Hydrolysed proteins

DOCUMENT M-CA, Section 5

TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Version history[1]

A) BIO

Date	Data points containing amendments or additions and brief description	Document identifier and version number
2005-26-06	Initial Document M version, submitted for application of approval of the active substance.	M-Hydr.Protein-AnnexII
2018-01-09	Two acute toxicity studies on *Daphnia sp* and on *Brachydanio rerio* (fish) are presented. Both studies were performed in a GLP complying laboratory and tested the active substance called "BIOCEBO" (35 % w/w hydrolysed proteins). A bibliographical analysis of the effects of hydrolysed proteins on aquatic organisms. These new data were presented to comply with Regulation 571/2012, amending Regulation (EU) No 540/2011, which establishes that for the hydrolysed proteins some additional information is required regarding the risk for aquatic organisms.	DOCUMENT M-CA, Section 5 CA Section 5.2 Acute Toxicology (Former Section 3A of the Document M, Annex II).

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

B) PHY

Date	Data points containing amendments or additions and brief description	Document identifier and version number

C) SIC

Date	Data points containing amendments or additions and brief description	Document identifier and version number

Table of Contents

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE (BIO)

Introduction

CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals

Hydrolysed proteins are natural compounds of degradation from the hydrolysis of living organisms tissues, that can have vegetable or animal origin. Proteins are the most abundant organic molecules in cells. They constitute the 50% of the dry weight of cells, or even more. They can be found in every single cell, since they are fundamental in all aspects of the cell structure and function (Lehninger, 1983).
The Animal and Vegetable cells are formed mainly by proteins, which constitute more than the half of the dry weight of the cell. Proteins determine the shape and structure of the cell and also function as an instrument of molecular recognition and of catalysis (Alberts, 1986).

Proteins have many different biological functions. The widest group of proteins are the enzymes whose function is about catalysing the biochemical processes that take place in the living organisms. Moreover, there are proteins of reservation of amino acids such as plant nutrients; transport proteins of specific molecules; proteins that work as essential elements of the motile and contractile systems; protective proteins that are present in the blood of the vertebrates such as antibodies; proteins that function as hormones and, finally, structural proteins (Lehninger, 1983).

The proteins that are found in food and eaten by human beings and mammals are normally degraded metabolically by means of enzymatic processes to give rise to more simple metabolites (peptides and amino acids) that are used by the living cells for the biosynthesis of new specific proteins. Therefore, they do not cause any danger to human beings and mammals in general. As it has been explained before, proteins appear in all biochemical processes that take place in every cell being, this way, essential compounds for human life.

Furthermore, hydrolysed proteins are authorized by the EU in order to be used as attractant in the elaboration of baits in combination with appropriate insecticides of the Organic Farming (Regulation **EC 889/2008 annex 2**). This shows the innocuousness of these compounds, since the practice of this kind of agriculture is very demanding with the use of products that can be harmful to human beings.

The active ingredient Hydrolysed proteins means polypeptides, peptides and amino acids and mixtures thereof obtained by hydrolysis of animal by-products. The main health hazard of concern with hydrolysed proteins derived from animal by-products is the risk of BSE/TSE (Bovine spongiform encephalopathy/transmissible spongiform encephalopathy) contamination or of microbial human pathogens contamination. In order to exclude the risk of contamination of the raw animal material, the manufacturing process and the plant facilities are in accordance to the

requirements of the Regulation 1069/2009, laying down health rules concerning animal by-products not intended for human consumption.

The Draft Assessment Report of Greece stated in 2008 that RMS accepted the argumentation that the active substance hydrolysed proteins derived from hydrolysis of animal tissues do not have any significant toxicity potential.

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

Not applicable.

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

Not applicable.

CA 5.2 Acute Toxicity

Regulation 571/2012, amending Regulation (EU) No 540/2011 as regards to the conditions of approval of the active substances aluminum silicate, hydrolysed proteins and 1,4-diaminobutane (putrescine), establishes that for the hydrolyzed proteins some additional information is required regarding the risk for aquatic organisms.

Moreover, in this mentioned Regulation, it is also stated that applicants shall submit the information requested to the European Commission through the Rapporteur European Member State (Greece in this case), by 1st of November 2013. BIOIBERICA S.A., as one of the applicants for the inclusion of Hydrolyzed proteins in Annex I of the derogated Directive 91/414, concerning the placing of plant protection products on the market, has accordingly prepared two acute toxicity studies on *Daphnia sp* and on *Brachydanio rerio* (fish). Both studies were performed in a GLP complying laboratory (Eurofins Biolab S.r.l., located in Italy) and tested the active substance called "BIOCEBO" (35 % w/w hydrolyzed proteins). Results showed that Hydrolysed proteins do not suppose any risk for aquatic organisms such as *Daphnia sp* or *Brachydanio rerio* in acute toxicity tests such as acute Immobilisation Test and Limit Test (equilibrium loss, irregular swimming, difficulties in respiratory functions and variation of pigmentation), respectively.

Moreover, BIOIBERICA, S.A. together with the other two applicants, requested to IRTA (Institute for Food and Agricultural Research and Technology) a bibliographical analysis of the effects of hydrolysed proteins on aquatic organisms. The results of this analysis concluded that there is no evidence for any effects of hydrolysed proteins on aquatic ecosystems in general and on aquatic organisms in particular.

The next pages present the results of these three studies. The complete reports are located in the J-Hydr.protein directory.

