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OPTIMISATION AND VALIDATION OF AN ALTERNATIVE MUCOSAL IRRITATION TEST

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LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
A. lusitanicus	*Arion lusitanicus*
ANOVA	analysis of variance
BAC	benzalkonium chloride
BCOP test	bovine corneal opacity and permeability test
Carb	Carbopol® 974P
CASRN	chemical abstract service registry number
Cat 1	irreversible effects on eyes (GHS category)
Cat 2	reversible effects on eyes (GHS category)
Cat 2A	irritating to eyes (GHS category)
Cat 2B	mildly irritating to eyes (GHS category)
Conc	concentration
DDWM	drum dried waxy maize starch
EC	European Commission
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EC/HO	European Commission and British Home Office
ECVAM	European Centre for the Validation of Alternative Methods
EU	European Union
GHS	globally harmonized system of classification and labelling of chemicals
HCE model	human corneal epithelium model of SkinEthic (Nice, France)
HCl	hydrochloride
HEC	hydroxyethyl cellulose
HET-CAM	hen's egg test on chorio-allantoic membrane
HT	heat treated
I	irritant

ICH	International Conference on Harmonisation
IU	international unit
LDH	lactate dehydrogenase
LDH2	lactate dehydrogenase release after the 2nd contact period
L. flavus	*Limax flavus*
L. maximus	*Limax maximus*
min.	minute
MMAS	modified maximum average score
Mod	moderate
n	number
N-9	nonoxynol-9
NAD^+	nicotinamide adenine dinucleotide
NaSF	sodium stearyl fumarate
NI	non irritant
NS	not sterilised
OECD	Organisation for Economic Cooperation and Development
OTC	over-the-counter
p	probability value
P1	protein release after the 1st contact period
P2	protein release after the 2nd contact period
PAA	linear polyacrylic acid
PEG	polyethylene glycol
PBS	phosphate buffered saline
R36	irritating to eyes (EU risk phrase)
R41	risk of serious damage to eyes (EU risk phrase)
S	sterilised
Sev	severe
SLS	sodium lauryl sulphate
SMI test	Slug Mucosal Irritation test
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

CHAPTER 1: SITUATION AND OBJECTIVES

1.1 INTRODUCTION

The mucosa has an important protective function by preventing entry of micro-organisms and noxious substances from the environment into the body (Marieb, 1995). The ocular, nasal, oral, gastro-intestinal, vaginal and rectal mucosae can be exposed accidentally or intentionally to pharmaceuticals, personal care products, cosmetics, and chemicals. However, many compounds can irritate the mucosa. Mucosal irritation and damage might increase the susceptibility to pathogens (Stephenson, 2000; Fichorova *et al.*, 2001; Van Damme *et al.*, 2002) and decrease the patient compliance. Because pharmaceuticals may be applied frequently over a period of months or years, it is extremely important to evaluate the mucosal irritation potential or mucosal tolerance of pharmaceutical formulations. The mucosal tolerance is evaluated in the research and development of new drugs because of several additional reasons. Firstly, safety testing is a helpful tool in the selection of lead candidates in the drug discovery and development phase (Curren and Harbell, 2002). The results of the safety evaluation can guide the drug development process, because mucosal irritation can be decreased or eliminated by adapting the formulation or dosage form (Fara and Myrback, 1990). Elimination of toxic compounds early in drug development is also of economical concern, because this contributes to saving of money and time (Vanparys, 2002). Furthermore, regulatory authorities require that the local tolerance of potential new medicinal products is assessed before the products are administered to humans (EC, 1990).

Pre-clinical studies to evaluate the ocular, nasal, oral, vaginal, and rectal tolerance of both the active agent and the clinical formulation are carried out in vertebrates in compliance with EU directives and guidelines (EC, 1965; EC, 1975a; EC, 1975b; EC, 1990). However, the use of vertebrates for safety studies is criticised based on ethical, scientific, economical and practical considerations (EC, 1986; CPMP, 1997). Directive 86/609/EEC stimulates

limitation of the use of laboratory animals without compromising human safety. This can be achieved by reduction, refinement or replacement of animal studies, known as the concept of the "Three Rs" (Balls *et al.*, 1995; CPMP, 1997). Consequently, there is a great interest in developing alternative methods such as *in vitro* methods and the use of 'lower' organisms as test organisms (Balls *et al.*, 1995). The development of a reliable and relevant alternative method for mucosal tolerance testing would be very useful for academic and pharmaceutical research centres to screen pharmaceuticals and ingredients. However, such a regulatory accepted alternative method is hitherto not available.

Within this scope, an alternative mucosal irritation test using invertebrates was developed at the Laboratory of Pharmaceutical Technology (Ghent University, Belgium). The terrestrial slug *Arion lusitanicus* was selected as test organism. Because slugs exposed to irritating substances produce mucus to protect the body wall, the mucus production of the slugs was selected as end point to evaluate the irritation potential of a substance. Additionally, tissue damage can be estimated by the release of proteins and enzymes from the slug body wall (Adriaens and Remon, 1999; Adriaens, 2000). A five-day procedure of the Slug Mucosal Irritation test seems to be a promising method for mucosal tolerance testing of dilutions of absorption enhancers and drugs on the one hand and for mucosal tolerance testing of neat ocular and nasal bioadhesive powder formulations on the other hand (Adriaens and Remon, 1999; Adriaens, 2000; Callens *et al.*, 2001; Ceulemans *et al.*, 2001).

When an alternative method has been developed, the relevance and the reliability of the test should be investigated using reference standards (Balls *et al.*, 1999). Because no list of reference standards intended for screening the mucosal tolerance is available, the relevance and reliability of the Slug Mucosal Irritation test were evaluated by means of eye irritation reference chemicals (Adriaens, 2000). A previous study showed that a two-day procedure enables to estimate the eye irritating and damaging properties of chemicals. The results were in agreement with the irritation data obtained after a single exposure of rabbit eyes to the chemicals (Adriaens and Remon, 2002). However, the test procedure needs to be further optimised and validated.

1.2 OBJECTIVES

On the one hand, this research aimed at the optimisation and validation of the test procedure and prediction model of the Slug Mucosal Irritation test for the evaluation of the eye irritation/damage potential of chemicals. The relevance and reliability of the developed

modified procedure and its prediction model were evaluated by using chemicals with known rabbit eye irritation data. The modified procedure was also used to evaluate the influence of the slug population and slug species on the end points of the test.

Furthermore, the five-day procedure of the Slug Mucosal Irritation test was further optimised for local tolerance testing of solid, semi-solid and liquid formulations intended for repeated administration via the ocular, buccal, nasal, rectal or vaginal route. Because no list of reference formulations intended for screening the mucosal tolerance is available, formulations with known human, animal or *in vitro* irritation data were selected for this purpose.

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CHAPTER 2: INTRODUCTION

2.1 ANIMAL USE AND LOCAL TOLERANCE TESTING

Before new chemicals, pharmaceuticals, and personal care products can be placed on the market, evaluation of their safety (such as ocular or mucosal tolerance) is required by regulatory authorities. Regulatory toxicology mainly aims at protecting public health against possible adverse effects posed by the use of pharmaceuticals and personal care products, ensuring the occupational safety of workers during the production process, and warning chemical users of potential health dangers. Regulatory authorities generally require that mucosal and ocular tolerance of products and chemicals is evaluated in vertebrates (EC, 1965; EC, 1967; EC, 1975a; EC, 1975b; EC, 1976; EC, 1990). Recent proposals for chemicals regulation in the EU will even lead to an increased use of vertebrates for the safety assessment of chemicals. The White Paper 'Strategy for a future chemicals policy' namely proposed to subject existing chemicals (marketed before 18 September 1981), for which less safety tests are required, and new chemicals (marketed after that date) to the same testing requirements. Adoption of the proposals in the White Paper would necessitate toxicity testing for around 30000 existing chemicals that are currently marketed in volumes greater than one ton per year by 2012 (EC, 2001; IEH, 2001).

Furthermore, the mucosal irritation and damage potential of chemicals or products is also assessed by academic researchers, pharmaceutical and chemical companies or research laboratories for fundamental research, product development purposes or in-house product safety evaluations. For example, the eye irritation potential is evaluated so that the workers' eyes can be adequately protected and occupational health can be ensured. The assessment of the local tolerance can be helpful in the selection of lead candidates for drug development. For this purposes, animal testing is not required by regulatory authorities (Curren and Harbell, 2002).

2.2 CONCEPT OF THE "THREE RS"

Ethical, scientific, economical and practical considerations require that the use of laboratory animals is restricted as much as possible (EC, 1986; CPMP, 1997). Consequently, there is a great interest in developing reliable and relevant alternative methods which can be used to reduce, refine or replace the use of animal experiments. The three types of alternative procedures, i.e. reduction, refinement and replacement methods, are known as the "Three Rs".

Russell and Burch (1959) defined reduction alternatives as methods which obtain comparable levels of information from the use of fewer animals, or more information from the same number of animals. Reduction of the number of animals can be achieved by the use of appropriate experimental design and statistics and by the international harmonisation of test protocols (Balls *et al.*, 1995b). Refinement alternatives alleviate or minimise potential pain, suffering and distress of the experimental animals (Russell and Burch, 1959). The animal well-being can be improved by the proper use of anaesthetics, analgesics, and tranquilisers and by the provision of optimal environmental and caging conditions (Balls *et al.*, 1995b). Replacement alternatives permit a given purpose to be achieved without using living vertebrate animals (Russell and Burch, 1959). Replacement alternatives encompass human studies, physicochemical techniques, mathematical and computer models, *in vitro* methods, assays based on foetal or embryonic forms of vertebrates, and methods that use 'lower' organisms – such as plants, micro-organisms and invertebrates – with limited sentience and/or not protected by laws regulating animal experiments (Balls *et al.*, 1995b).

It is interesting to note that a particular alternative test can serve both as partial replacement test and as reduction and refinement test. For example, an *in vitro* test can replace the use of the animal test for safety testing of certain classes of test substances on the one hand and contribute to a reduction or refinement of the animal use when it is used in a stepwise testing strategy on the other hand (Worth and Balls, 2004).

The "Three Rs" are embedded in Directive 86/609/EEC, which regulates the use of animals for experimental and other scientific purposes in the EU. The latter directive defines an experimental animal as, unless otherwise qualified, any live non-human vertebrate, including free-living larval and/or reproducing larval forms, but excluding foetal or embryonic forms, used or to be used in experiments. Moreover, the directive states that an experiment using experimental animals may not be performed, if a scientifically satisfactory alternative method is available (EC, 1986).

2.3 VALIDATION OF ALTERNATIVE TESTS

Before an alternative toxicity test is accepted by researchers and regulators, it has to be proved that the test provides a reliable level of protection which equals at least that provided by the conventional animal test (Balls *et al.*, 1990; CPMP, 1997; Liebsch and Spielmann, 2002). The validation of an alternative method has been defined as the process by which the reliability and relevance of an alternative method are established for a particular purpose (Balls *et al.*, 1990).

Archer *et al.* (1997) described that an alternative method for the (partial) replacement of an animal test consists of a test system on the one hand and a prediction model on the other hand. In case of a toxicity test, the test system generates safety related data for substances of interest, whereas the prediction model is a tool for the conversion of these data into *in vivo* toxicity predictions (Archer *et al.*, 1997). By preference, prediction models are developed based on both in-house experience and statistical methods. However, they can also be formulated on the basis of either in-house experience or statistical analysis (Worth and Balls, 2001). Classification prediction models make predictions on a categorical scale, whereas mathematical models make predictions on a continuous scale (Worth and Balls, 2001).

Consequently, validation of a (partial) replacement test involves an independent evaluation of both the test system and its prediction model (Worth and Balls, 2001). Reliability has been defined by Balls *et al.* (1990, 1995a) as establishment of the reproducibility of test results both within and between laboratories and over time. Assessment of the relevance has been defined as establishing the scientific meaningfulness and usefulness of results from an alternative method for a particular purpose (Balls *et al.*, 1990; Balls *et al.*, 1995a). In case of a replacement test method, the relevance of both the test system (scientific relevance) and its associated prediction model (predictive relevance) has to be demonstrated (Figure 2.1) (Worth and Balls, 2001). Evaluation of the predictive relevance involves the establishment of the accuracy of the predictions made by the prediction model (Worth and Balls, 2001). Evidence for the relevance of an alternative method is currently provided by demonstrating sufficient agreement between the data of the alternative method on the one hand and those obtained with the accepted *in vivo* test (Holzhütter *et al.*, 1996; CPMP, 1997) or with *in vitro* validated assays (Hothorn, 2002) on the other hand.

