EXAMINATION OF EYE IRRITANCY BY USING TWO ALTERNATIVE METHODS

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Abstract

Agrochemicals must undergo numerous toxicological tests before registration. To get knowledge about eye irritation, nowadays only the *in vivo* Draize-test is accepted, which is one of the most criticized methods because of the injuries of the test animals. Therefore, several *in vitro* tests have been developed to replace *in vivo* eye irritation testing. Two of these alternative methods, the MTT-Assay and the HET-CAM test (Hen's Egg Test – Chorioallantois Membrane) were used in our comparative screening, with a set of agrochemicals to establish parallel data on *in vitro* and *in vivo* (Draize) results. The examined products were:

Systhane 12 E (miclobuthanyl, 125 g/l), Clinic 480 SL (glyphosate isopropylamin salt 360 g/l), Targa Super 5 EC (quizalofop-P-ethyl

5%), Trend™ (isodecyl acetate), Silwet L-77 (polyalkylenoxid 84%, iso-propylene 16%) and Substral (66 g/l nitrogen, 30 g/l P_2O_5 water-soluble phosphate, 67 g/l water-soluble K_2O).

In these experiments, the same results were obtained in the alternative tests and in the Draize test which shows that the methods applied may be possible the replace the *in vivo* test as a test system in the future.

Keywords: chorioallantoic membrane, *in vitro*, rabbit, Draize-test, HET-CAM test, MTT-Assay

Összefoglalás

A mezőgazdasági vegyi anyagok forgalomba hozatalát megelőző, előírt toxikológiai vizsgálatokban a szemirritáció meghatározására jelenleg az élő nyulakon elvégzett vizsgálatok eredményeit fogadják el. Mivel a módszer a kísérleti állatokra nézve erős fájdalommal járhat, az erősödő állatvédő mozgalmak hatására több olyan *in vitro* módszert is kidolgoztak, amely nem csak csökkentheti az ecélra felhasznált emlős állatok számát, hanem esetleg teljes mértékben ki is válthatja ezek alkalmazását. A lehetséges alternatív módszerek közé tartoznak a tyúktojás chorioallantois membránját (CAM) használó tesztek és az *in vitro* citotoxicitási tesztek.

A kísérletekben 6 mezőgazdasági vegyi anyagot használtunk fel, a kísérleti anyagok károsító hatásait az alternatív tesztrendszerrel (HET-CAM, MTT-Assay) és *in vivo* (Draize-teszt) módszerrel vizsgáltuk. Az alternatív tesztekből származó eredményeket összevetettük az élő állaton végzett teszt eredményeivel. Az irritációs kategóriákba történt besorolás után az *in vitro* és az *in vivo* módszerek eredményei jelen esetben teljes egyezést mutattak, ami alapján feltételezhető, hogy a későbbiekben az alternatív tesztek tesztrendszerként alkalmasak lehetnek

a szemirritáció mértékének becslésére, így a kísérletekben felhasznált állatok számának csökkentésére.

Introduction

Recently, all chemicals have to be tested before putting on the market. In this process toxicological examinations play an important role, because they can show several features of the ingredients that preclude the possibility of their distribution in the European Union. During drug and pesticide research and development different toxicological test are used for evaluation of their safety. These tests are necessary to determine the potential risk of chemicals for humans, pets and livestock animals, and to investigate their possible deleterious effects on the living organisms. The people can get into direct contact with the chemicals during the manufacture or using, and the animals can be contaminated with these agents accidentally during the authorised application or due to the irregular storage. The contact of these poisonous materials with the eyes can even lead to irreversible changes or blindness (Bordás, 1971). To detect the ocular irritation, only the Draize eye irritation test (OECD 405, 2002) is accepted now that is one of the most criticized methods because of the pain induced to the test animals. Several *in vitro* methods have been used to investigate the toxicity of potential eye irritants with a view to replacing *in vivo* eye irritation testing. To detect severe irritancy or corrosivity, the Isolated Chicken Eye Test is acceptable (OECD no. 438, 2009)

The HET-CAM (Hen's egg test – chorioallantoic membrane) test, using the chorioallantois membrane (CAM) of the chick embryo, is an alternative test to replace the Draize rabbit eye test (Walum et al., 1992). The CAM of the chick embryo has been used extensively for many years in various fields of biological research including virol-

ogy, bacteriology and toxicology, as it is a complete tissue including arteries, capillaries and veins, and is technically easy to study. In this test chemicals are placed in direct contact with the chorioallantoic membrane of the hen's egg. The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique as an indication of the likelihood that a chemical would damage mucous membranes (especially the eye) *in vivo* (Leighton et al., 1985).