STUDY NUMBER 1. Acute toxicity on aquatic organisms (Daphnia sp): EC50 TEST ON "BIOCEBO"

Introduction:

The aim of this study was to determine the ecotoxicological effects "BIOCEBO" (hydrolyzed proteins ≥35 % w/w), on biotic systems putting as a model the aquatic organism *Daphnia magna* as a test system to perform the EC_{50} test.

This acute immobilization test was evaluated according to the OECD guideline N.202.

Report:	S-2013-01827 AM
Title:	Acute toxicity on aquatic organisms (Daphnia sp): EC50 TEST ON "BIOCEBO"
Test facilities:	Eurofins Biolab S.r.l. of Vimodrome (MI)-via B. Buozzi n.2 (Italy)
Guidelines:	OECD Guidelines for the testing of Chemicals/Section 2: Effects on Biotic Systems Test Nº. 202: Daphnia sp. Acute Immobilisation Test 2004
GLP	Yes

Material and Methods:

The organisms were exposed to 5 different solutions of BIOCEBO for a total period of 48 hours, the number of immobilized organisms and/or possible abnormal behaviors were observed.

120 Daphnie were used, 100 of them treated with different concentrations and 20 as a control (no Biocebo addition). 4 replications for every condition were prepared, afterwards Daphnie was added to the vessels of the assay sample.

Dissolved oxygen, pH and temperature of the assay were measure at the beginning and at the end of the test.

Validity criteria:

- The immobilization of control animals must not be higher than 10% at the end of the test.
- pH values must not change for more than 1.5 units
- The concentration of dissolved oxygen in the vessels must not go below 2 mg/l.

Results:

Table 1: Number of Daphnia immobilized at 24 and 48 hours in treated and control conditions

Group	Replication Nº	Concentration of BIOCEBO administered	Nº Daphnia individuals immobilized at 24h	Nº Daphnia individuals immobilized at 48h
Treated	1	1.00 g/l	1/5	2/5
	2		0/5	1/5
	3		1/5	1/5
	4		0/5	0/5
Treated	1	0.50 g/l	0/5	0/5
	2		0/5	1/5
	3		0/5	1/5
	4		0/5	1/5
Treated	1	0.25 mg/l	0/5	0/5
	2		0/5	0/5
	3		0/5	0/5
	4		0/5	0/5
Treated	1	0.125 mg/l	0/5	0/5
	2		0/5	0/5
	3		0/5	0/5
	4		0/5	0/5
Treated	1	0.063 mg/l	0/5	0/5
	2		0/5	0/5
	3		0/5	0/5
	4		0/5	0/5
Control	1	0.00 mg/l	0/5	0/5
	2		0/5	0/5
	3		0/5	0/5
	4		0/5	0/5

n=5 for each condition

Table 2: Concentration of dissolved oxygen (mg/l) in treated (1 g/l) and control at the beginning and at the end of the test (48 hours).

	pH		**Dissolved oxygen**	
	Beginning of the test	End of test	Beginning of the test	End of test
Control				
Replication nº 1	8.18	8.72	6.61	5.65
Replication nº 2	8.20	8.74	6.64	5.69
Replication nº 3	8.15	8.70	6.58	5.61
Replication nº 4	8.17	8.71	6.67	5.67
Concentration 1.00 g/l				
Replication nº 1	7.40	8.14	7.21	3.75
Replication nº 2	7.43	8.12	7.17	3.78
Replication nº 3	7.39	8.16	7.22	3.73
Replication nº 4	7.41	8.15	7.25	3.77

Findings:

- pH values were not significantly different between the two treatment conditions.
- Dissolved oxygen was lower at the end of the test with the highest concentration of BIOCEBO but validity criteria was satisfied.
- Temperature did not change during the test.
- The obtained results showed that the *Daphnia magna* EC_{50} after 48 hours at the concentration of 100 mg/l of the test item “BIOCEBO” is higher than 1.00 g/l.
- All the test parameters satisfied the validity criteria.

Conclusion/endpoint:

Results showed that BIOCEBO supposes no risk for aquatic organisms such as *Daphnia*.

STUDY NUMBER 2. *Brachydanio rerio*, acute toxicity test-limit test: on "BIOCEBO".

Introduction:

The aim of this study was to determine the ecotoxicological effects "BIOCEBO" (hydrolyzed proteins ≥35 % w/w), on biotic systems putting as a model the aquatic organism *Brachydanio rerio* as a test system to perform a limit test.

This acute immobilization test was evaluated according to the OECD guideline N.203.

Report:	S-2013-01828 AM
Title:	Brachydanio rerio, ACUTE TOXICITY TEST-LIMIT TEST: ON "BIOCEBO".
Test facilities:	Eurofins Biolab S.r.l. of Vimodrome (MI)-via B. Buozzi n.2 (Italy)
Guidelines:	OECD Guidelines for the testing of Chemicals/Section 2: Effects on Biotic Systems Test Nº. 203: Fish, Acute Toxicity Test 1992
GLP	Yes

Material and Methods:

The organisms were exposed to 100 mg/l of BIOCEBO or control solutions for a total period of 96 hours. All visible abnormalities such as equilibrium loss, irregular swimming, difficulties in respiratory functions and variation of pigmentation were measured.

14 fishes were used, 7 of them treated with the BIOCEBO with a concentration of 100 mg/l and other 7 were used as control, in the same assay conditions than without adding BIOCEBO.

At the different observations intervals pH, dissolved oxygen and assay water temperature were measured.