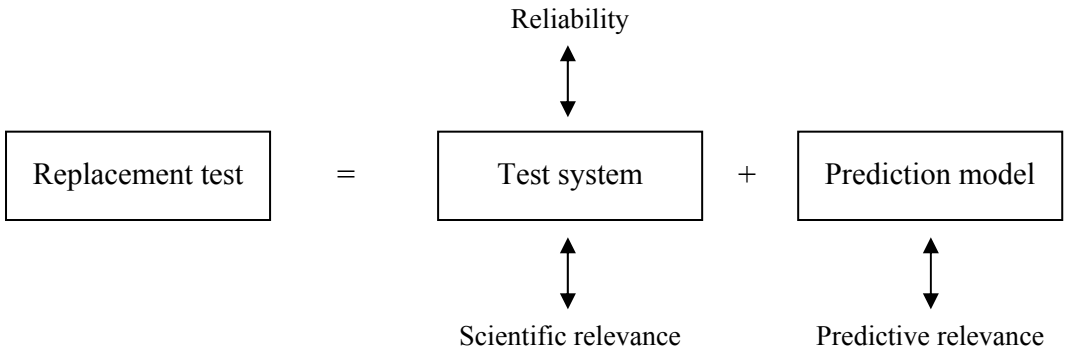

Figure 2.1 A schematic representation of a replacement test and its performance properties (based on Worth and Balls, 2001)

The relevance and the reliability of an alternative test should be investigated using reference standards, i.e. substances with a known degree of toxicity *in vivo* (Balls *et al.*, 1999). Substances are considered suitable reference standards, if they can be readily obtained in a chemically pure and stable form, they cover the entire range of *in vivo* toxicity, and they are associated with reproducible *in vivo* toxicity data of sufficient quality (Balls *et al.*, 1999). Human data would be the ideal benchmark. However, human data are collected in studies which differ with respect to their purpose, exposure conditions, and means and time points of assessing safety outcomes. Consequently, it is not easy to compare the available human data and to develop a uniform reference database based on human data (Balls *et al.*, 1990).

Balls *et al.* (1995a) stated that the following five main stages have to be successfully passed through before an alternative test is accepted by regulators: test development, prevalidation, formal validation, independent assessment, and regulatory acceptance. A prevalidation study is an inter-laboratory study aimed at refining the protocol and prediction model of the alternative test method and at investigating the relevance and reliability of the test by using a small number of reference standards. The prevalidation process consists of three successive phases. Phase I (method refinement) involves the refinement of the protocol and prediction model of a test method and the evaluation of the reproducibility of the test results in a single laboratory. During phase II (method transfer), the transferability of the method to a second laboratory is assessed and any necessary refinements to the protocol and prediction model are made. If the results of phase II are acceptable, the method can proceed to phase III (method performance) which involves the assessment of the relevance and reliability of the test under blind conditions in three or more laboratories (Curren *et al.*, 1995; Worth and Balls, 2002). In a formal validation study – also an inter-laboratory study – a larger number of

chemicals are tested under blind conditions in order to obtain a more definitive assessment of relevance and reliability (Worth and Balls, 2002). The validation of alternative methods is coordinated at EU level by the European Centre for the Validation of Alternative Methods (ECVAM) (EC, 1991). Once a method is successfully validated, the appropriate data can be submitted to an expert group for an independent assessment. If the expert group states that the criteria for test development and validation are satisfied, the validated alternative test is submitted to the EU authorities for consideration for regulatory acceptance (Worth and Balls, 2002). It is important to note that adoption of an alternative test solely by the EC will not result in a reduction in overall animal use or suffering, because chemicals and formulations will still be tested outside the EU according to other regulatory requirements. In order to be effective all over the world, the alternative test needs therefore to be adopted at the level of the Organisation for Economic Cooperation and Development (OECD) or the International Conference on Harmonisation (ICH) (IEH, 2001; Liebsch and Spielmann, 2002).

ECVAM proposed to make the validation process more flexible without compromising its high standards. For this purpose, the validation process is divided into seven independent modules (test definition, intra-laboratory variability, transferability, inter-laboratory variability, predictive capacity, applicability domain, and performance standards). The information needed for completing each module is defined. This information can be provided via a prospective or retrospective approach or via a combination of both. Because the modules are considered to be separate aspects, they can be completed at different time points. Once all the modules are satisfactorily completed, the suitability of a test to enter the peer-review process is determined (Hartung *et al.*, 2004).

Hitherto, only a few formally validated *in vitro* tests have been accepted at the EU level and incorporated into the EU official test methods. Two *in vitro* tests replacing the *in vivo* skin corrosion test (rat skin transcutaneous electrical resistance test and human skin model tests) and an *in vitro* test for phototoxicity testing (3T3 neutral red uptake phototoxicity test) (EC, 2000) are accepted at the EU level and are also adopted in the OECD Test Guidelines. Consequently, the methods are recognised internationally (OECD, 2004).

Several factors contribute to the fact that only a few alternative tests have been hitherto formally validated despite several years of intensive research and validation effort. Firstly, the conventional animal tests have not been formally validated (Balls *et al.*, 1995b). Safety related end points in animals are mostly subjectively assessed, which results in variable data (Balls *et al.*, 1999). The lack of availability of compounds with *in vivo* data of sufficiently high quality hampers the evaluation of the relevance of alternative tests (Balls *et*

al., 1995b). Furthermore, the use of the statistical approaches for the data analysis is often not appropriate. The development of protocols and prediction models of the alternative tests is also sometimes insufficient (Balls *et al.*, 1999).

It is interesting to note that a replacement alternative does not need to be formally validated in case the test is adopted for fundamental research, product development purposes or in-house product safety evaluations. The replacement test can be selected based on independent peer reviewed publications that report the test's relevance and reliability for a particular purpose (Balls *et al.*, 1995b; Balls and Fentem, 1999). For regulatory purposes, however, successful formal validation of alternative toxicity tests is required in order to ensure the protection of workers, patients, and consumers on the one hand, and the protection of the regulatory authorities themselves and of the companies involved in the production, transport and/or marketing of chemicals and products on the other hand. Furthermore, regulatory acceptance of an alternative test that afterwards points out to be not relevant and reliable would have disadvantageous consequences for further development of alternative tests (Balls and Fentem, 1999).

2.4 ALTERNATIVE TESTS FOR LOCAL TOLERANCE TESTING

2.4.1 *In vitro* tests

Freshly harvested tissue slices, cryopreserved tissues, primary cell cultures, immortalised cell lines, and reconstructed three dimensional tissue models are used *in vitro* to evaluate the irritation potential of chemicals and pharmaceuticals. Besides the saving of animals, the increasing popularity of *in vitro* tests to evaluate the local tolerance of chemicals and pharmaceuticals can be explained by the following advantages. Firstly, *in vitro* models are generally faster and more standardised than *in vivo* models (Agu *et al.*, 2002). Furthermore, only small amounts of the test substance are necessary in *in vitro* studies (Agu *et al.*, 2002). The latter is important in view of the low availability of test compounds in the drug discovery and development phase. Moreover, the use of tissues or cells from human origin can reduce the problem of data extrapolation from animals to humans (Agu *et al.*, 2002; Zucco *et al.*, 2004). Consequently, the use of *in vitro* tests during the discovery phase can result in a better and earlier selection of lead candidates for drug development and can contribute to high throughput screening (Agu *et al.*, 2002; Curren and Harbell, 2002; Vanparys, 2002).

However, complete replacement of *in vivo* methods by *in vitro* methods is not possible (Agu *et al.*, 2002; Vanparys, 2002). At present, one of the main challenges is the *in vitro* reconstruction of the complex *in vivo* tissue composition and characteristics (Quadir *et al.*, 1999; Agu *et al.*, 2002; Zucco *et al.*, 2004). For example, the normal overlaying protective mucous layer is generally absent in *in vitro* mucosal models (Merkus *et al.*, 1991; Furrer *et al.*, 2002; Lebe *et al.*, 2004). Furthermore, the isolated material and cell cultures generally have a restricted life span, so that repeated exposure to pharmaceutical compounds or formulations can not be investigated (Agu *et al.*, 2002).

An overview of the currently used *in vitro* tests for local tolerance testing of ocular, buccal, nasal, rectal and vaginal products is given in the beginning of the following chapters.

2.4.2 Slug Mucosal Irritation test

2.4.2.1 Background and principle

In order to decrease the number of vertebrates used for mucosal tolerance testing, an alternative mucosal irritation test was developed using invertebrates not protected by legislation controlling animal experiments. The terrestrial slug *A. lusitanicus* (phylum Mollusca, class Gastropoda, subclass Pulmonata, order Stylommatophora, suborder Sigmurethra, and family Arionidae) was selected as test organism, because the mucosal tissue of interest is located at the outside of the slug. Consequently, effects on the mucosal tissue caused by substances can be easily observed (Adriaens, 2000). Moreover, the slug mucosa histologically resembles the human mucosa (Adriaens, 2000). The soft, non-keratinised body wall of slugs namely comprises an outer single-layered epithelium consisting of ciliated and non-ciliated microvillous epithelial cells, mucus secreting cells, and mucous gland cells overlying connective tissue (Dyson, 1964; Lainé, 1971; Prior *et al.*, 1994). Examination of the ventral foot epithelium of terrestrial slugs revealed three longitudinal bands. The medial band is composed of ciliated microvillous epithelial cells, mucus secreting cells, and mucous glands. This band is specialised for locomotion by generating waves of muscular contractions. The lateral absorptive bands consist of mucus secreting cells, mucous glands, and microvillous epithelial cells (Prior *et al.*, 1994).

The body wall of slugs is vulnerable to mechanical or chemical damage (South, 1992). Mucus secretions serve not only for the locomotion, the lubrication of the slug body wall and the prevention of dehydration of the slug, but also for the protection of the body wall against

damage (South, 1992; Deyrup-Olsen and Luchtel, 1998). The unique properties of mucus are mainly based on the presence of the complex glycoproteins or mucins. The slug mucins are packaged in the Golgi system of very large mucus secreting cells (up to 500 µm in length) into membrane-bound granules (up to 10 µm axis) (Deyrup-Olsen *et al.*, 1983, 1992; Luchtel *et al.*, 1991; Deyrup-Olsen and Luchtel, 1998). It seems probable that chemical and mechanical stimulation of the slug results in contractions of the smooth muscle cells (situated near by the mucus secreting cells in the slug body wall) which cause in turns apocrine secretion of the mucus granules by the mucus secreting cells (Deyrup-Olsen and Martin, 1982; South, 1992; Deyrup-Olsen and Luchtel, 1998). Once the granules enter the extracellular environment and meet appropriate triggering conditions, the properties of the granule membrane change so that ions and water can flow across it. Consequently, the contained mucins hydrate and swell 100-fold or more within fractions of a second (Verdugo *et al.*, 1987; Deyrup-Olsen and Luchtel, 1998).

Several studies with the Slug Mucosal Irritation test identified the mucus production of the slugs as end point with high predictive power for the irritation potential of substances (Adriaens and Remon, 1999; Adriaens, 2000; Adriaens and Remon, 2002). Other authors also supported the mucus secretion as an indicator of irritation (Whitmore *et al.*, 1979; Morris and Wallace, 1981; Morris and Harding, 1991). Furthermore, the tissue damaging potential of the substances was studied via microscopic examination of the slug body wall, evaluation of permeability of propidium iodide, and measurement of the release of several markers like proteins, enzymes (Adriaens, 2000), prostaglandins, and interleukins (data not published) from the slug body wall. The release of proteins and enzymes is also used in various other studies to evaluate tissue damage (Whitmore *et al.*, 1979; Ichijima *et al.*, 1992; Shao and Mitra, 1992; Shao *et al.*, 1992; Krishnamoorthy *et al.*, 1995; Marttin *et al.*, 1995; Tengamnuay *et al.*, 2000). Finally, proteins, lactate dehydrogenase and alkaline phosphatase released from the body wall of the slugs were selected as the most relevant markers to evaluate mucosal damage caused by substances (Figure 2.2) (Adriaens and Remon, 1999). Damage of the single-layered epithelium and the underlying connective tissue namely results in leakage of proteins and enzymes out of the cells and eventually in leakage of haemolymph (Adriaens, 2000). In case of cell damage, the release of the cytosolic enzyme lactate dehydrogenase occurs before the release of the membrane-bound enzyme alkaline phosphatase, because alkaline phosphatase is not present in the single-layered epithelium but in the underlying connective tissue (Adriaens, 2000).