The MTT-Assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a simple method to determine the viability/number of cells in culture, through the formation of a coloured product to which the cell membrane is impermeable. Determination of the ability of cells to reduce MTT to the formazan product after exposure to test compounds enables the relative toxicity of test chemials to be assessed.

In our studies the HET-CAM test (Luepke and Kemper, 1986) and the MTT were used as *in vitro* methods. The results from the *in vitro* tests were compared with results from *in vivo* Draize rabbit eye test.

Materials and methods

Test materials

The test materials were: Systhane 12 E (miclobuthanyl 125 g/l), Clinic 480 SL (glyphosate isopropylamin salt 360 g/l), Targa Super 5 EC (quizalofop-P-ethyl 5%), Trend™ (isodecyl acetate), Silwet L-77 (polyalkylenoxid 84%, iso-propylene 16%) and Substral (66 g/l nitrogen, 30 g/l P_2O_5 water-soluble phosphate, 67 g/l water-soluble K_2O).

All of the chemicals were applied in their original form in the HET-CAM test and in the Draize rabbit eye irritation test. In the MTT assay, the test materials were tested in different dilutions.

Methods

HET-CAM Test

Shaver Rusticbrow chicken eggs were used in the study, incubated in a Ragus incubator. The temperature was 37 ^{o}C, the relative humidity was 60-70%. The eggs were rotated for 8 days to prevent the attachment of the embryo to one side of the egg. The eggs were prepared for assaying on day 10. The section of shell above the air chamber was removed with scissors. The membrane was moistened carefully with 0.9% NaCl solution.

Two eggs were used as control, treated with 0.9% NaCl, 2 eggs as standards, treated with 1% Sodium dodecyl sulphate and 0.1 M NaOH. The test materials were applied on 6 eggs, on 4 separated replicates.

The membrane was removed carefully with tapered forceps. 0.1 ml of test pesticide was added to the chorioallantoic membrane and the effect was observed over a period of up to 300 seconds. Hemorrhage, vascular lysis or coagulation can be seen on the chorioallantoic membrane. Each reaction occurred were recorded in seconds. A computer software was used to evaluate data (Invittox Protocol no. 47). The computer software uses the following algorithm:

$$RI = \frac{301\text{-}secH}{300} \times 5 + \frac{301\text{-}secL}{300} \times 7 + \frac{301\text{-}secC}{300} \times 9$$

Where H = haemorrhage, L = vascular lysis, C = coagulation, RI = irritation index, and sec = start second.

The irritation categories can be seen in Table 1.

Irritation index	Irritation category
0-0.9	no irritation
1-4.9	weak irritation
5-8.9	moderate irritation
9-21	severe irritation

Table 1. Classification of HET-CAM test

MTT-Assay

Maintained fibroblast-like cells from the kidney of the African green monkey (Vero-Hektor cells, ECACC No.: 03092503) were used for the MTT assay, according to Invittox protocol No.: 17. The cells were distributed into the wells of a microtiter plate 24 hours before the test. Test samples were diluted with cell culture medium, diluted 10, 20, 40, 80, 160, 320, 640 and 1280 times and added to the cells. After 24 hours dissolved MTT (Sigma M 5655) was added, which in turn was converted to water insoluble purple formazan by mitochondrial dehydrogenases. At the end of the incubation period of two hours the formazan was dissolved in methanol and its absorbance was measured at a wavelength of 570 nm. Reduction of the value (OD570- OD630) compared to the same value of non-treated cells indicates cell destruction due to the test chemical. For comparison among the test chemicals LCC_{50} (50% lethal concentration for cells) was assessed. For the assessment approximately linear correlation was supposed between concentration and effect in the vicinity of LCC_{50}. The irritation categories were detected based on effects of the dilution used (Table 2).