Validity criteria:

- The mortality in the control animals should not exceed one fish at the end of the test.
- The dissolved oxygen concentration must have been at least 60 percent of the air saturation value throughout the test.

Results:

Table 1: Number of dead fishes in control and treated during the assay

Group	Nº of animals	Concentration of BIOCEBO administered	24 hours	48 hours	72 hours	96 hours
Treated	7	100 mg/l	0	0	0	0
Control	7	0.00 mg/l	0	0	0	0

Table 2: Concentration of dissolved oxygen (mg/l) in treated (1 g/l) and control at the beginning and at the end of the test (48 hours).

Group	0 hours	24 hours	48 hours	72 hours	96 hours
pH values					
Control	7.90	8.20	8.30	8.18	8.11
Treated (100 mg/L)	7.75	8.08	8.14	8.10	8.10
% of saturation of dissolved oxygen					
Control	70.0	67.4	67.1	66.7	66.5
Treated (100 mg/L)	71.8	65.7	64.5	63.7	62.8

Findings:

- pH values remained within the required limits (6,0-8,5).
- The percentage of saturation has remained for the whole length of the assay above 60% both in control and in treated conditions.
- Temperature remained in the interval required for the species.
- The obtained results showed that no case of mortality was declared in treated animals and in control ones. No toxic symptom was detected neither.
- All the test parameters satisfied the validity criteria.

Conclusion/endpoint:

The obtained results, in compliance with assay validity criteria, showed that no dead fishes at 100 mg/l of BIOCEBO after 96 hours were observed.
Results showed that BIOCEBO supposes no risk for aquatic organisms such as Brachydanio.

STUDY NUMBER 3. Bibliographical analysis of the effects of hydrolysed proteins on aquatic organisms

Introduction:

The objective and scope of this study was to make a complete and systematic technical and scientific bibliographical review of the effects that the use of protein hydrolysate baits may have on the aquatic organisms.

Report:	-
Title:	Bibliographical analysis of the effects of hydrolysed proteins on aquatic organisms.
Test facilities:	IRTA: Institute for Food and Agricultural Research and Technology
Guidelines:	Not applicable
GLP	Not applicable

Material and Methods:

To do so, the following database and open access search engine and repositories have been consulted: Web of Knowledge, Google Scholar, AGRIS, Aquatic Commons, OceanDocs, OAister WorldCat and OpenDOAR.

The search strategy consisted on different key words used such as “hydrolysed protein” and “aquatic organisms” and the Boolean operators applied to combine them.

Results:

- Through the different searches, several records were found but none of them was relevant to the subject.

Conclusion/endpoint:

The obtained results of the systematical bibliographical search showed no effects of any kind of the use of hydrolysed protein baits on aquatic organisms.

Therefore, there is no evidence for any adverse effects of hydrolysed proteins on aquatic ecosystems in general and on aquatic organisms in particular.

CA 5.2.1 Oral

CA 5.2.2 Dermal

CA 5.2.3 Inhalation

CA 5.2.4 Skin irritation

CA 5.2.5 Eye irritation

CA 5.2.6 Skin sensitisation

CA 5.2.7 Phototoxicity

CA 5.3 Short-Term Toxicity

CA 5.3.1 Oral 28-day study

CA 5.3.2 Oral 90-day study

CA 5.3.3 Other routes

CA 5.4 Genotoxicity Testing

CA 5.4.1 *In vitro* studies

CA 5.4.2 *In vivo* studies in somatic cells

CA 5.4.3 *In vivo* studies in germ cells

CA 5.5 Long-Term Toxicity and Carcinogenicity

CA 5.6 Reproductive Toxicity

CA 5.6.1 Generational studies

CA 5.6.2 Developmental toxicity studies

CA 5.7 Neurotoxicity Studies

CA 5.7.1 Neurotoxicity studies in rodents

CA 5.7.2 Delayed polyneuropathy studies

CA 5.8 Other Toxicological Studies

CA 5.8.1 Toxicity studies of metabolites

CA 5.8.2 Supplementary studies on the active substance

CA 5.8.3 Endocrine disrupting properties

CA 5.9 Medical Data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

CA 5.9.2 Data collected on humans

CA 5.9.3 Direct observations

CA 5.9.4 Epidemiological studies

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

In case of skin contact, wash with water. In case of irritation, seek medical advice.
In case of eye contact, rinse immediately with plenty of potable water/sterile eye wash solution
In case of ingestion, drink plenty of water

CA 5.9.7 Expected effects of poisoning

In case of poisoning call a doctor for the usual first aid measures, showing him the label of the product (insecticide) which has been mixed with the respective hydrolysed proteins.

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE (PHY)

Introduction

PHYTOPHYL manufactures “Hydrolysed Protein” which is made of Beet molasses and Urea. Both of them are used very widely for many years and have not ever classified as dangerous substances.

Beet molasses are a natural by-product of the sugar industry, defined as the end product of sugar manufacture or refining from which no more sugar may be economically crystallized by conventional means.

Beet molasses mainly used for two purposes, Animal feed additive and Alcohol Production.

There is no evidence in bibliography that Beet molasses are for some reason toxic, irritant or ecologically unsafe.

Urea and beet molasses are substances widely used as feed additives for decades without problems and are permitted in EC, US and many other countries.

PHYTOPHYL & FORESTRY COMISSION notified urea according to 91/414 and the substance is now approved under Reg. (EC) No 1107/2009. No toxicity studies were submitted but literature data about the toxicity of urea indicated limited toxicological potential.