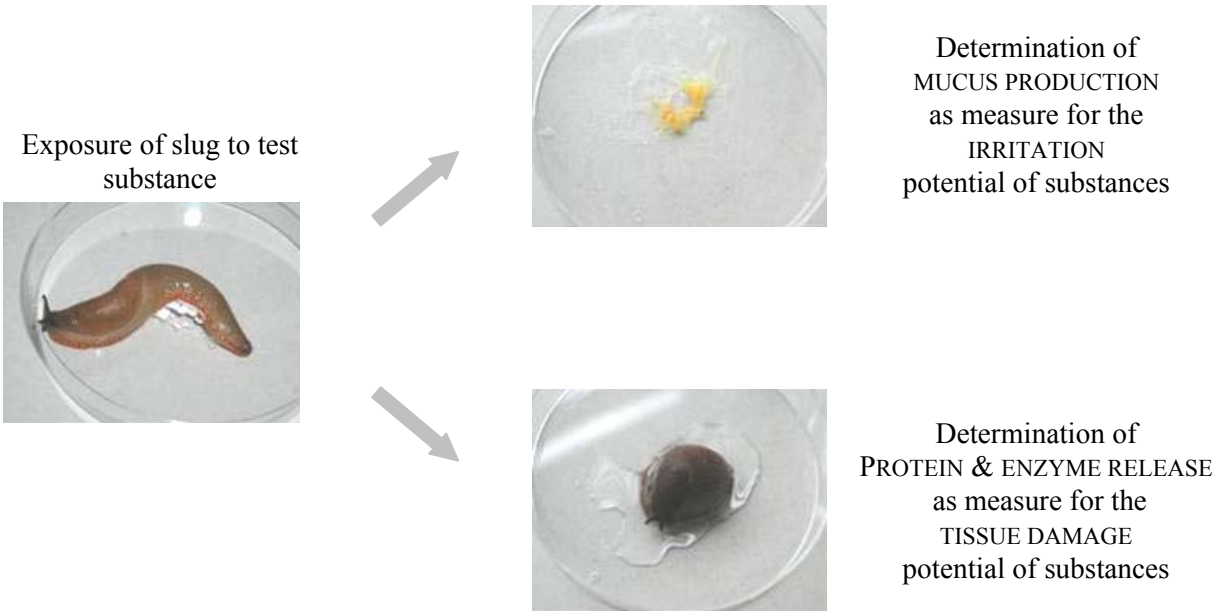

Figure 2.2 A schematic representation of the Slug Mucosal Irritation test

2.4.2.2 Test procedures

The Slug Mucosal Irritation test was developed for screening the irritation potential of absorption enhancers and drugs on mucosal surfaces. The slugs were put on 1% (w/v) dilutions of absorption enhancers, β-blocking agents or local anaesthetics for 15 minutes during five successive days (Adriaens and Remon, 1999; Adriaens, 2000). The effect on the mucosal tissue was determined from the mucus production and the reduction of the slug body weight during the contact period, and from the release of proteins and enzymes from the slug body wall after the treatment. The results of the Slug Mucosal Irritation test were in agreement with the data of various *in vivo* and *in vitro* models (Adriaens and Remon, 1999).

This five-day test procedure of the Slug Mucosal Irritation test was utilised to evaluate the mucosal tolerance of bioadhesive powder formulations. The effect of the powders on the mucosal tissue was determined by placing the slugs on the neat powder for 15 minutes during five successive days (Adriaens, 2000). The mucus production and the release of proteins and enzymes induced by a repeated treatment with the powders were used to evaluate the mucosal tolerance. However, the Slug Mucosal Irritation test underestimated the irritation potential of the irritating powders with regard to nasal irritation data obtained in rabbits. Therefore, the duration of the contact period was prolonged to 30 minutes (Dhondt, 2001). Additional data obtained with the modified test procedure were in agreement with *in vivo* rabbit and human data. Therefore, it was concluded that the modified test procedure seems to be promising to

evaluate the mucosal tolerance of powder formulations early in the development process (Callens *et al.*, 2001; Ceulemans *et al.*, 2001).

Because no list of reference standards for screening the mucosal tolerance is available, the relevance of the five-day Slug Mucosal Irritation test procedure was investigated using 15 reference chemicals for eye irritation. The chemicals were tested as 1% (w/v) dilutions for 15 minutes on five successive days. The study showed that the chemicals were accurately classified into two eye irritation categories (non-irritant and irritant) based on the mucus production during the first contact period. A concordance, sensitivity, and specificity of 87%, 83% and 100% was obtained (Adriaens, 2000). In order to reduce the number of false negatives, the contact period was prolonged to 60 minutes (personal communication E. Adriaens, Ghent University, Belgium, 2001). The modified test procedure was evaluated by means of 12 of the previous tested chemicals and 16 additional chemicals (among which nine alcohols). The irritation potential of several eye reference chemicals was correctly estimated based on the amount of mucus produced during a 60-minute contact period with a 1% (w/v) dilution of the chemical. However, treatment of the slugs with some *in vivo* irritating chemicals (especially alcohols) did not result in an increased mucus production. This was probably due to the fact that the irritating alcohols altered the mucus production by anaesthetizing the slugs. However, the latter chemicals induced an increased protein release. For severely irritating chemicals, tissue damage could be detected immediately, but for moderately irritating chemicals the process was delayed and an additional 60-minute treatment with a 1% (w/v) dilution of the test substance was needed on the next day. Using this two-day test procedure, 89% of the 28 chemicals were correctly classified into two irritation categories with a sensitivity and specificity of 88% and 92%, respectively (Adriaens and Remon, 2002).

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CHAPTER 3: EVALUATION OF THE EYE IRRITATION/ DAMAGE POTENTIAL OF CHEMICALS

Based on Adriaens, E., Dhondt, M.M.M. and Remon, J.P. (2005).

3.1 INTRODUCTION

Accidental or intentional exposure of the eye to certain chemicals can cause severe eye irritation and damage and can even result in permanent blindness (Wilhelmus, 2001). Eye irritation has been defined as the production of changes in the eye following the application of a test substance to the anterior surface of the eye, which are fully reversible within 21 days of application. Serious eye damage or corrosion has been defined as the production of tissue damage in the eye or serious physical decay of vision, following the application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application (United Nations, 2003; EC, 2004).

In order to protect the eyes of workers during the production of chemicals and to warn chemical users of potential dangers for the eye, evaluation of the eye irritation and damage potential of new chemicals is required by regulatory authorities before new chemicals can be introduced to the market (EC, 1967, EC, 2004; OECD, 2004).

3.2 OCULAR TISSUES

From ophthalmic and toxicological point of view, the cornea, conjunctiva, and iris are important and vulnerable ocular tissues. The cornea covers the iris and pupil at the anterior side of the eyeball. Because the cornea is normally transparent due to the absence of blood vessels, it allows light to pass into the eye. From anterior to posterior, the cornea is composed of the following five layers: the stratified squamous epithelium, the Bowman's membrane, the stroma, the Descemet's membrane, and the simple squamous endothelium. The epithelium and endothelium protect the intraocular contents and avoid the entrance of additional water into the cornea, which would cause swelling and opacity. In contrast with the endothelium,

the epithelium is extremely capable of regeneration and repair (Marieb, 1995; Curren and Harbell, 1998; Furrer *et al.*, 2002; Beresford, 2005).

The conjunctiva lines the inside of the eyelids and the visible part of the sclera (Marieb, 1995; Curren and Harbell, 1998). It consists of a non-keratinised stratified squamous mucous membrane containing mucus secreting cells (Marieb, 1995; Curren and Harbell, 1998; York and Steiling, 1998; Beresford, 2005). Furthermore, the conjunctiva is highly vascularized (Curren and Harbell, 1998).

The iris is located between the cornea and the lens. By varying the pupil size, the iris influences the amount of light that can enter the eye (Curren and Harbell, 1998).

3.3 EVALUATION OF THE EYE IRRITATION/DAMAGE POTENTIAL OF CHEMICALS

3.3.1 Draize rabbit eye test

At present, the only regulatory accepted method for the evaluation of eye irritation/ damage of substances requires the application of the compound of interest to the eyes of albino rabbits, as described in the OECD test guideline 405 (OECD, 2004) and in the EU official test method B5 (EC, 2004). The rabbit eye irritation test is generally referred to as the Draize eye test. In the standard version, 0.1 ml or 0.1 g of the test substance is applied to the conjunctival sac of one of rabbit' eyes, whereas the untreated eye serves as control. The test is usually performed without the use of anaesthetics. The extent of eye irritation/corrosion is evaluated by visual scoring the effects on cornea, conjunctiva, and iris at regular time points after treatment during maximum 21 days. For the cornea, the degree of opacity and corneal area involved are scored. The conjunctiva is evaluated for redness, chemosis, and discharge. The iris is examined for inflammation. The scores are then weighted and added up to give a maximum score of 110 (Draize *et al.*, 1944). The modified maximum average score (MMAS) is obtained by averaging the individual animal weighted scores at 24 hours (or more) after application of the test substance and then selecting the maximum average score (ECETOC, 1998).

Based on these scores, different eye irritation classification systems have been developed. The EU eye irritation classification system assigns the risk phrase R41 to substances that result in severe reversible changes or irreversible changes of cornea, conjunctiva or iris; whereas chemicals that cause significant (non-severe) changes of cornea, conjunctiva or iris which are reversible within 21 days are labelled R36 (EC, 2001). Recently,

the OECD, the United Nations, and other international regulatory authorities have developed a globally harmonised system (GHS) for classification and labelling of chemicals (United Nations, 2003). The GHS eye irritation classification system assigns category 1 to substances which cause lesions of the cornea, conjunctiva or iris which are not expected to reverse or are not fully reversible within an observation period of normally 21 days. Substances that have the potential to induce (non-severe) reversible eye irritation are classified as category 2. Reversible effects are further subclassified based on the duration of persistence as category 2A (reverses within 21 days, irritating to eyes) or category 2B (reverses within 7 days, mildly irritating to eyes) (United Nations, 2003). Throughout this thesis, it is focused on the EU classification, because this classification is hitherto more frequently used throughout Europe.

3.3.2 Alternative eye irritation tests

The rabbit eye irritation test is criticised because of ethical reasons for considerable time. Ocular testing of severe eye irritants is namely associated with pain and suffering of the test animals due to the fact that the eye is very sensitive. In order to reduce testing of severely eye irritating substances in rabbits and to decrease the number of animals used, a stepwise strategy for eye irritation testing and evaluation is recommended. The stepwise strategy combines evidence of eye and skin irritation/damage from existing human or animal studies and structure-activity relationship methods as well as physicochemical properties, pH data and the results of validated *in vitro* or *ex vivo* skin and eye irritation tests (United Nations, 2003; EC, 2004; OECD, 2004). Four alternative methods (bovine corneal opacity and permeability test, hen's egg test on chorio-allantoic membrane, isolated rabbit eye test and isolated chicken eye test) are accepted by a number of EU member states for the hazard identification of severe ocular irritants without animal testing (Worth and Balls, 2002). Chemicals with sufficient evidence of their eye irritation/damage potential are classified without the need to perform further tests in laboratory animals. Only for chemicals that are identified as non-irritant in all the preceding steps, the rabbit eye irritation test has to be conducted using at most three rabbits in order to confirm that the chemicals are not irritating to rabbits' eyes (United Nations, 2003; EC, 2004; OECD, 2004).

The rabbit eye irritation test is not only criticised based on ethical grounds, but also based on several scientific grounds. Firstly, the use of rabbits to evaluate eye irritation of substances may not be appropriate due to differences between rabbit and human eye anatomy and response (Wilhelmus, 2001; Curren and Harbell, 2002). Furthermore, the data obtained

with the rabbit eye irritation test are highly variable (Weil and Scala, 1971; Earl *et al.*, 1997; Ohno *et al.*, 1999) because of the subjective scoring of the tissue lesions, the spillage of test substance from the animal eyes, the inadequate number of the animals used, and the oversimplification of the overall irritation response by the use of the MMAS (York and Steiling, 1998). Moreover, the rabbit eye irritation test has not been formally validated (ICCVAM and NICEATM, 2005).