LCC_{50} (mL/L)	**Irritation category**
> 10.000	no irritation
5.000 – 1.250	weak irritation
1.25 – 0.078	moderate irritation
< 0.078	severe irritation

Table 2. Irritation categories from the MTT-Assay

Draize Rabbit Eye Test

The test was performed on 2 or 3 New-Zealand rabbits, depending on the symptoms observed. Using of separated control group is not necessary as the untreated right eye serves as control. Rabbits were kept in individual cages of a climatic animal room. The temperature was 22-25 ^{o}C and the relative humidity was 50-70%. The animals were fed with laboratory rabbit diet, and tap water was served *ad libitum* (OECD Guidelines for Testing of Chemicals, Number 405, 2002). The animal study was performed by the permission of the Animal Testing Work Committee.

A volume of 0.1 ml agrocemical was instilled into the conjunctival sac of each rabbit. Ocular irritation on the treated eye was evaluated at 1 hour, then 1, 2, 3 and 7 days post installation (Draize et al., 1944). Individual scores were recorded for each animal. The time interval with the highest mean score (Maximum Mean Total Score – MMTS) for all rabbits was used to classify the test substance by the system of Kay and Calandra (1962), (Table 3).

MAXIMUM MEAN SCORE		PERSISTENCE OF SCORE	DESCRIPTION RATING (AND CLASS)
0.0 to 0.5	Group mean total score at 24 hours = 0 Group mean total score at 24 hours > 0		Non-irritant (1) Practically non-irritant (2)
0.5 to 2.5	Group mean total score at 24 hours = 0 Group mean total score at 24 hours > 0		Practically non-irritant (2) Minimal irritant (3)
2.5 to 15	Group mean total score at 48 hours = 0 Group mean total score at 48 hours > 0		Minimal irritant (3) Mild irritant (4)
15 to 25	Group mean total score at 72 hours = 0 Group mean total score at 72 hours > 0		Mild irritant (4) Moderate irritant (5)
		More than half of the individual total scores at 7 days 10 or less	Moderate irritant (5)
25 to 50	Group mean total score at 7 days 20 or less	More than half of the individual total scores at 7 days > 10 but no individual total score at 7 days > 30	Moderate irritant (5)
		More than half of the individual total scores at 7 days > 10 and any individual score at 7 days > 30	Severe irritant (6)
	Group mean total score at 7 days > 20		Severe irritant (6)
		More than half of the individual total scores at 7 days 30 or less	Severe irritant (6)
50 to 80	Group mean total score at 7 days 40 or less	More than half of the individual total scores at 7 days > 30 but no individual total scores at 7 days > 60	Severe irritant (6)

Table 3.

Classification of eye irritation scores from Draize eye irritation test

		More than half of the individual total scores at 7 days > 30 and individual total score at 7 days > 60	Very severe irritant (7)
	Group mean total score at 7 days > 40		Very severe irritant (7)
		More than half of the individual total scores at 7 days 60 or less	Very severe irritant (7)
80 to 100	Group mean total score at 7 days 80 or less	More than half of the individual total scores at 7 days > 60 but no individual total score at 7 days > 100	Very severe irritant (7)
		More than half of the individual total scores at 7 days > 60 and individual total score at 7 days > 100	Extremely severe irritant (8)
	Group mean total score at 7 days > 80		Extremely severe irritant (8)
100 to 110	Group mean total score at 7 days 80 or less Group mean total score at 7 days > 80		Very severe irritant (7) Extremely severe irritant (8)

Table 3.

Classification of eye irritation scores from Draize eye irritation test (Continued)

Results

Results of the HET-CAM Test

The numerical data can be seen in Table 4. After the treatment with Systhane 12 E lysis was observed between 5 and 12 sec, and haemorrhage between 14 and 28 sec. By these results the irritation index is 11.50 and the fungicide is severely irritative.