During this first notification and inclusion Urea was not registered to ECHA but now has a full registration, the dossier is evaluated and there are 163 active registrants as a high volume chemical (production of 10.000 000 – 100.000.000 TONNES per year).

PHYTOPHYL & FORESTRY COMISSION submitted also confirmatory data about the risk for operators, workers and bystanders concerning the application of urea to low volume bait sprays in olive trees in mixture with insecticides to control Olive Fruit Fly *Bactrocera oleae.*

In this report performed a preliminary and indicative exposure assessment for urea based on all available data for urea in literature and ECHA registration database. All application scenarios and application rates as detailed in GAP have concluded in acceptable exposure when appropriate Personal Protective Equipment is assigned as required.

There are not any new studies submitted by this dossier only an open literature review on MC-A Section 9 which will give more data about scientific knowledge during the last decade for hydrolysed proteins concerning, toxicity studies, relevant data and the potential risk for man and the environment.

More detailed literature information on urea had given in the first assessment report of the substance and will be found also in the Urea renewal dossier which will be submitted at the same time with this dossier by PHYTOPHYL and FORESTRY COMISSION.

In next pages of this section giving data about urea and we are presenting all the end point summaries found on Urea registration dossier from ECHA site to support the limited toxicological potential of Beet molasses - Urea hydrolysates.

CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals

Urea is widely distributed within the natural world, as a by-product in protein synthesis in ureotelic animals, including mammals whence it is excreted in urine (humans 25g - 30g/day).

All humans are exposed to this material from birth, and its toxicity has been studied over many years. A lot of literature studies exist about the metabolism of urea in mammals.

From ECHA site for urea:

Toxicokinetics

A waiver is proposed. Urea is produced in large quantities by the human body as a product of normal metabolism and is excreted unchanged in the urine. Further studies characterising the toxicokinetics of urea are not required.

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

From ECHA site for urea:

Dermal absorption values of 7.2-9.5% is reported for urea.

Urea is present at appreciable levels in the human epidermis, where it may play a role as a humectant, maintaining hydration of the stratum corneum. At very high levels of exposure, urea may act as a denaturant and may enhance the dermal absorption of other compounds. Bronaugh et al (1982), report a dermal absorption value of 7.2%, based on the results of a study in the rat in vivo and comparable results in vitro.

The Cosmetic Ingredient Review (CIR) Expert Panel (2005) reviewed the available data on the dermal absorption of urea in vitro and vivo in various test systems and conclude that a value of 9.5% derived from a study in intact human skin in vitro is appropriate. The review also notes that the absorption of urea through abraded skin is considerably more extensive and that urea is known to enhance the dermal penetration of other substances.

CA 5.2 Acute Toxicity

No extra data for the acute toxicity of hydrolysed protein presenting. Because of very low toxicity of beet molasses only for a.s. urea presenting below the ECHA endpoint summary of each section.

CA 5.2.1 Oral

ECHA endpoint summary for urea:

Urea is of very low acute oral toxicity in the rat and mouse. Sato et al (1977) report LD50 values of 14.3 (12.9 -15.9) and 15.0 (13.4 -16.8) g/kg bw in male and female rats; LD50 values of 11.5 (10.6 -12.5) and 13.0 (11.0 -15.4) g/kg bw in the mouse. Urea is of generally low acute oral toxicity

in most species but higher toxicity is noted in ruminants due to the generation of ammonia by gastric flora. Stiles et al (1970) report an LDlo of approximately 600 mg/kg bw in cattle.

CA 5.2.2 Dermal

ECHA endpoint summary for urea:

No data are available: a waiver is proposed for this endpoint. Urea is demonstrated to be of very low acute toxicity by the oral, subcutaneous and intravenous routes in the rat and mouse. Testing for acute dermal toxicity is not justified on scientific grounds and for reasons of animal welfare. Specifically, the very low toxicity of urea by the subcutaneous and intravenous routes indicates that dermal toxicity would also be very low, even assuming rapid and total dermal penetration.

CA 5.2.3 Inhalation

ECHA endpoint summary for urea:

No data are available: a waiver is proposed for this endpoint. The substance is a non-volatile solid and is produced as crystals with a particle size of >100 µm. There is therefore no potential for inhalation exposure. In addition, the substance has been demonstrated to be of very low toxicity by other routes of exposure. Testing for acute inhalation toxicity is therefore not justified on scientific grounds or based on exposure considerations.

CA 5.2.4 Skin irritation

ECHA endpoint summary for urea:

Frosch & Kligman (1977) (cited in WHO/JECFA evaluation) exposed human volunteers to three daily applications of urea (dissolved in water) at concentrations of between 7.5 -30%; applications were made to intact and scarified skin. On abraded skin, slight irritation was seen with 7.5% urea; marked irritation was seen with 30% urea. No effects were seen on intact skin. In a study by Lashmar et al (1989) application of 10% urea for 24 hours induced no discernible change in the histological appearance of the skin. No evidence of skin irritation was seen in a modern guideline study (Hooiveld, 2003).

It is notable that skin creams, commonly containing urea at concentrations of between 5 -10% but also at concentrations of up to 25% and higher are widely used for the treatment of dry/irritant skin conditions, therefore it can be predicted that urea is not a skin irritant. Urea is also naturally present in the stratum corneum at a level of approximately 1%. It is also notable that no signs of local irritation were noted in 28 -day and 25 -week repeated dose dermal toxicity studies in the rat (Sato et al, 1977)

CA 5.2.5 Eye irritation

ECHA endpoint summary for urea:

Urea was found to be a mild eye irritant in a guideline-compliant study (Kirsch & Kersebohm, 1988), which would not require a classification according to DSD, however require a classification according to CLP.