Therefore, the search for alternative eye irritation tests has been the subject of considerable research for many years. Various methods based on isolated organs (Burton *et al.*, 1981; Muir, 1984; Gautheron *et al.*, 1992; Prinsen, 1994), organotypic models (Luepke, 1985; Gilleron *et al.*, 1997), cell based tests (Borenfreund and Puerner, 1985; Pape *et al.*, 1987), tissue based models (Kahn *et al.*, 1993; Stern *et al.*, 1998; Van Goethem *et al.*, 2005), tests based on lower organisms (Kappler and Kristen, 1987; Silverman and Pennisi, 1987), and structure activity relationship models (Cronin *et al.*, 1994; Abraham *et al.*, 1998) have been developed. However, recently completed validation studies could not satisfactorily establish the scientific validity of one or more replacement tests for eye irritation (Balls *et al.*, 1995; Brantom *et al.*, 1997; Balls *et al.*, 1999). Several factors are responsible for the outcomes of these studies. The main reason is the high variability of the rabbit eye irritation test. Other possible contributing factors are the inability of *in vitro* tests to completely model the *in vivo* complexity of the eye, the use of the variable MMAS as *in vivo* end point to evaluate the validity of the alternative tests (Balls *et al.*, 1999), and the factors described (in Chapter 2) to be responsible for the low number of formally validated alternative tests.

Three alternative eye irritation tests to which the results of the Slug Mucosal Irritation test were compared in the discussion are specified below.

3.3.2.1 Bovine corneal opacity and permeability test

In the bovine corneal opacity and permeability (BCOP) test which is based on the method of Muir (1984, 1985), bovine corneas are used to assess objectively the eye irritation potential of substances (Gautheron *et al.*, 1992). After fresh collection of bovine eyes from a local slaughterhouse, the corneas are dissected and placed in special holders, which consist of an anterior and a posterior chamber. Liquids and surfactants are applied for 10 minutes to the anterior chamber of the holder as 100% and 10% dilutions, respectively, whereas solids are tested for 4 hours as 20% dilutions. After treatment, the corneas are washed and two end points are investigated, namely corneal opacity and permeability. The opacity is determined

by using an opacitometer that measures the difference in light transmission passing through a treated cornea on the one hand and a control cornea on the other hand. Immediately after the opacity measurement, the permeability of the cornea is determined by exposure of the anterior corneal side to sodium fluorescein solution for 90 minutes followed by the assessment of the optical density of the medium in the posterior chamber. Based on the results of both corneal opacity and permeability, an *in vitro* score is calculated as follows: *in vitro* score = opacity + 15 • optical density value (Vanparys *et al.*, 1993; Gautheron *et al.*, 1994; Gautheron, 1996). Substances with an *in vitro* score ≤ 25.0 are classified as non or mild irritant, substances with a score between 25.1 and 55.0 are classified as moderate irritants and substances with an *in vitro* score ≥ 55.1 are predicted as severe irritants (Gautheron *et al.*, 1994).

The regulatory authorities of France, Germany, United Kingdom, The Netherlands, Belgium, and Ireland accept the BCOP test for identifying severely eye irritants (i.e. substances labelled with risk phrase R41) (Worth and Balls, 2002). Recently, an expert panel confirmed that the BCOP assay is an accurate and reliable method for identification of ocular corrosive and severely irritating substances other than alcohols, ketones, and solids when used in a stepwise testing scheme (ICCVAM and NICEATM, 2005). The discrepancies with the *in vivo* results may be due to the fact that the BCOP assay can not predict effects on the conjunctiva (Gilleron *et al.*, 1996).

3.3.2.2 Hen's egg test on chorio-allantoic membrane

In the hen's egg test on chorio-allantoic membrane (HET-CAM) which is originally developed by Luepke (1985), test substances are placed on the vascularized non-innervated chorio-allantoic membrane of fertilized hen's eggs in order to assess their eye irritating properties. It is namely reported that the chorio-allantoic membrane can be used to predict the effects of chemical exposure on the conjunctiva (Weterings and Van Erp, 1987). To perform the test the egg shell and the inner membrane of 10-day old chicken eggs are removed, so that the chorio-allantoic membrane that separates the embryo from the air chamber becomes visible. After application of the test substance directly onto the chorio-allantoic membrane, the presence of coagulation, haemorrhage, and lysis of the membrane and its blood vessel network is examined microscopically (Gilleron *et al.*, 1997). Several HET-CAM protocols are available to enable evaluation of substances with different physicochemical properties (Spielmann, 1997). In the most frequently used reaction time method, the presence of coagulation, lysis, and haemorrhage is scored for five minutes after the application of

generally undiluted test substance (Spielmann, 1992; Gilleron *et al.*, 1997). The reaction times for each end point are combined in an *in vitro* eye irritation score, ranging from 0 to 21 (Kalweit *et al.*, 1990). Substances with an *in vitro* score < 5 are classified as non-irritants or mild irritants, whereas those with a score ≥ 5 are classified as moderate, severe or extreme eye irritants (Spielmann *et al.*, 1991).

The HET-CAM assay has been accepted by the British, French, Dutch and German authorities for the classification of severe eye irritants (Worth and Balls, 2002). An expert panel concluded that the HET-CAM has been shown to be useful for identification of severe or corrosive ocular irritants in a stepwise testing strategy. However, the panel stated that the generation of a high number of false positive results is a limiting factor (ICCVAM and NICEATM, 2005).

3.3.2.3 Reconstituted human corneal epithelial model

The reconstituted human corneal epithelial (HCE) model (SkinEthic Laboratories, Nice, France) is a three-dimensional model and consists of immortalized human corneal epithelial cells. The construct histologically resembles the stratified epithelium of the normal human corneal epithelium. Chemicals are applied directly onto the surface of the epithelial culture for 10 minutes. After treatment, the cell viability is measured using the MTT assay. Furthermore, histological sections are prepared and the release of enzymes and cytokines is determined. The classification prediction model can discriminate between eye irritants and non-irritants and is defined based on a viability cut-off value of 60%. When the viability after 10 minutes exposure is < 60%, the test chemical is considered irritant. A recent study showed that the interlaboratory reproducibility of the HCE model is good (Van Goethem *et al.*, 2005).

3.4 OBJECTIVES

Previous research demonstrated that the two-day procedure of the Slug Mucosal Irritation test can be used as an alternative for screening the eye irritation and damage potential of chemicals (e.g. in a stepwise scheme for eye irritation testing). The chemicals were first classified based on the amount of mucus produced during a 60-minute contact period with a 1% (w/v) dilution of the chemical. Chemicals that induced a mucus production higher than 6.3% of the body weight were classified as R41, whereas chemicals that induced 2.2% to 6.3% mucus production were classified as R36. Test substances that induced less than

2.2% mucus were additionally tested at a 1% (w/v) concentration on the next day and were then classified into the EU eye irritation categories based on the protein release (Adriaens and Remon, 2002). The fact that the described method needs two days for completion can be a limiting factor for high throughput screening. Therefore, the objective of this study was to reduce the test procedure to one day and to determine whether the modified assay is a relevant and reliable method to screen the eye irritation potential of test substances.

Because several *in vivo* irritating chemicals were identified based on the amount of mucus produced during a 60-minute contact period with a 1% (w/v) concentration, this step of the test procedure was maintained. Indeed, a lot of data related to the mucus production and protein and enzyme release induced by 1% (w/v) dilutions of chemicals are available. In a first phase, it was investigated if increasing the concentration of the *in vivo* irritants that were underestimated based on the mucus production, induced a faster onset on the tissue damage. To evaluate the influence of an increasing concentration on the specificity of the test, the concentration response effect of six *in vivo* non-irritating chemicals was also investigated.

Once the test concentration for the second contact period was selected, the modified test procedure was evaluated with a set of 28 reference chemicals that had already been tested in a previous study (Adriaens and Remon, 2002). Phosphate buffered saline (PBS) and benzalkonium chloride were respectively used as negative and positive control like in previous studies. Indeed, incubation of freshly excised pig corneas in PBS did not change the corneal cell structure and tissue integrity (Berdy *et al.*, 1992; Baydoun *et al.*, 2004). Furthermore, treatment of the slugs with PBS caused neither irritation nor tissue damage as was demonstrated by the low mucus production and protein release (Adriaens and Remon, 1999; Adriaens, 2000; Adriaens *et al.*, 2001; Adriaens and Remon, 2002). Benzalkonium chloride was selected as a positive control, because the toxicity of benzalkonium chloride to the eye is demonstrated in *in vivo* and *in vitro* eye irritation tests (Vanparys *et al.*, 1993; Gilleron *et al.*, 1997; ECETOC, 1998; Van Goethem *et al.*, 2005). Benzalkonium chloride is also used as positive control in other alternative eye irritation tests such as the HET-CAM (Gilleron *et al.*, 1997). Moreover, treatment of the slugs with this chemical resulted in severe irritation and damage of the slug mucosa as was demonstrated by an increased mucus production and the release of proteins and enzymes from the slug body wall (Adriaens and Remon, 1999; Adriaens, 2000; Adriaens *et al.*, 2001; Adriaens and Remon, 2002).

Based on the end points of the Slug Mucosal Irritation test on the one hand and on the eye irritation categories derived from the rabbit eye irritation data on the other hand, a classification prediction model was developed to classify the chemicals into the EU eye

irritation categories. It was not opted to develop a mathematical prediction model to predict the MMAS score of the chemicals, because the MMAS scores do not take into account the irreversibility of the effects in individual eye tissues. Furthermore, the MMAS values are variable, especially in the middle part of the irritation scale (Prinsen, 1999).

Next, the reliability and the relevance of the modified test procedure and prediction model were assessed. For this purpose, the chemicals were tested on five separate occasions. Both the intra-laboratory reproducibility of the test results and the intra-laboratory reproducibility of the EU classifications obtained with the Slug Mucosal Irritation test were determined. The results obtained with the Slug Mucosal Irritation test were compared with the EU classification obtained with the rabbit eye irritation test and the concordance, specificity, sensitivity, positive and negative predictive values were determined.

Additionally, the relevance of the modified assay was evaluated by means of 12 reference chemicals that were not used for the development of the prediction model. Only those chemicals were selected with sufficient and as much as possible unambiguous data from animal and *in vitro* models. Furthermore, the selected test chemicals were distributed uniformly across the EU eye irritation categories.

3.5 MATERIALS AND METHODS

3.5.1 Chemicals

The 28 reference chemicals used for the optimisation and evaluation of the test procedure and prediction model and the 12 reference chemicals used for the evaluation of the relevance of the modified assay are presented in Tables 3.1 and 3.2, respectively. The chemicals belong to various chemical classes. They cover the entire irritancy range and were selected from the eye irritation reference chemicals data bank. This data bank contains the individual rabbit eye irritation scores generated according to OECD Test Guideline 405 as well as the MMAS (ECETOC, 1998).

The individual rabbit eye irritation data (ECETOC, 1998) were used to classify the chemicals into the EU eye irritation classes (Prinsen, 1999; EC, 2001) and the GHS eye irritation classes (United Nations, 2003). Chemicals which could not be classified as irritant or corrosive to the eye were classified as non-irritant (NI). All of the chemicals classified as R41 were also classified as category 1. With the exception of one chemical, all of the chemicals classified as R36 were also classified as category 2. Sodium lauryl sulphate was

classified as R36 on the basis of the EU criteria, whereas it was classified as category 1 on the basis of the GHS criteria. Furthermore, 2-methyl-1-pentanol and ethanol were classified as NI on the basis of the EU criteria, but as category 2B on the basis of the GHS criteria.

