, liquid scintillation counting.

1.4 DESCRIPTION OF THE METHOD

1.4.1 **Preparations**

1.4.1.1 *Selection of animal species*

Rats are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study the weight variation of animals should be minimal and not exceed ± 20% of the mean weight for each sex.

1.4.1.2 *Housing and feeding conditions*

General conditions referred in the General Introduction to Part B are applied although the aim for humidity should be 50-60%.

1.4.1.3 *Preparation of the animals*

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

1.4.1.4 *Test substance/Preparation*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2 **Test conditions**

1.4.2.1 *Solvent/vehicle*

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2 *Controls*

Concurrent positive and negative controls (solvent/vehicle) should be included in each independently performed part of the experiment. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the animals in the treated groups.

Positive controls should be substances known to produce UDS when administered at exposure levels expected to give a detectable increase over background. Positive controls needing metabolic activation should be used at doses eliciting a moderate response (4). The doses may be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include:

Sampling Times	Substance	CAS No.	EINECS No.
Early sampling times (2-4 hours)	N-Nitrosodimethylamine	62-75-9	200-249-8
Late sampling times (12-16	N-2-Fluorenylacetamide (2-AAF)	53-96-3	200-188-6

Other appropriate positive control substances may be used. It is acceptable that the positive control should be administered by a route different from the test substance.

1.5 PROCEDURE

1.5.1 **Number and sex of animals**

An adequate number of animals should be used, to take account of natural biological variation in test response. The number of animals should be at least 3 analysable animals per group. Where a significant historical database has been accumulated, only 1 or 2 animals are required for the concurrent negative and positive control groups.

If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex, preferably males, will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2 **Treatment schedule**

Test substances are generally administered as a single treatment.

1.5.3 **Dose levels**

Normally, at least two dose levels are used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. In general, the lower dose should be 50% to 25% of the high dose.

Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study.

The highest dose may also be defined as a dose that produces some indication of toxicity in the liver (e.g. pyknotic nuclei).

1.5.4 **Limit test**

If a test at one dose level of at least 2000 mg/kg body weight, applied in a single treatment, or in two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected, based upon data from structurally related substances, then a full study may not be necessary. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5 **Administration of doses**

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure may be acceptable where they can be justified. However, the intraperitoneal route is not recommended as it could expose the liver directly to the test substance rather than via the circulatory system. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

1.5.6 **Preparation of liver cells**

Liver cell are prepared from treated animals normally 12-16 hours after dosing. An additional earlier sampling time (normally 2-4 hours post-treatment) is generally necessary unless there is a clear positive response at 12-16 hours. However, alternative sampling times may be used when justified on the basis of toxicokinetic data.

Short-term cultures of mammalian liver cells are usually established by perfusing the liver *in situ* with collagenase and allowing freshly dissociated liver cells to attach themselves to a suitable surface. Liver cells from negative control animals should have a viability (5) of at least 50 percent.

1.5.7 **Determination of UDS**

Freshly isolated mammalian liver cells are incubated usually with medium containing ^{3}H-TdR for an appropriate length of time, e.g. 3-8 hours. At the end of the incubation period, medium should be removed from the cells, which may then be incubated with medium containing excess unlabelled thymidine to diminish unincorporated radioactivity (“cold chase”). The cells are then rinsed, fixed and dried. For more prolonged incubation times, cold chase may not be necessary. Slides are dipped in autoradiographic emulsion, exposed in the dark (e.g. refrigerated for 7-14 days), developed, stained, and exposed silver grains are counted. Two to three slides are prepared from each animal.

1.5.8 **Analysis**

The slide preparations should contain sufficient cells of normal morphology to permit a meaningful assessment of UDS. Preparations are examined microscopically for signs of overt cytotoxicity (e.g. pyknosis, reduced levels of radiolabelling).

Slides should be coded before grain counting. Normally 100 cells are scored from each animal from at least two slides; the scoring of less than 100 cells/animal should be justified. Grain counts are not scored for S-phase nuclei, but the proportion of S-phase cells may be recorded.

The amount of ^{3}H-TdR incorporation in the nuclei and the cytoplasm of morphologically normal cells, as evidenced by the deposition of silver grains, should be determined by suitable methods.

Grain counts are determined over the nuclei (nuclear grains, NG) and nucleus equivalent areas over the cytoplasm (cytoplasmic grains, CG). CG counts are measured by either taking the most heavily labelled area of cytoplasm, or by taking an average of two to three random cytoplasmic grain counts adjacent to the nucleus. Other counting methods (e.g. whole cell counting) may be used if they can be justified (6).

2. **DATA**

2.1 TREATMENT OF RESULTS

Individual slide and animal data should be provided. Additionally, all data should be summarised in tabular form. Net nuclear grain (NNG) counts should be calculated for each cell, for each animal and for each dose and time by subtracting CG counts from NG counts. If “cells in repair” are counted, the criteria for defining “cells in repair” should be justified and based on historical or concurrent negative control data. Numerical results may be evaluated by statistical methods. If used, statistical tests should be selected and justified prior to conducting the study.

2.2 EVALUATION AND INTERPRETATION OF RESULTS

Examples of criteria for positive/negative responses include:

positive	(i)	NNG values above a pre-set threshold which is justified on the basis of laboratory historical data;
or	(ii)	NNG values significantly greater than concurrent control.
negative	(i)	NNG values within/below historical control threshold;
or	(ii)	NNG values not significantly greater than concurrent control.

The biological relevance of data should be considered: i.e. parameters such as inter-animal variation, dose-response relationship and cytotoxicity should be taken into account. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

A positive result from the UDS test with mammalian liver cells *in vivo* indicate that a test substance induces DNA damage in mammalian liver cells *in vivo* that can be repaired by unscheduled DNA synthesis *in vitro*. A negative result indicates that, under the test conditions, the test substance does not induce DNA damage that is detectable by this test.

The likelihood that the test substance reaches the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. REPORTING

TEST REPORT

The test report must include the following information :

Solvent/Vehicle

— justification for choice of vehicle;

— solubility and stability of the test substance in solvent/vehicle, if known.

Test animals:

— species/strain used;

— number, age and sex of animals;

— source, housing conditions, diet, etc.;

— individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group;

Test conditions :

— positive and negative vehicle/solvent controls;

— data from range-finding study, if conducted;

— rationale for dose level selection;

— details of test substance preparation;

— details of the administration of the test substance;

— rationale for route of administration;

— methods for verifying that test agent reached the general circulation or target tissue, if applicable;

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;

— details of food and water quality;

— detailed description of treatment and sampling schedules;

— methods for measurement of toxicity;

— method of liver cell preparation and culture;

— autoradiographic technique used;

— number of slides prepared and numbers of cells scored;

— evaluation criteria;

— criteria for considering studies as positive, negative or equivocal;

Results:

— individual slide, animal and group mean values for nuclear grains, cytoplasmic grains, and net nuclear grains;

— dose-response relationship, if available;

— statistical evaluation if any;

— signs of toxicity;

— concurrent negative (solvent/vehicle) and positive control data;

— historical negative (solvent/vehicle) and positive control data with range, means and standard deviations;

— number of "cells in repair" if determined;

— number of S-phase cells if determined;

— viability of the cells.

Discussion of results.

Conclusions.

4. REFERENCES

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