Medical surveillance data of 10 urea producing facilities were collected, which showed no cases of eye irritation or related adverse eye effects resulting from exposure to urea. (Borealis Agrolinz Melamine, 2013).

CA 5.2.6 Skin sensitisation

ECHA endpoint summary for urea:

Endpoint conclusion:

No adverse effect observed (not sensitising)

Additional information:

No data are available: a waiver is proposed for this endpoint. Urea is naturally present at relatively high concentrations in human skin (up to 1% by weight) and is widely used in skin creams for the treatment of dry and irritant skin conditions without any reports of sensitisation reactions (Loden et al, 2002). A survey of 1905 patients does not reveal any reports of sensitisation (Stuttgen, 1992). A human volunteer study (Alchangian et al, 1986) does not report any sensitisation reactions. It is therefore considered to be very unlikely to be a skin sensitiser.

Migrated from Short description of key information:

Urea is naturally present at relatively high concentrations in human skin (up to 1% by weight) and is widely used in skin creams for the treatment of dry and irritant skin conditions without any reports of sensitisation reactions. It is therefore considered to be very unlikely to be a skin sensitiser.

CA 5.2.7 Phototoxicity

Not available

CA 5.3 Short-Term Toxicity

ECHA endpoint summary for urea:

Endpoint conclusion

Dose descriptor: NOAEL 2 250 mg/kg bw/day

Mode of Action Analysis / Human Relevance Framework

Additional information

Repeated dose oral toxicity

In 12 -month carcinogenicity screening assays (Fleischman et al, 1980), F-344 rats and C57BL/6 mice (50/sex/group) were exposed to urea in the diet at concentrations of 4500, 9000 or 45000 ppm for 12 months. Five animals/sex/group were sacrificed at the end of the 365-day exposure period and a comprehensive list of tissues was investigated histopathologically; interim deaths were similarly investigated. All remaining animals were sacrificed after the 4-month recovery

period and investigated histopathologically. There were no signs of toxicity. Survival and bodyweights were unaffected by treatment. Gross and microscopic pathology did not reveal any treatment-related effects. It is concluded that urea is of very low chronic toxicity. Using default conversion factors, the dose level of 45000 ppm is calculated to be equivalent to approximately 2250 mg/kg bw/d in the rat and 6750 mg/kg bw/d in the mouse.

Repeated dose dermal toxicity

In 4 -week and 25 -week dermal toxicity studies, urea (formulated as an ointment) was applied to the shorn dorsal skin of groups of male and female Wistar rats. Bodyweights were measured; food and water consumption were assessed. Clinical chemistry, urinalysis and haematological parameters were investigated. At necropsy, organ weights were recorded; gross necropsy and histopathology were performed. No dose-dependent toxicity was observed. Bodyweights, food and water consumption were unaffected by treatment. Clinical chemistry, haematology and urinalysis parameters were comparable in all groups. There was no effect of treatment on organ weights or pathology (Sato et al, 1977).

Repeated exposure inhalation toxicity

Urea is demonstrated to be of very low toxicity by the oral and subcutaneous routes. The substance is a non-volatile solid produced as crystals with particle sizes of >0.1 mm. There is therefore no potential for inhalation exposure. The data requirement is therefore waived on scientific grounds and on exposure considerations. Testing is additionally not justified on animal welfare grounds.

Repeated dose toxicity by other routes of exposure

Twelve unilaterally nephrectomised dogs were injected subcutaneously with 10% urea solution (3000-4000 mg/kg bw) every 8 hours over a period of 45 days. Administration led to increased diuresis, plasma urea levels were 200 - 700 mg/100ml. The dogs displayed mild drowsiness. Haematocrit, platelet counts and EEG were not affected. The study indicates that urea is of very low toxicity in the dog following repeated administration (Balestri et al, 1971).

CA 5.3.1 Oral 28-day study

CA 5.3.2 Oral 90-day study

CA 5.3.3 Other routes

CA 5.4 Genotoxicity Testing

ECHA endpoint summary for urea:

Genotoxicity in vitro

Ames tests

The potential mutagenicity of urea was investigated in a screening assay performed in S. typhimurium TA98, TA100 and TA1537 according to the published method of Ames et al (1977),

with a pre-incubation step and in the absence and presence of an exogenous source of metabolic activation. No evidence of mutagenicity was seen under the conditions of this assay (Ishidate et al, 1981).

The mutagenicity of urea was determined in the Ames test using Salmonella typhimurium and Escherischia coli, with and without S9 metabolic activation. The substance was not mutagenic at any of the 7 concentrations tested (Shimizu et al, 1985).

The potential mutagenicity of urea was investigated in a guideline-comparable Ames test (pre-incubation assay). Triplicate cultures of S. typhimurium TA97, TA98, TA100, TA1535 and TA1537 were exposed to urea (dissolved in water) at five concentrations between 10 -10000 ug/plate in the presence and absence of an exogenous metabolic activation system (Aroclor 1254 -induced male Sprague-Dawley rat and male Syrian hamster liver S9;). Exposure to urea caused cytotoxicity in some strains. The numbers of revertant colonies were not increased by exposure to urea. Appropriate positive controls confirmed the sensitivity of the assay (Mortelmans et al, 1986).