Table 3.1 Eye reference chemicals selected for the optimisation and evaluation of the test procedure and prediction model

Test substance	Chemical information			Draize eye test				
	CASRN	Supplier	Purity (%)	Conc (%)	MMAS	EU class	GHS class	n
PBS		Sigma						
3,3-Dimethylpentane	562-49-2	Aldrich	99	100	0.0	NI	NI	3
PEG 400	25322-68-3	α Pharma	-	100	0.0	NI	NI	6
3-Methoxy-1,2-propanediol	623-39-2	Aldrich	98	100	0.0	NI	NI	3
Propylene glycol	57-55-6	Fraver	-	100	1.3	NI	NI	6
Glycerol	56-81-5	Sigma	99	100	1.7	NI	NI	6
PEG 600	25322-68-3	Merck	-	100	2.0	NI	NI	6
Methylcyclopentane	96-37-7	Aldrich	98	100	3.7	NI	NI	6
Tween 20®	9005-64-5	α Pharma	-	100	4.0	NI	NI	4
Methyl isobutyl ketone	108-10-1	Aldrich	> 99	100	4.8	NI	NI	4
Toluene	108-88-3	Vel	99	100	9.0	NI	NI	4
2-Methyl-1-pentanol	105-30-6	Aldrich	99	100	13.0	NI	Cat 2B	3
Ethanol	64-17-5	Merck	99.2	100	24.0	NI	Cat 2B	3
Ammonium nitrate	6484-52-2	Sigma	> 99.5	100	18.3	R36	Cat 2B	3
1-Octanol	111-87-5	Aldrich	99	100	41.0	R36	Cat 2B	3
4-Carboxybenzaldehyde	619-66-9	Aldrich	> 97	100	50.3	R36	Cat 2A	3
2-Ethyl-1-hexanol	104-76-7	Aldrich	> 99	100	51.3	R36	Cat 2B	4
Sodium lauryl sulphate	151-21-3	Sigma	99	15	59.2	R36	Cat 1	6
1-Hexanol	111-27-3	Fluka	≥ 99	100	64.8	R36	Cat 2A	4
Acetone	67-64-1	Vel	99	100	65.8	R36	Cat 2A	4
Triton X-100®	9002-93-1	Sigma	> 99	10	68.7	R36	Cat 2A	6
p-Fluoroaniline	371-40-4	Aldrich	99	100	69.8	R36	Cat 2	6
Lauric acid	143-07-7	Merck	> 99	100	38.0	R41	Cat 1	3
Imidazole	288-32-4	Sigma	99	100	59.3	R41	Cat 1	3
Sodium oxalate	62-76-0	Fluka	≥ 99	100	61.3	R41	Cat 1	3
Cyclohexanol	108-93-0	Aldrich	99	100	79.8	R41	Cat 1	4
Cetylpyridinium bromide	140-72-7	Sigma	> 99	10	89.7	R41	Cat 1	6
Sodium hydroxide	1310-73-2	Vel	≥ 98	10	108.0	R41	Cat 1	1
Benzalkonium chloride	8001-54-5	Sigma	> 98	10	108.0	R41	Cat 1	3

CASRN, Chemical Abstract Service Registry Number; Cat 1, irreversible effects on eyes; Cat 2, reversible effects on eyes; Cat 2A, irritating to eyes; Cat 2B, mildly irritating to eyes; MMAS, Modified Maximum Average Score (ECETOC, 1998); NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes.

Table 3.2 Additional eye reference chemicals selected for the evaluation of the relevance of the modified assay

Test substance	Chemical information			Draize eye test				
	CASRN	Supplier	Purity (%)	Conc (%)	MMAS	EU class	GHS class	n
Potassium tetrafluoroborate	14075-53-7	Aldrich	> 99.99	100	0.0	NI	NI	3
Tetra-aminopyrimidine sulphate	5392-28-9	Aldrich	97	100	10.3	NI	NI	3
Methyl amyl ketone	110-43-0	Aldrich	99	100	10.5	NI	NI	4
Ethyl acetate	141-78-6	Aldrich	> 99.5	100	15.0	NI	NI	4
Methyl acetate	79-20-9	Aldrich	99	100	39.5	R36	Cat 2B	4
Isopropanol	67-63-0	Aldrich	> 99.5	100	30.5	R36	Cat 2B	4
γ-Butyrolactone	96-48-0	Fluka	≥ 99	100	43.0	R36	Cat 2B	3
1-Butanol	71-36-3	Merck	≥ 99	100	60.8	R36	Cat 2B	4
Pyridine	110-86-1	Fluka	≥ 99.8	100	48.0	R41	Cat 1	3
Promethazine hydrochloride	58-33-3	Sigma	98	100	71.7	R41	Cat 1	3
Chlorhexidine	55-56-1	Aldrich	98	100	82.3	R41	Cat 1	3
Trichloroacetic acid	76-03-9	Sigma	99	30	106	R41	Cat 1	1

CASRN, Chemical Abstract Service Registry Number; Cat 1, irreversible effects on eyes; Cat 2B, mildly irritating to eyes; Conc, concentration; MMAS, Modified Maximum Average Score (ECETOC, 1998); NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes.

The dilutions of the chemicals were prepared in phosphate buffered saline (PBS, pH 7.4; Sigma, St. Louis, MO, USA). PBS and benzalkonium chloride were respectively used as negative and positive control.

3.5.2 Eye irritation/damage test procedure of the Slug Mucosal Irritation test

The parenteral slugs of *A. lusitanicus* were collected in local gardens along Varsenare and Aalter (Belgium). The slugs were bred in plastic containers in the laboratory at 18-22°C and fed with lettuce, cucumber, carrots, and commercial dog food. The slugs were carefully examined and those presenting defects, such as a damaged body wall, were discarded. Slugs weighing between 3 g and 6 g were isolated two days before the start of an experiment and placed in a plastic box lined with a paper towel (moistened with PBS) at 18-22°C.

The concentration response effect of 13 reference chemicals on the mucosal tissue of the slugs was investigated. Dilutions ranging from 1% to 10% (w/v) prepared in PBS were tested. The slugs on the one hand and the Petri dishes containing a membrane filter (cellulose acetate 0.45 µm, 90 mm; Sartorius AG, Goettingen, Germany) moistened with 2 ml of the test medium on the other hand were weighed at the beginning of the experiment. Subsequently,

the slugs were placed individually during 60 minutes in a Petri dish on the test medium. For each concentration, five slugs were used. After this contact period, the amount of mucus produced was measured by reweighing the Petri dishes containing the test medium (without the slugs). The mucus production was expressed as percentage (w/w) of the body weight. Next, the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. One hour later, the PBS samples were collected with a micropipette. The samples were analysed immediately for the presence of proteins, lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) released from the slug body wall.

In the next steps, 28 reference chemicals and 12 additional reference chemicals were tested using the modified test procedure. Each experiment contained five negative control slugs (PBS), five positive control slugs (benzalkonium chloride), and six series of five slugs each treated with the test chemicals. At the beginning of the experiment, the slugs on the one hand and the Petri dishes containing a membrane filter moistened with 2 ml of a 1% (w/v) dilution of the test chemical on the other hand were weighed. Subsequently, the slugs were placed individually during 60 minutes in a Petri dish on the test medium. After this first contact period, the mucus production was measured, the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. One hour later, the PBS samples were collected with a micropipette. Two hours after the end of the first contact period, the slugs were placed during 60 minutes in a Petri dish on a membrane filter moistened with 2 ml of the more concentrated dilution of the chemical. After this second contact period, the mucus production was measured, the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. The PBS samples were collected after 60 minutes. The samples were analysed immediately for the presence of proteins, LDH, and ALP released from the body wall of the slugs.

3.5.3 Analytical procedures

3.5.3.1 Protein determination

The protein concentration in the samples was determined with a NanoOrange® protein quantitation kit (Molecular Probes, Leiden, The Netherlands). The NanoOrange® reagent allows accurate detection of proteins in solution at concentrations between 10 ng/ml and 10 µg/ml. The NanoOrange® working solution was prepared by diluting 500-fold the NanoOrange® protein quantitation reagent (component A) in NanoOrange® diluent. The NanoOrange® diluent was made by diluting NanoOrange® component B 10-fold in distilled

water. The protein concentration in each sample was determined by adding 3 µl sample to 247 µl working solution in a 96-well plate (polystyrene, OptiPlate™-96F, PerkinElmer, Zaventem, Belgium). In order to convert fluorescence to µg/ml protein and to control for day-to-day variation in the performance of the fluorometer, also ten standards covering the full assay range (0-10 µg/ml) were prepared. The standards were prepared in a 96-well plate using bovine serum albumin (NanoOrange® component C, 2 mg/ml) and working solution. Immediately after preparation, the 96-well plates with samples and standards were covered with aluminium foil and heated to 90-96°C for 10 minutes. After cooling down the standards and samples to room temperature, fluorescence measurements were carried out on a fluorometer (Wallac 1420 multilabel counter Victor 2, PerkinElmer, Turku, Finland) using excitation/emission wavelengths of 485/590 nm. The protein release of the slugs was expressed as µg/ml per gram body weight.

3.5.3.2 Lactate dehydrogenase determination

The lactate dehydrogenase activity (LDH, EC 1.1.1.27) was measured using commercial kits (DG 1340-UV, Sigma Diagnostics, St. Louis, MO, USA and LDH/HBDH 2.8, ABX Diagnostics, Montpellier, France). LDH catalyzes the interconversion of pyruvate and lactate. During reduction of pyruvate, an equimolar amount of NADH is oxidized to NAD^+. The oxidation of NADH results in a decrease in the absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directionally proportional to LDH activity in the sample. The LDH reagents measure the enzyme activity using an optimised method based on the standard method recommended by the German Society for Clinical Chemistry (DGKC, 1972). One international unit (IU) of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol/l of NAD^+ per minute under the conditions of the assay. The LDH activity measurements were conducted at 37°C on a Cobas Plus analyser (ABX, Brussels, Belgium) of which the performance was daily checked with standard solutions (Accutrol™ Normal, Sigma Diagnostics, St. Louis, MO, USA and N-Control, ABX Diagnostics, Montpellier, France). The detection limit of the method was 4 IU/l. The LDH release of the slugs was expressed as IU/l per gram body weight.

3.5.3.3 Alkaline phosphatase determination

The alkaline phosphatase activity (ALP, EC 3.1.3.1) was measured using commercial kits (DG 1245-UV, Sigma Diagnostics, St. Louis, MO, USA and ALP 6, ABX Diagnostics, Montpellier, France). ALP hydrolyzes *p*-nitrophenyl phosphate to *p*-nitrophenol and inorganic phosphate. The hydrolysis occurs at alkaline pH and the *p*-nitrophenol formed shows an absorbance maximum at 405 nm. The rate of increase in absorbance at 405 nm is directly proportional to ALP activity in the sample. The ALP reagents of Sigma Diagnostics measure the enzyme activity using an optimised method based on the standard method recommended by the German Society for Clinical Chemistry (DGKC, 1972), whereas the ALP reagents of ABX Diagnostics measure the enzyme activity using an optimised method based on the method recommended by the French Clinical Biology Society (Mathieu *et al.*, 1982). One international unit (IU) of ALP activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol/l of *p*-nitrophenol per minute under the conditions of the assay. The ALP activity measurements were conducted at 37°C on a Cobas Plus analyser (ABX, Brussels, Belgium) of which the performance was daily checked with standard solutions (Accutrol™ Normal, Sigma Diagnostics, St. Louis, MO, USA and N-Control, ABX Diagnostics, Montpellier, France). The detection limit of the method was 4 IU/l. The ALP release of the slugs was expressed as IU/l per gram body weight.

3.5.4 Data analysis

For each slug, the mucus production during each contact period and the protein, LDH, and ALP release after each contact period were calculated. These data were used for the statistical analyses (SPSS version 12.0; SPSS, Chicago, IL, USA). A probability value (p) < 0.05 was considered statistically significant.

Pearson correlations between the end points of the Slug Mucosal Irritation test and the MMAS of the Draize eye test were determined in order to select the most predictive end points. Based on these end points, a classification prediction model for the determination of the EU eye irritation category of chemicals was developed. For this purpose, the data of the Slug Mucosal Irritation test on the one hand and the EU eye irritation categories based on the rabbit eye irritation data on the other hand were analysed using linear discriminant analysis and cut-off values for the classification of the chemicals were calculated. Various random subsets of the Slug Mucosal Irritation test data were used to calculate cut-off values. On the

basis of these results and in-house experience, cut-off values were established and the chemicals were classified into one of the corresponding EU categories.

The repeatability of the test results and the classifications obtained in the five runs was evaluated using various statistical methods as suggested by Balls *et al.* (1995), Bruner *et al.* (1996), Holzhütter *et al.* (1996), and Fentem *et al.* (2001). Firstly, the coefficients of variation were calculated. Ideally, the overall mean coefficient of variation should be < 30% (Fentem *et al.*, 2001). Furthermore, statistically significant differences between the test results obtained in the repeated experiments were determined for each chemical using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal, the data were transformed to their logarithm. To compare further the effects of the repeated experiments, a multiple comparison among pairs of means was performed with a Scheffé test. The repeatability of the test results was also assessed using the Pearson correlation coefficient and the slope of the linear regression line. The agreement between the predicted categories of the repeated experiments was evaluated using the κ-statistics. A κ-value of 0.75 or higher suggests strong agreement above chance (Fleiss, 1981).