On the chorioallantois membranes treated with Clinic 480 SL vascular lysis started between 2 and 7 sec, and there was slight haemor-

rhage from 7 to 19 sec. Based on irritation index of 11.74, it is a severe irritative pesticide.

Vascular lysis was noted between 4 and 6 sec after treatment with Targa Super 5 EC, and haemorrhage was found from 8 to 14 sec. On the basis of the 11.70 irritation index, Targa Super 5 EC is severely irritative.

During the observation period there was vascular lysis from 3 to 10 sec after treatment with Trend™, followed by haemorrhage started between 9 and 21 sec. The irritation index of Trend™ is 11.66, thus it is a severely irritative agent.

On the chorioallantois membranes treated with Silwet L-77 vascular lysis started between 2 and 7 sec, and there was mild haemorrhage from 7 to 16 sec. On the base of the 11.75 irritation index, this pesticide is severely irritative.

During the observation period mild haemorrhage was observed from 8 to 19 sec after the treatment with Substral. Based on the irritation index of 4.80, Substral is a weakly irritative pesticide.

Pesticide	**Irritation index**
Systhane 12 E	11.50
Clinic 480 SL	11.74
Targa Super 5 EC	11.70
Trend™	11.66
Silwet L-77	11.75
Substral	4.80
NaOH 0.1 M + SDS 1%	11.53

Table 4. Irritation indices from HET-CAM test

The obtained irritation indices can be evaluated using the classification categories of Table 1.

Results from the MTT-assay

The numerical data can be seen in Table 5.

The destruction of the cells treated with Systhane 12 E was over 50% in all the dilutions applied up to 1280 times dilution. The pesticide showed high citotoxicity.

Clinic 480 SL also showed high citotoxicity. More than 50% of the cells were destroyed by the treatment with the highest dilution.

More than 50% of the cells treated with 1280 times diluted Targa Super 5 EC died after the treatment, which shows the herbicide to be highly citotoxic.

The destruction of the cells treated with Trend™ was over 50% in all the dilutions applied up to 1280 times dilution. The pesticide showed high citotoxicity.

More than 50% of the cells treated with 1280 times diluted Silwet L-77 died after the treatment, which shows the pesticide to be higly citotoxic.

Substral, applied 10, 20 and 40 times diluted, destroyed more than 50% of the cells. The test material diluted 80 times caused no destruction of the cells which shows the chemical to be less citotoxic.

Test material/ Dilution	Systhane 12 E	Clinic 480 SL	Targa Super 5 EC	Trend™	Silwet L-77	Substral
10x	98%	98%	86%	98%	96%	98%
20x	98%	96%	97%	98%	97%	92%
40x	98%	97%	96%	98%	98%	60%
80x	98%	97%	96%	98%	98%	0%
160x	98%	96%	96%	98%	98%	0%
320x	98%	95%	97%	98%	98%	0%
640x	96%	97%	96%	97%	96%	0%
1280x	87%	97%	97%	97%	97%	0%

Table 5. Destruction of cells in different dilutions

The results show that the test materials with higher irritation potential were toxic to the cells in higher dilution as well.

Results from the Draize rabbit eye irritation test

The obtained numerical data are summarised in Table 6. After instillation of Systhane 12 E, positive conjunctival responses with severe redness and, chemosis, and strong discharge were noted until the end of the observation period. The iris lesion was seen within 1 hour. On the cornea moderate opacity was noted from the first day which was not reversible during the 7 days observation period. On the basis of the irritation index (78.33), Systhane 12 E is very severely irritative to the rabbit eye.

Severe redness and chemosis, and strong discharge were recorded during 7 days after treatment with Clinic 480 SL. Iris lesion was seen from day 1 or 2 and it was not reversible during the observation period. On the cornea slight opacity was noted from 1 hour in all animals until the end of the observation period. Clinic 480 SL is very severely irritative to the rabbit eye on the basis of the irritation index (56.67).