Clastogenicity

Ishidate et al (1981) report the results of a chromosomal aberration assay performed in CHL cells with a number of chemicals, including urea. Approximately 10e5 cells were plated and exposed to concentrations of urea up to the concentration causing 50% growth inhibition, in the absence and presence of PCB-induced Wistar rat liver S9-fraction. Cells were harvested at 24 and 48 hours (-S9) or at 24 hours following a 3-hour pre-incubation step (+S9). Chromosomal aberrations (including numerical aberrations) were scored from 100 well-spread metaphases per concentration. A positive result is reported in this assay, however the DT20 value (the concentration at which 20% of cells or approximately 4x background) of 13.0 mg/mL or 216 mM is well in excess of the limit concentration of 5 mg/ml or 10 mM recommended by OECD 473 (1997). The authors note a very low clastogenic potential. Considering the high concentrations of urea required to produce a response in this assay, which are well in excess of the limit concentration, it cannot be concluded that urea is clastogenic. The finding in this study is very likely to be a false positive due to osmotic effects.

The same group (Ishidate & Odashima, 1977) tested urea for the ability to cause chromosomal aberrations in a screening assay in CHL cells in vitro in the absence of metabolic activation and at concentrations up to those causing 50% growth inhibition. A positive result is reported at a concentration of 266.4 mMol/L, which is well in excess of the limit concentration of 10 mM. The result is considered to be a false positive and is attributable to the effects of osmolarity.

Mammalian cell mutation

The potential mutagenicity of urea was investigated in a mouse lymphoma assay in the absence of metabolic activation. A weak positive response was seen at concentrations of 265 -662 mMol/L, concentrations which also caused cytotoxicity and which are well in excess of the limit concentration of 10 mMol/L recommended in OECD 476 (1997). The result is considered to be a false positive. The authors conclude that the effect is due to the influence of high concentrations of urea on the osmolarity of the culture medium (Wangenheim & Bolcsfoldi, 1988).

Other studies

The ability of urea to cause DNA damage was assessed in two DNA-unwinding/alkaline elution assays. Garberg et al (1987), report positive effects in mouse lymphoma cells at high concentrations of 628 and 718 mMol/L, however negative results are reported by Sina et al (1983) at concentrations of up to 3 mMol/L in cultured rat hepatocytes.

Genotoxicity in vivo

Chaurasia & Sinha (1987) investigated the potential of urea to cause chromosomal aberrations in the bone marrow of Swiss mice. Mice (number unspecified) were administered urea in the diet at a dose level of 500 mg/day for 5 days. Animals were sacrificed after a recovery period of 7 days and the bone marrow harvested. A total 300 metaphases from treated animals and untreated controls were assessed for chromosomal aberration. A marked increase in the incidence of chromosomal aberrations was seen in the treated group (7x controls). However the dose level administered in this study is equivalent to 16 -17 g/kg bw/day and is thus far in excess of the limit dose of 1000 mg/kg bw. Signs of toxicity are not reported, but marked toxicity can be predicted at this dose level.

Conclusion

Positive results obtained in vitro are associated with concentrations well in excess of the recommended limit concentrations are not considered to be of biological relevance. A positive result in vivo is also associated with an excessive dose level. Considering the physiological role and presence of substantial quantities of urea in the human body, it is not considered likely that this substance is genotoxic. Further testing for genotoxicity is not proposed.

Short description of key information:

Negative results are reported in three Ames tests. Positive results are reported in assays for mutagenicity and clastogenicity in mammalian cells, however the value of these studies are limited by the extremely high test concentrations. A positive result is reported in a mouse bone marrow assay of unconventional design, however this study is not considered to be reliable. Based on its physiological role and presence in the body at high concentrations, urea is not considered to be genotoxic.

Endpoint Conclusion: No adverse effect observed (negative)

CA 5.4.1 *In vitro* studies

CA 5.4.2 *In vivo* studies in somatic cells

CA 5.4.3 *In vivo* studies in germ cells

CA 5.5 Long-Term Toxicity and Carcinogenicity

ECHA endpoint summary for urea:

Carcinogenicity: via oral route

Endpoint conclusion

Dose descriptor: NOAEL 2 250 mg/kg bw/day

Additional information

The carcinogenicity of urea was investigated in NCI 12 -month screening studies in the rat and mouse (Fleischman et al, 1980). No evidence of carcinogenicity or toxicity was seen in either study at the very high dose level of 45000 ppm (4.5% in the diet).

F344 rats (50/sex/group) were exposed to urea in the diet at concentrations of 4500, 9000 or 45000 ppm for 12 months. Five animals/sex/group were sacrificed at the end of the 365-day exposure period and a comprehensive list of tissues was investigated histopathologically; interim deaths were similarly investigated. All remaining animals were sacrificed after the 4-month recovery period and investigated histopathologically. There were no signs of toxicity. A significant linear trend in the incidence of interstitial cell tumours was noted in male rats. The incidence was 21/50 in controls, 27/48, 25/48 and 35/50 in the low, intermediate and high dose groups respectively. The authors do not consider this finding to be of biological significance as the background incidence of this tumour type is noted to be up to 100% in F344 rats.

Using default conversion factors, the dose level of 45000 ppm is calculated to be equivalent to approximately 2250 mg/kg bw/d in the rat and 6750 mg/kg bw/d in the mouse.