The relevance of the assay was evaluated using several statistical methods as proposed by Balls *et al.* (1990), Holzhütter *et al.* (1996), and Hothorn (2002). The extent of agreement between the eye irritation categories predicted by the alternative test and the categories assigned on the basis of the Draize eye test was assessed by constructing contingency tables. For this purpose, the overall predicted category for each chemical was used. If there was any discrepancy between the categories obtained in the different runs, then the category obtained in three or four runs was used. Concordance, sensitivity, and specificity were calculated as defined by Cooper *et al.* (1979). Concordance is the number of compounds correctly classified by the alternative test, divided by the total number of compounds tested. Sensitivity (evaluation of false negatives) is the total number of irritants correctly classified by the alternative test, divided by the total number of *in vivo* irritants tested. Specificity (evaluation of false positives) is the total number of non-irritants correctly predicted by the alternative test, divided by the total number of *in vivo* non-irritants tested. Fentem *et al.* (2001) stated that the sensitivity and specificity should be > 60%. Furthermore, the positive predictive value (likelihood that a positive outcome in the alternative test correctly identifies an irritant) and the negative predictive value (likelihood that a negative outcome in the alternative test correctly identifies a non-irritant) were calculated. The positive predictive value is the total number of irritants correctly classified by the alternative test, divided by the total number of

irritants identified by the alternative test. The negative predictive value is the total number of non-irritants correctly classified by the alternative test, divided by the total number of non-irritants identified by the alternative test. To determine the sensitivity, specificity, positive and negative predictive value of the alternative test, the chemicals were divided into two classes: non-irritant (NI) and irritant (R36 and R41).

In order to provide criteria for test validity, acceptance criteria for the negative and positive control were calculated as defined by Hahn and Meeker (1991).

3.6 RESULTS

3.6.1 Modification of the test procedure using concentration response experiments

Historical data from the negative control (45 experiments) showed that when slugs are placed during 60 minutes on PBS, the amount of mucus is typically less than 2% of the body weight. Furthermore, negative control slugs showed low protein levels after the contact period (less than 50 µg/ml.g) and no LDH release was detected. Therefore, chemicals that induce a response that is comparable to the negative control slugs are considered to be non-irritant. Taking this into account, the concentration response effect of several *in vivo* irritants that were underestimated by the mucus production and of some *in vivo* non-irritating chemicals was investigated. 1%, 2.5%, 5% and 10% (w/v) dilutions of each chemical were tested. Because an increasing concentration of the chemicals influenced especially the mucus production and protein release, these data are presented in Table 3.3 and Table 3.4, respectively. The data of the ALP release are not mentioned, because ALP release was only induced after treatment with 5% or 10% (w/v) dilutions of a few chemicals.

PEG 400 and lauric acid induced no concentration response effect, the mucus production and protein release were low at all tested concentrations and no LDH release was detected. Increasing concentrations of glycerol and propylene glycol resulted in an increased mucus production (> 4% of the body weight) which is an indication of irritation, this effect started at a 5% and a 10% (w/v) concentration, respectively. However, the protein levels were low and no LDH release was detected indicating that these chemicals induced no tissue damage at the tested concentrations. Tween 20® had no effect on the mucus production and the LDH release was below the detection limit, while a concentration response effect was present for the protein release (low to moderate release). A 10% (w/v) dilution of ethanol induced severe irritation (increased mucus production, protein and LDH release), whereas

concentrations of 5% (w/v) and lower had no effect. No concentration response effect was found on the mucus production for 2-ethyl-1-hexanol, 1-hexanol, and cyclohexanol, while 1-octanol induced an increased mucus production from a 2.5% (w/v) concentration on. A 2.5% (w/v) dilution of acetone and a 10% (w/v) dilution of toluene resulted in an increased mucus production (> 6% of the body weight), whereas other concentrations had no effect on this end point. All those chemicals induced a concentration dependent tissue damage. Acetone and cyclohexanol resulted in tissue damage at concentrations of 5% (w/v) and higher, whereas toluene, 1-octanol, 2-ethyl-1-hexanol, and 1-hexanol resulted in tissue damage starting at a 2.5% (w/v) concentration. For benzalkonium chloride, maximum mucus production, protein and LDH release data were obtained at a 2.5% (w/v) concentration.

From the results of the concentration response experiments, it is clear that all *in vivo* irritating chemicals, except lauric acid, can be identified based on the protein and LDH release when they are tested as a 5% (w/v) dilution. Therefore, it was decided to select a 5% (w/v) concentration for the second contact period. However, preliminary experiments showed that the specificity of the test decreased. Consequently, the test concentration of the second contact period was reduced to 3.5% (w/v).

Table 3.3 Concentration response effect of the reference chemicals on the mucus production as assessed with the Slug Mucosal Irritation test

Test substance	Draize eye test		Slug Mucosal Irritation test Mucus production (%) [a]			
	EU class	MMAS	1.0%	2.5%	5.0%	10.0%
PEG 400	NI	0.0	-1.3 ± 1.3	-1.2 ± 0.8	-1.1 ± 0.5	1.2 ± 3.0
Propylene glycol	NI	1.3	1.4 ± 2.3	1.9 ± 1.7	1.1 ± 1.8	4.3 ± 3.2
Glycerol	NI	1.7	2.3 ± 2.4	2.9 ± 4.8	5.4 ± 2.1	6.7 ± 5.5
Tween 20®	NI	4.0	0.1 ± 1.2	1.0 ± 2.0	-1.4 ± 0.7	-0.9 ± 0.5
Toluene	NI	9.0	-0.5 ± 1.2	2.4 ± 5.0	2.2 ± 2.3	10.6 ± 12.6
Ethanol	NI	24.0	-0.5 ± 1.1	-1.1 ± 0.8	-0.7 ± 1.6	9.4 ± 3.4
1-Octanol	R36	41.0	-1.2 ± 1.4	3.7 ± 3.5	13.5 ± 6.5	6.5 ± 6.3
2-Ethyl-1-hexanol	R36	51.3	-0.6 ± 0.7	1.0 ± 1.3	0.6 ± 0.6	1.3 ± 0.7
1-Hexanol	R36	64.8	0.5 ± 1.0	1.0 ± 2.0	1.0 ± 0.5	2.0 ± 2.0
Acetone	R36	65.8	0.4 ± 2.6	6.5 ± 2.0	2.0 ± 0.8	1.5 ± 2.4
Lauric acid	R41	38.0	-1.9 ± 1.9	-1.3 ± 2.1	-1.1 ± 0.8	0.1 ± 1.7
Cyclohexanol	R41	79.8	0.6 ± 0.6	0.6 ± 0.8	-0.3 ± 0.9	0.6 ± 1.4
Benzalkonium chloride	R41	108.0	20.3 ± 9.5	30.4 ± 5.1	32.3 ± 6.6	31.1 ± 4.1

MMAS, modified maximum average score (ECETOC, 1998); NI, non-irritant; R36, irritating to eyes, R41, risk of serious damage to eyes.

[a] Values are the mean ± standard deviation of 5 slugs.

Table 3.4 Concentration response effect of the reference chemicals on the protein release as assessed with the Slug Mucosal Irritation test

Test substance	Draize eye test		Slug Mucosal Irritation test Protein release (µg/ml.g) [a]			
	EU class	MMAS	1.0%	2.5%	5.0%	10.0%
PEG 400	NI	0.0	9 ± 12	12 ± 15	7 ± 2	1 ± 1
Propylene glycol	NI	1.3	13 ± 13	15 ± 10	46 ± 71	12 ± 11
Glycerol	NI	1.7	21 ± 23	8 ± 4	9 ± 5	35 ± 45
Tween 20®	NI	4.0	37 ± 29	65 ± 47	67 ± 36	83 ± 33
Toluene	NI	9.0	8 ± 8	141 ± 104	149 ± 97	135 ± 108
Ethanol	NI	24.0	3 ± 3	14 ± 21	7 ± 3	210 ± 203
1-Octanol	R36	41.0	50 ± 72	255 ± 102	420 ± 113	295 ± 49
2-Ethyl-1-hexanol	R36	51.3	58 ± 25	76 ± 11	75 ± 37	136 ± 30
1-Hexanol	R36	64.8	4 ± 4	92 ± 69	99 ± 48	167 ± 142
Acetone	R36	65.8	18 ± 18	18 ± 20	482 ± 206	488 ± 65
Lauric acid	R41	38.0	19 ± 19	13 ± 6	17 ± 20	12 ± 7
Cyclohexanol	R41	79.8	14 ± 6	17 ± 22	67 ± 60	166 ± 29
Benzalkonium chloride	R41	108.0	323 ± 207	490 ± 134	628 ± 183	522 ± 52

MMAS, modified maximum average score (ECETOC, 1998); NI, non-irritant; R36, irritating to eyes, R41, risk of serious damage to eyes.

[a] Values are the mean ± standard deviation of 5 slugs.

3.6.2 Validation of the modified test procedure

3.6.2.1 Evaluation of the modified test procedure

Table 3.5 summarizes the data obtained for the 28 reference chemicals and the negative control. The ALP release data are not mentioned, because ALP release was only induced after the first contact period for one out of the 28 reference chemicals (imidazole) and after the second contact period for seven out of the 28 reference chemicals.

Table 3.5 Irritation potential of the reference chemicals as assessed with the Draize eye test and the Slug Mucosal Irritation test

Test substance	Draize eye test		Slug Mucosal Irritation test						
	EU class	MMAS	1st 60-min. contact period [a]			2nd 60-min. contact period [b]			n
			Mucus (%)	Protein (µg/ml.g)	LDH (IU/l.g)	Mucus (%)	Protein (µg/ml.g)	LDH (IU/l.g)	
PBS			-1.3	19	-	-1.9	18	-	25
3,3-Dimethylpentane	NI	0.0	-2.6	13	-	0.8	24	-	5
PEG 400	NI	0.0	-0.1	8	-	-0.9	11	-	5
3-Methoxy-1,2-propanediol	NI	0.0	-0.5	24	-	-0.1	10	-	5
Propylene glycol	NI	1.3	-1.4	11	-	-0.4	17	-	5
Glycerol	NI	1.7	0.8	13	-	0.3	11	-	5
PEG 600	NI	2.0	-2.4	31	-	-1.7	12	-	5
Methylcyclopentane	NI	3.7	-0.4	21	-	-1.7	18	-	5
Tween 20®	NI	4.0	-2.6	24	-	-1.3	56	-	5
Methyl isobutyl ketone	NI	4.8	3.1	313	-	6.2	47	0.4	5
Toluene	NI	9.0	-1.9	20	-	-0.7	208	-	5
2-Methyl-1-pentanol	NI	13.0	0.5	5	-	-1.2	194	9.0	5
Ethanol	NI	24.0	-0.1	27	-	-4.0	10	-	5
Ammonium nitrate	R36	18.3	4.3	29	-	15.4	277	2.8	5
1-Octanol	R36	41.0	5.6	172	1.1	3.6	259	13.2	5
4-Carboxybenzaldehyde	R36	50.3	2.8	45	-	4.6	38	2.6	5
2-Ethyl-1-hexanol	R36	51.3	-0.1	78	1.5	-3.4	238	5.8	5
Sodium lauryl sulphate	R36	59.2	19.0	67	-	12.2	437	11.9	5
1-Hexanol	R36	64.8	-2.6	14	-	-2.4	230	8.7	5
Acetone	R36	65.8	0.0	97	-	-1.3	122	-	5
Triton X-100®	R36	68.7	13.0	44	-	15.0	251	8.3	5
p-Fluoroaniline	R36	69.8	2.0	29	-	-4.0	53	3.1	5
Lauric acid	R41	38.0	-1.8	15	-	-3.8	30	-	5
Imidazole	R41	59.3	6.1	270	-	22.8	415	3.9	5
Sodium oxalate	R41	61.3	12.5	178	6.6	20.4	255	21.9	5
Cyclohexanol	R41	79.8	0.7	103	-	-3.4	63	1.5	5
Cetylpyridinium bromide	R41	89.7	13.8	177	0.9	5.7	370	9.6	5
Sodium hydroxide	R41	108.0	15.8	80	-	6.4	88	0.9	5
Benzalkonium chloride	R41	108.0	20.2	282	8.2	8.2	496	66.5	25

MMAS, modified maximum average score (ECETOC, 1998); NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes; -, below the detection limit.

[a] Treatment with a 1% (w/v) dilution of the chemical.

[b] Treatment with a 3.5% (w/v) dilution of the chemical.