After instillation of Targa Super 5 EC, positive conjuctival responses with moderate redness and chemosis, and strong discharge were found after the first hour. Iris lesion was seen at 24 hours after the treatment and the inflammation was present until the end of the observation period. Slight opacity was seen on the cornea from 1 hour that was not reversible but stronger until day 7. On the base of the irritation index (69.00), Targa Super 5 EC was very severe irritative to the rabbit eye. Based on the symptoms observed on the first and second animal, the third rabbit was not treated due to animal welfare reasons.

Moderate redness and chemosis, and strong discharge were noted at the first observation post instillation of Trend™ and the symptoms became stronger on day 2 and 3. The iris was changed after 1 day and

did not recover until the end of the observation period. Moderate opacity was noted on the cornea from the first hour until the end of the observation period. Based on the irritation index (64.00), Trend™ has very severe irritative potential to the rabbit eye. Based on the symptoms observed on the first and second animal, the third rabbit was not treated due to animal welfare reasons.

Positive conjuctival responses with moderate redness and chemosis, and strong discharge were observed during the observation post instillation of Silwet L-77. Iris lesion was seen after 1 hour. Slight opacity was noted on the cornea from the first hour and it became stronger during the observation period. On the basis of its irritation index (70.70), Silwet-L-77 was very severely irritative to the rabbit eye.

Moderate redness and slight chemosis were noted in the first hour post instillation of Substral. After 24 hours the treated eyes returned to normal. The iris remained normal. On the cornea no opacity was noted during the observation period. On the basis of these results, Substral was minimally irritative to the rabbit eye.

Pesticide	**Irritation index**	**Category**
Systhane 12 E	78.33	Very severe
Clinic 480 EC	56.67	Very severe
Targa Super 5 EC	69.00	Very severe
Trend™	64.00	Very severe
Silwet L-77	70.70	Very severe
Substral	3.30	Minimal

Table 6. Irritation indices from Draize rabbit eye test with corresponding irritation categories

Discussion

The HET-CAM test and the MTT-Assay, as *in vitro* methods have several advantages including their rapidity, simplicity, sensitivity, ease of performance and their relative cheapness. One of the disadvantages of the HET-CAM test is the subjective nature of the evaluation of the results. In addition, the evaluation can not be done in case of opaque and colour chemicals, because these materials cover up the membrane and make the emulsion colourful. To test powder or granulate formulations by these methods is more complicated.

In case of chemicals tested in present study, all the results from the *in vitro* MTT-Assay and HET-CAM test were equal with the *in vivo* data from Draize rabbit eye test. Based on these data, both of these methods may be appropriate to predict the prospective irritation caused by liquid agrochemicals. The results in comparison can be seen in Table 7.

Pesticide	**Category from HET-CAM test**	**LCC_{50} (mL/L) from cytotoxicity test**	**Category from Draize rabbit eye test**
Systhane 12 E	Severe	<0.078	Very severe irritative
Clinic 480 EC	Severe	< 0.078	Very severe irritative
Targa Super 5 EC	Severe	< 0.078	Very severe irritative
Trend™	Severe	< 0.078	Very severe irritative
Silwet L-77	Severe	< 0.078	Very severe irritative
Substral	Weak	1.25–2.50	Minimal irritative

Table 7. Irritation categories by HET-CAM test and MTT-Assay in comparison to irritation categories by Draize rabbit eye test

In several previous experiments a good correlation was found between the results from *in vitro* HET-CAM test and the results from *in vivo* Draize eye irritation test (Luepke and Wallat, 1987; Budai and Várnagy, 2000; Budai et al., 1995, 1997, 1998, 2002). In 1992 the German authorities accepted the use of HET-CAM data for the classification of R41 chemicals in the notification of new industrial chemicals (Balls et al., 1999).

In this study a good (100%) correlation was seen between the data from *in vitro* and *in vivo* methods. The HET-CAM test or the MTT-Assay could not replace the currently used Draize eye irritation toxicological test, but it could diminish investigations in mammals as well as limit or eliminate pain and damage in animal experiments. In this form the HET-CAM test and the MTT-Assay can be useful for prescreen methods as part of an *in vitro* system.

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