B6C3F1 mice (50/sex/group) were exposed to urea in the diet at concentrations of 4500, 9000 or 45000 ppm for 12 months. Five animals/sex/group were sacrificed at the end of the 365-day exposure period and a comprehensive list of tissues was investigated histopathologically; interim deaths were similarly investigated. All remaining animals were sacrificed after the 4-month recovery period and investigated histopathologically. There were no signs of toxicity. A significantly increased incidence of haematopoietic tumours (malignant lymphoma) was seen in female rats in the mid-dose group. The incidence of this finding was 10 -92 in controls; 7/43, 10/38 and 9/50 in low, mid and high dose group animals, respectively. There is no relationship to treatment in the absence of a dose-response relationship.

Justification for classification or non-classification

No classification is proposed for carcinogenicity. There is no evidence from animal studies that urea is carcinogenic. The physiological role of urea and level of production by the human body indicates that the substance is not carcinogenic.

CA 5.6 Reproductive Toxicity

CA 5.6.1 Generational studies

ECHA endpoint summary for urea

Effects on fertility

Description of key information: No additional information is available.

Additional information

Large quantities of urea are formed naturally in the human body as a consequence of normal protein catabolism. Urea is shown to be essentially without toxicity in the available studies and no effects (organ weight, gross pathology, histopathology) were observed on the reproductive organs of rats and mice exposed to urea at very high dietary levels for 12 months (Fleischman et al, 1980). The level of any primary, occupational or secondary exposure to urea is likely to be insignificant compared to the quantities (20-50 g/day) produced by normal metabolism and present at high concentrations in the blood. It is therefore considered that urea is very unlikely to be a reproductive toxin and testing cannot be justified scientifically.

Short description of key information:

No standard studies are available. It is considered extremely unlikely that occupational, primary or secondary exposure to urea will result in any effects on fertility as the levels of exposure will be insignificant compared to those present in the body as a result of protein catabolism.

CA 5.6.2 Developmental toxicity studies

ECHA endpoint summary for urea

Description of key information:

In a key prenatal developmental toxicity study, urea was administered orally to female pregnant rats by gavage at dose levels of 100, 300 or 1000 mg/kg bw/day from the 6th to 20th day of pregnancy. In the dams, there were no item-related effects on the maternal and reproductive parameters. In the fetuses, there was also no test item-related influence on the prenatal fetal development and no malformations nor variations were noted during the macroscopic, skeletal and soft tissue examinations. In conclusion, the NOAEL was above 1000 mg Urea/kg bw/day for maternal developmental and foetal toxicity as well as for teratogenicity. It is considered extremely unlikely that occupational, primary or secondary exposure to urea will result in developmental toxicity as the levels of exposure will be insignificant compared to those present in the maternal and foetal circulation as a result of protein catabolism.

Effect on developmental toxicity: via oral route

Endpoint conclusion: no adverse effect observed

Dose descriptor: NOAEL 1000 mg/kg bw/day

Study duration: subacute

Species: rat

Quality of whole database: reliable

Effect on developmental toxicity: via inhalation route

Endpoint conclusion: no study available

Effect on developmental toxicity: via dermal route

Endpoint conclusion: no study available

Additional information

In a key prenatal developmental toxicity study (Hansen, 2017), the test item Urea was administered orally to female pregnant CD® / Crl:CD(SD) rats by gavage at dose levels of 100, 300 or 1000 mg/kg bw/day from the 6th to 20th day of pregnancy. The measured actual concentrations of the test item in the test item vehicle mixtures were between 101.0% and 105.8% of the nominal concentrations. On gestation day 21, the dams were laparotomised and examined for implantation sites, resorptions in the uterus and for the condition of the fetuses. In the dams, no premature death, nor changes in behaviour, body weight (gain) and food/water consumption were noted between the control group and the treatment groups. No test item-related changes were noted during the macroscopic inspection nor for the gravid uterus or carcass weights of the dams at necropsy. No test item-related influence was noted on the reproductive parameters (number of implantation sites, resorptions and fetuses). In the fetuses, no test item-related influence on the prenatal fetal development with respect to the number of resorptions, number of live fetuses, fetal and placental weights, the values calculated for the post-implantation loss and the sex distribution of fetuses. No malformations nor variations were noted during the macroscopic, skeletal and soft tissue examinations. Under the conditions of the study, Urea did not show any teratogenic potential. In conclusion, the NOAEL was above 1000 mg Urea/kg bw/day for maternal developmental and foetal toxicity as well as for teratogenicity.

In a supporting dose range finding study (Hansen, 2016), urea was administered once daily by oral gavage from gestation day 6 until gestation day 20 to 4 groups of pregnant CD® / Crl:CD(SD) rats, consisting of 3 animals per group, at dose levels of 0, 100, 300 and 1000 mg/kg bw/day. On gestation day 21, the dams were laparotomised and examined for implantation sites, resorptions in the uterus and for the condition of the fetuses. Two litters per dose group were examined. In the dams, no premature death, no changes in behavior, body weight (gain) and food/water consumption were noted between the control group and the treatment groups. No test item-related changes were noted during the macroscopic examination at necropsy nor gravid uterus weight and carcass weight. In the fetuses, no test item-related effects were observed. Laparotomy revealed no dead fetuses, runts, malformations or variations at external examination. The dose levels for the main study were selected as 0, 100, 300 and 1000 mg Urea/kg bw/day, p.o.