The results of the Slug Mucosal Irritation test were compared to the EU categories. Because nine out of the 16 *in vivo* irritants induced an increased mucus production (> 4%) during the first contact period with a 1% (w/v) dilution of the test substance, this end point can be used as a first step to discriminate between irritants and non-irritants. However, seven *in vivo* irritating chemicals resulted in a mucus production that was comparable to the negative controls. Three of those chemicals resulted in some tissue damage after the first contact period and by increasing the test concentration during the second contact period to 3.5% (w/v), six out of the seven *in vivo* irritants that were underestimated by the mucus production were detected based on the protein and LDH release. Only the *in vivo* irritating lauric acid induced neither mucus production nor increased release of proteins or enzymes. The amount of mucus secreted during the second contact period did not give any additional information. However, the modified assay also affected the false positive results. Eleven of 12 *in vivo* non-irritants resulted in a mucus production that was comparable to the negative control slugs and nine *in vivo* non-irritants did not result in tissue damage and can therefore be considered non-irritant. One *in vivo* non-irritant (methyl isobutyl ketone) induced a slight increase of the mucus production as well as an increased protein release after the first contact period. Two other *in vivo* non-irritants (toluene and 2-methyl-1-pentanol) induced tissue damage after the second contact period.

3.6.2.2 Development of prediction model

The results obtained with the Slug Mucosal Irritation test were compared with the MMAS values and with the EU categories based on the rabbit eye irritation data (ECETOC, 1998; EC, 2001). Based on statistical analyses and in-house experience, it was decided to work out a prediction model that classifies the chemicals first based on the amount of mucus produced during the first contact period with a 1% (w/v) dilution of the chemical. Chemicals that induced ≤ 3% mucus production are tested during a second contact period with a 3.5% (w/v) dilution of the chemical. Those compounds are then classified based on the tissue damage which is expressed as a score. The score for tissue damage takes into account the protein release after the first contact period (P1), and the protein and LDH release after the second contact period (P2, LDH2) and is calculated as follows: P1 + P2 + 30 • LDH2. The classification prediction model is presented in Figure 3.1.

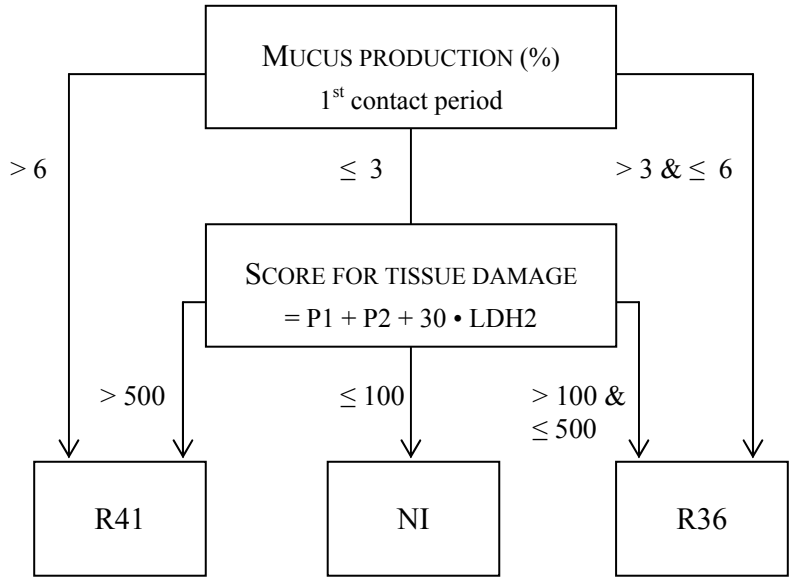

Figure 3.1 Classification prediction model of the Slug Mucosal Irritation test for the determination of the EU eye irritation category of chemicals using *A. lusitanicus* as test organism (P1 and P2, protein release after 1st and 2nd contact period; LDH2, LDH release after 2nd contact period; NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes).

3.6.2.3 Reliability of the modified test procedure and prediction model

In order to investigate the repeatability of the modified procedure, all chemicals were tested independently on five separate occasions. Tables 3.6 and 3.7 show for each of the 28 reference chemicals the mucus productions (of the first contact period) and the scores for tissue damage obtained in the repeated experiments and the mean of the five runs. Because several compounds induced a mucus production that was close to zero, the coefficients of variation for the end point mucus production were large and misleading. Consequently, it was decided to not present the coefficients of variation. For each chemical, a one-way ANOVA test was performed to detect statistically significant differences between the test results obtained in the five runs. For 16 out of the 28 test substances, significantly different mucus productions were detected in the five runs ($p < 0.05$). However, only for four out the latter 16 substances, the average mucus production of the five runs was more than 1%. For seven out of the 28 test substances, significantly different scores were detected in the five runs ($p < 0.05$).

Table 3.6 Intra- and inter-experiment variability for the mucus production (%)

Test substance	Run 1	Run 2	Run 3	Run 4	Run 5	**Average**
3,3-Dimethylpentane	-2.6 ± 1.5 [a]	-1.5 ± 1.2 [a, b]	-0.1 ± 0.6 [b]	-0.1 ± 0.6 [b]	0.0 ± 1.3 [b]	**-0.8 ± 1.2**
PEG 400*	-0.1 ± 1.1	0.5 ± 0.9	1.4 ± 1.7	0.7 ± 0.9	1.3 ± 0.7	**0.8 ± 0.7**
3-Methoxy-1,2-propanediol	-0.5 ± 1.0 [a, b]	0.2 ± 0.9 [a, b]	-0.9 ± 1.7 [a]	2.5 ± 0.6 [c]	1.6 ± 0.7 [b, c]	**0.6 ± 1.4**
Propylene glycol	-1.4 ± 1.0 [a]	-0.2 ± 0.9 [a, b, c]	-0.8 ± 1.5 [a, b]	1.3 ± 1.0 [b, c]	1.6 ± 0.5 [c]	**0.1 ± 1.3**
Glycerol*	0.8 ± 2.1	1.4 ± 1.0	-0.4 ± 2.0	1.9 ± 0.5	1.4 ± 0.7	**1.0 ± 0.9**
PEG 600	-2.4 ± 1.9 [a]	-0.6 ± 0.8 [a, b]	-0.1 ± 0.5 [a, b]	0.4 ± 0.7 [b]	0.7 ± 1.5 [b]	**-0.4 ± 1.2**
Methylcyclopentane	-0.4 ± 0.6 [a, b]	0.2 ± 0.5 [a, b]	-1.7 ± 0.8 [a]	0.8 ± 1.4 [b]	0.7 ± 1.0 [b]	**-0.1 ± 1.0**
Tween 20®	-2.6 ± 1.4 [a]	-0.4 ± 1.9 [a, b]	-0.8 ± 0.8 [a, b]	1.5 ± 1.1 [b]	-0.3 ± 1.2 [a, b]	**-0.5 ± 1.4**
Methyl isobutyl ketone*	3.1 ± 1.7	4.4 ± 4.4	1.9 ± 2.7	5.0 ± 5.2	1.0 ± 0.9	**3.1 ± 1.7**
Toluene	-1.9 ± 1.0 [a]	0.0 ± 0.3 [a, b]	-1.1 ± 1.1 [a]	1.3 ± 0.9 [b, c]	2.5 ± 1.3 [c]	**0.2 ± 1.8**
2-Methyl-1-pentanol	0.5 ± 2.0 [a]	0.0 ± 0.6 [a]	-1.6 ± 1.0 [a, b]	-2.9 ± 0.9 [b]	1.0 ± 0.8 [a]	**-0.6 ± 1.6**
Ethanol	-0.1 ± 1.2 [a, b]	-0.1 ± 0.5 [a, b]	-1.1 ± 0.5 [a]	0.9 ± 0.4 [b]	0.9 ± 0.9 [b]	**0.1 ± 0.9**
Ammonium nitrate*	4.3 ± 3.4	6.0 ± 1.1	4.9 ± 0.6	4.3 ± 0.5	5.0 ± 1.7	**4.9 ± 0.7**
1-Octanol	5.6 ± 2.5 [a]	0.6 ± 1.0 [b]	1.1 ± 1.2 [b]	2.5 ± 1.0 [b]	2.3 ± 0.9 [b]	**2.4 ± 2.0**
4-Carboxybenzaldehyde	2.8 ± 2.6 [a]	3.6 ± 3.0 [a]	6.1 ± 2.5 [a, b]	9.4 ± 3.1 [b]	5.4 ± 1.8 [a, b]	**5.5 ± 2.6**
2-Ethyl-1-hexanol	-0.1 ± 1.7 [a, b]	0.8 ± 1.0 [a, b]	0.4 ± 1.3 [a, b]	-1.1 ± 0.6 [a]	2.2 ± 1.0 [b]	**0.4 ± 1.2**
Sodium lauryl sulphate*	19.0 ± 4.4	18.4 ± 7.2	23.6 ± 5.6	18.0 ± 3.8	16.9 ± 3.7	**19.2 ± 2.6**
1-Hexanol	-2.6 ± 1.3 [a]	0.0 ± 0.6 [b, c]	-0.9 ± 1.3 [a, b]	-2.6 ± 0.9 [a]	1.6 ± 0.9 [c]	**-0.9 ± 1.8**
Acetone*	0.0 ± 2.0	1.1 ± 1.0	-0.4 ± 1.1	-0.9 ± 1.4	1.4 ± 0.3	**0.2 ± 1.0**
Triton X-100®	13.0 ± 2.6 [a]	8.1 ± 3.9 [a, b]	6.6 ± 3.1 [b]	11.6 ± 1.6 [a, b]	9.2 ± 2.5 [a, b]	**9.7 ± 2.6**
p-Fluoroaniline	2.0 ± 1.8 [a, b, c]	3.0 ± 0.6 [b, c]	0.3 ± 1.4 [a, b]	0.1 ± 1.5 [a]	4.1 ± 1.1 [c]	**1.9 ± 1.7**
Lauric acid	-1.8 ± 1.2 [a]	-0.5 ± 0.5 [a, b]	-1.0 ± 0.7 [a, b]	0.1 ± 1.4 [a, b]	1.1 ± 0.8 [b]	**-0.4 ± 1.1**
Imidazole*	6.1 ± 3.4	7.2 ± 0.4	11.2 ± 14.7	5.5 ± 1.0	5.6 ± 2.2	**7.1 ± 2.4**
Sodium oxalate*	12.5 ± 4.1	9.4 ± 1.4	10.1 ± 2.7	12.9 ± 2.2	12.0 ± 2.6	**11.4 ± 1.5**
Cyclohexanol*	0.7 ± 1.3	0.4 ± 0.8	0.5 ± 0.8	1.7 ± 0.8	1.8 ± 0.6	**1.0 ± 0.7**
Cetylpyridinium bromide*	13.8 ± 6.5	6.4 ± 2.0	7.9 ± 3.9	10.1 ± 6.7	8.4 ± 0.7	**9.3 ± 2.9**
Sodium hydroxide*	15.8 ± 7.9	6.4 ± 2.5	11.7 ± 4.0	11.8 ± 4.8	14.9 ± 3.5	**12.1 ± 3.7**
Benzalkonium chloride*	14.0 ± 10.4	25.0 ± 8.6	16.7 ± 6.4	18.9 ± 4.0	24.8 ± 2.8	**19.9 ± 4.9**

Values are the mean data ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 5 runs.

* For this test substance, there are no significant differences between the mucus productions of the 5 runs ($p > 0.05$, One-way ANOVA).