A study of limited design also did not indicate any teratogenicity or effects on renal development in the rat (Seipelt et al, 1969), however slightly lower pup weights were seen at the dose level of 500 mg/kg bw/d in this study. The findings were observed in a small group of 6 animals and were overruled by the key developmental toxicity study; the dose of 500 mg/kg bw can be considered as NOAEL from a conservative viewpoint. Finally, a screening assay in chick eggs is considered to be of limited value (Mora et al, 1991).

Large quantities of urea are formed naturally in the human body as a consequence of normal protein catabolism. Urea is shown to be essentially without toxicity in the available studies. The level of any primary, occupational or secondary exposure to urea is likely to be insignificant compared to the quantities (20-50 g/day) produced by normal metabolism and present at high concentrations in the maternal and foetal circulation.

A developmental toxicity study in a second non-rodent species is waived based on a weight-of-evidence approach providing scientific reasons. Urea is endogenous in humans and animals, and is eliminated mainly via the kidney. Urea was negative for developmental and foetal toxicity in the rat developmental toxicity study up to the limit dose, and it is not expected to result in developmental and foetal toxicity in other species. Uraemia, which is a high blood concentration of urea either due to physiological or pathological conditions, is not tolerated very well by all species. Rabbits are different from other species in various viewpoints towards urea gastro-intestinal tolerance and systemic elimination. Uraemia in rabbits can become problematic due to gastro-intestinal (especially caecum) impactation of high urea or ammonium concentrations, leading to increased pH and dysbiosis at the caecum and diarrhea. In addition, once systemically absorbed, rabbits are characterised by a lack of kidney urea concentration, leading to dehydration and bad condition due to the osmotic activity of urea. These specific rabbit issues may complicate toxicological testing, especially in pregnant female rabbits in developmental toxicity studies which are more susceptible to stress. Therefore the rabbit is not considered appropriate to be used as a second species for developmental toxicity testing. The same applies to guinea-pigs. For animal welfare reasons, higher animal species are not considered.

Justification for classification or non-classification

Developmental toxicity testing in rats dosed orally up to 1000 mg/kg bw did not result in adverse effects. There are no studies in animals showing clear evidence of reproductive effects. The results of the available studies do not trigger classification according to Directive 67/548/EEC.

CA 5.7 Neurotoxicity Studies

CA 5.7.1 Neurotoxicity studies in rodents

CA 5.7.2 Delayed polyneuropathy studies

CA 5.8 Other Toxicological Studies

CA 5.8.1 Toxicity studies of metabolites

CA 5.8.2 Supplementary studies on the active substance

CA 5.8.3 Endocrine disrupting properties

CA 5.9 Medical Data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

CA 5.9.2 Data collected on humans

CA 5.9.3 Direct observations

CA 5.9.4 Epidemiological studies

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

CA 5.9.7 Expected effects of poisoning

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE (SIC)

Introduction

CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals

According to EFSA (EFSA Journal 2012; 10 (29:2545), the hydrolysed proteins are derived by the hydrolysis of tissues from organisms that can be of plant or animal origin. Hydrolysed proteins *per se* are likely to be of low toxicological concern provided hydrolysed proteins of animal origin are pathogen-free. On this basis no risks to human health could be expected from its use as a plant protection product and data waivers for specific toxicological studies were initially supported.
The manufacturing process and the manufacturing plants of collagen protein hydrolysate are in accordance with the Regulation EC n. 1069/2009 . Hydrolysed protein is thus free from any BSE/TSE risk and it is considered safe from use.

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

CA 5.2 Acute Toxicity

CA 5.2.1 Oral

Confidential

CA 5.2.2 Dermal

Confidential

CA 5.2.3 Inhalation

Confidential

CA 5.2.4 Skin irritation

Confidential

CA 5.2.5 Eye irritation

Confidential

CA 5.2.6 Skin sensitisation

Confidential

CA 5.2.7 Phototoxicity

CA 5.3 Short-Term Toxicity

CA 5.3.1 Oral 28-day study

CA 5.3.2 Oral 90-day study

CA 5.3.3 Other routes

CA 5.4 Genotoxicity Testing

CA 5.4.1 *In vitro* studies

CA 5.4.2 *In vivo* studies in somatic cells

CA 5.4.3 *In vivo* studies in germ cells

CA 5.5 Long-Term Toxicity and Carcinogenicity

CA 5.6 Reproductive Toxicity

CA 5.6.1 Generational studies

CA 5.6.2 Developmental toxicity studies

CA 5.7 Neurotoxicity Studies

CA 5.7.1 Neurotoxicity studies in rodents

CA 5.7.2 Delayed polyneuropathy studies

CA 5.8 Other Toxicological Studies

CA 5.8.1 Toxicity studies of metabolites

CA 5.8.2 Supplementary studies on the active substance

CA 5.8.3 Endocrine disrupting properties

CA 5.9 Medical Data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

CA 5.9.2 Data collected on humans

CA 5.9.3 Direct observations

CA 5.9.4 Epidemiological studies

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

No remarkable particular symptoms and effects

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

After inhalation: If breathed, move person from danger area and provide for fresh air and seek medical advice. If not breathing give artificial respiration.

After skin contact: Wash with water.

After eye contact: Rinse with copious quantities of clean water keeping the eyelids well open in order to assure an adequate rinsing and seek medical advice.

After swallowing: Rinse out the mouth with copious quantity of water and seek medical advice. Never give anything by mouth to an unconscious person.

Self-protection of the first aider: Follow good working practice. No remarkable particular indication.

indication of any immediate medical attention and special treatment needed: No remarkable particular indication.

CA 5.9.7 Expected effects of poisoning

No remarkable particular symptoms and effects