[a, b, c] Mucus productions of the slugs treated with this test substance marked with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

Table 3.7 Intra- and inter-experiment variability for the score for tissue damage

Test substance	Run 1	Run 2	Run 3	Run 4	Run 5	**Average**
3,3-Dimethylpentane*	37 ± 25	57 ± 53	62 ± 112	77 ± 56	15 ± 13	**50 ± 24**
PEG 400*	19 ± 12	13 ± 9	36 ± 33	25 ± 5	25 ± 21	**24 ± 9**
3-Methoxy-1,2-propanediol	34 ± 24 [b, c]	8 ± 4 [a]	53 ± 42 [c]	84 ± 52 [c]	11 ± 3 [a, b]	**38 ± 32**
Propylene glycol	28 ± 12 [a]	10 ± 4 [a, b]	9 ± 9 [b]	24 ± 11 [a, b]	9 ± 5 [a, b]	**16 ± 9**
Glycerol*	24 ± 28	15 ± 21	4 ± 3	22 ± 10	23 ± 23	**18 ± 8**
PEG 600*	43 ± 63	30 ± 18	39 ± 72	29 ± 10	18 ± 7	**32 ± 9**
Methylcyclopentane	39 ± 13 [b]	13 ± 4 [a, b]	9 ± 9 [a]	19 ± 7 [a, b]	28 ± 17 [a, b]	**21 ± 12**
Tween 20®*	81 ± 20	118 ± 66	93 ± 40	71 ± 28	78 ± 14	**88 ± 18**
Methyl isobutyl ketone*	373 ± 109	333 ± 141	490 ± 211	445 ± 212	493 ± 365	**427 ± 71**
Toluene*	228 ± 87	173 ± 118	144 ± 68	249 ± 80	115 ± 86	**182 ± 56**
2-Methyl-1-pentanol*	471 ± 225	190 ± 135	1002 ± 950	595 ± 368	312 ± 195	**514 ± 313**
Ethanol*	37 ± 35	18 ± 8	18 ± 13	19 ± 8	20 ± 10	**22 ± 8**
Ammonium nitrate*	389 ± 209	254 ± 83	117 ± 76	231 ± 89	218 ± 108	**242 ± 97**
1-Octanol*	828 ± 633	447 ± 256	549 ± 444	334 ± 176	409 ± 166	**513 ± 192**
4-Carboxybenzaldehyde*	163 ± 76	207 ± 196	162 ± 81	215 ± 158	35 ± 33	**156 ± 72**
2-Ethyl-1-hexanol*	491 ± 171	217 ± 66	569 ± 362	213 ± 90	408 ± 285	**380 ± 161**
Sodium lauryl sulphate*	860 ± 730	364 ± 135	607 ± 323	387 ± 217	310 ± 220	**506 ± 228**
1-Hexanol	506 ± 190 [a, b]	197 ± 130 [a]	906 ± 850 [b]	1755 ± 581 [b]	185 ± 123 [a]	**710 ± 654**
Acetone*	219 ± 219	63 ± 29	250 ± 309	135 ± 152	102 ± 117	**154 ± 79**
Triton X-100®	545 ± 448 [a]	427 ± 252 [a]	1719 ± 713 [b]	846 ± 351 [a, b]	451 ± 369 [a]	**798 ± 541**
p-Fluoroaniline*	174 ± 80	244 ± 114	305 ± 232	94 ± 49	103 ± 33	**184 ± 91**
Lauric acid*	45 ± 20	48 ± 47	26 ± 24	42 ± 40	34 ± 22	**39 ± 9**
Imidazole*	803 ± 369	574 ± 202	1198 ± 789	667 ± 331	848 ± 254	**818 ± 239**
Sodium oxalate*	1088 ± 465	683 ± 154	887 ± 807	502 ± 50	446 ± 134	**721 ± 268**
Cyclohexanol*	209 ± 85	139 ± 171	145 ± 75	106 ± 181	43 ± 35	**128 ± 61**
Cetylpyridinium bromide	836 ± 625 [b]	283 ± 83 [a, b]	659 ± 151 [b]	886 ± 643 [b]	188 ± 214 [a]	**570 ± 319**
Sodium hydroxide*	195 ± 104	136 ± 81	147 ± 83	270 ± 134	342 ± 317	**218 ± 87**
Benzalkonium chloride	2489 ± 1512 [a, b]	1889 ± 687 [a, b]	3446 ± 738 [b]	1851 ± 631 [a, b]	1310 ± 360 [a]	**2197 ± 813**

Values are the mean data ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 5 runs.

* For this test substance, there are no significant differences between the scores of the 5 runs ($p > 0.05$, One-way ANOVA).

[a, b, c] Scores of the slugs treated with this test substance marked with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

The repeatability of the test results was also evaluated using the Pearson correlation coefficient and the slope of the regression line. Generally, the data are highly correlated between the different trials for both end points (Tables 3.8 and 3.9). For the mucus production, the slope of the regression line is close to one suggesting that the data are of the same magnitude, whereas for the score for tissue damage the magnitude of the data varies between the different trials.

Table 3.8 Repeatability of the mucus production data (of the first 60-minute contact period)

	Run 1	Run 2	Run 3	Run 4	Run 5
Run 1	-	0.83	0.90	0.91	0.88
Run 2	0.89 (0.30)	-	0.91	0.90	0.95
Run 3	0.92 (0.41)	0.87 (0.66)	-	0.92	0.90
Run 4	1.00 (-0.62)	0.91 (-0.21)	0.98 (-0.70)	-	0.92
Run 5	0.95 (-0.91)	0.95 (-0.83)	0.95 (-1.06)	0.91 (-0.09)	-

Values above the dashes are the Pearson correlation coefficients. Values below the dashes are the slopes of the linear regression lines while the values between the brackets are the intercepts (n = 28). The correlation and regression coefficients are significant ($p < 0.001$).

Table 3.9 Repeatability of the score for tissue damage data

	Run 1	Run 2	Run 3	Run 4	Run 5
Run 1	-	0.96	0.91	0.78	0.89
Run 2	1.35 (56)	-	0.92	0.72	0.91
Run 3	0.65 (87)	0.47 (28)	-	0.84	0.90
Run 4	0.84 (96)	0.56 (53)	1.17 (4)	-	0.70
Run 5	1.56 (35)	1.13 (-11)	2.20 (-29)	1.13 (98)	-

Values above the dashes are the Pearson correlation coefficients. Values below the dashes are the slopes of the linear regression lines while the values between the brackets are the intercepts (n = 28). The correlation and regression coefficients are significant ($p < 0.001$).

The reference chemicals can be classified into one of the EU eye irritation categories using the classification prediction model described in Figure 3.1. The agreement between the classifications of the repeated experiments was assessed using the κ-statistics. The classification prediction model shows a high reproducibility with κ-values ranging from 0.63 to 0.84 (Table 3.10). Eighteen chemicals were classified the same during the five different trials. For six compounds, the classification differed between the R36 and R41 and for another four compounds, there was a switch between the R36 and NI category.

Table 3.10 Repeatability of the classification prediction model of the Slug Mucosal Irritation test

Test substance	Predicted EU category [a]				
	Run 1	Run 2	Run 3	Run 4	Run 5
3,3-Dimethylpentane	NI	NI	NI	NI	NI
PEG 400	NI	NI	NI	NI	NI
3-Methoxy-1,2-propanediol	NI	NI	NI	NI	NI
Propylene glycol	NI	NI	NI	NI	NI
Glycerol	NI	NI	NI	NI	NI
PEG 600	NI	NI	NI	NI	NI
Methylcyclopentane	NI	NI	NI	NI	NI
Tween 20®	NI	R36	NI	NI	NI
Methyl isobutyl ketone	R36	R36	R36	R36	R36
Toluene	R36	R36	R36	R36	R36
2-Methyl-1-pentanol	R36	R36	R41	R41	R36
Ethanol	NI	NI	NI	NI	NI
Ammonium nitrate	R36	R36	R36	R36	R36
1-Octanol	R36	R36	R41	R36	R36
4-Carboxybenzaldehyde	R36	R36	R41	R41	R36
2-Ethyl-1-hexanol	R36	R36	R41	R36	R36
Sodium lauryl sulphate	R41	R41	R41	R41	R41
1-Hexanol	R41	R36	R41	R41	R36
Acetone	R36	NI	R36	R36	R36
Triton X-100®	R41	R41	R41	R41	R41
p-Fluoroaniline	R36	R36	R36	NI	R36
Lauric acid	NI	NI	NI	NI	NI
Imidazole	R41	R41	R41	R36	R36
Sodium oxalate	R41	R41	R41	R41	R41
Cyclohexanol	R36	R36	R36	R36	NI
Cetylpyridinium bromide	R41	R41	R41	R41	R41
Sodium hydroxide	R41	R41	R41	R41	R41
Benzalkonium chloride	R41	R41	R41	R41	R41
Agreement between the runs			κ-value		
Run 1	-				
Run 2	0.84 ± 0.09	-			
Run 3	0.79 ± 0.09	0.63 ± 0.11	-		
Run 4	0.79 ± 0.10	0.63 ± 0.12	0.78 ± 0.10	-	
Run 5	0.84 ± 0.09	0.78 ± 0.10	0.63 ± 0.11	0.73 ± 0.11	-

NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes.

[a] EU eye irritation categories were assigned using the classification prediction model described in Figure 3.1 and the data presented in Tables 3.6 and 3.7.

3.6.2.4 Relevance of the modified assay based on 28 chemicals

Next, the EU eye irritation categories predicted by the Slug Mucosal Irritation test were compared with the EU categories based on the rabbit eye irritation data (ECETOC, 1998; EC, 2001). Acetone, *p*-fluoroaniline, and cyclohexanol were falsely predicted non-irritant in one of the five trials, while they were predicted R36 in the other cases. For Tween 20®, a false positive result was obtained in the second trial, whereas it was predicted correctly in the other trials. Sodium lauryl sulphate and Triton X-100® were overestimated in the five trials, while cyclohexanol was underestimated in the five trials in comparison with the EU label. To evaluate the concordance, sensitivity, and specificity of the Slug Mucosal Irritation test, the overall obtained classification was compared with the EU risk phrase of the reference chemicals (Figure 3.2 and Table 3.11). Only one chemical (lauric acid) was predicted non-irritant in the five different trials resulting in a sensitivity of 94%. Methyl isobutyl ketone, toluene, and 2-methyl-1-pentanol were overestimated (false positives) in the five trials resulting in a specificity of 75%.

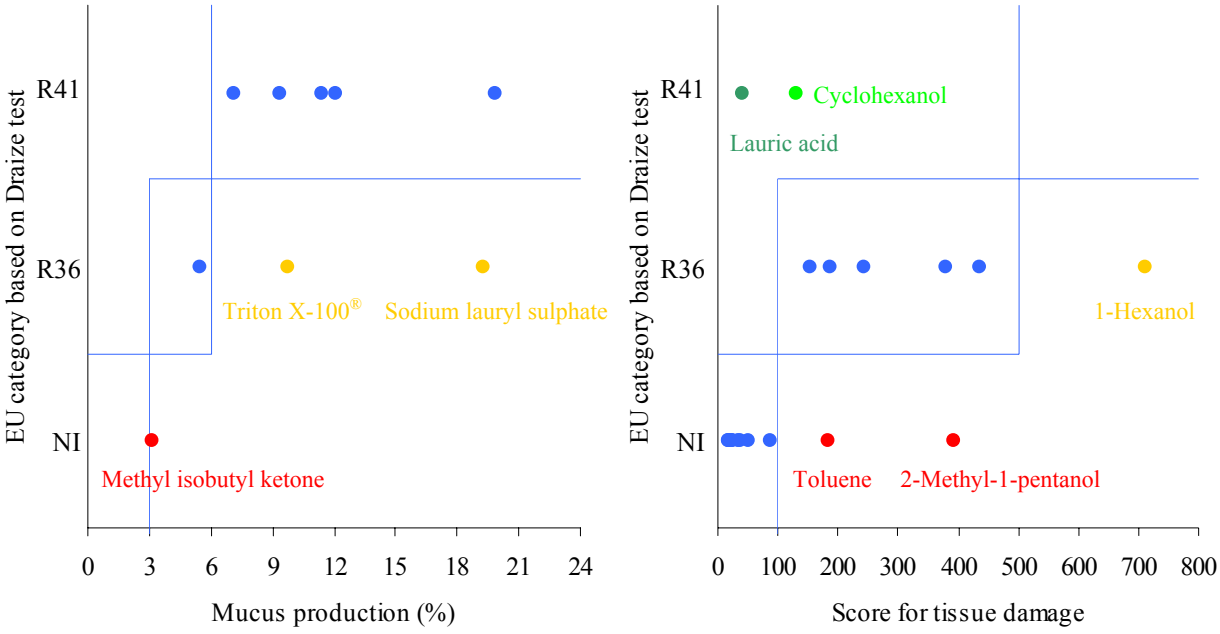

Figure 3.2 Left-graph shows the agreement between the mucus production and the EU eye irritation category based on the Draize eye test for substances that induced > 3% mucus production. Right-graph shows the agreement between the score for tissue damage and the Draize EU eye irritation category for substances that induced ≤ 3% mucus production. Data points are presented as the mean values of the 5 runs. Correctly classified (●), false positive (●), false negative (●), overestimated (●), and underestimated (●) test substances are indicated.

Table 3.11 Agreement of the EU classification of the chemicals obtained with the Slug Mucosal Irritation test and the Draize rabbit eye test

Slug Mucosal Irritation test	Draize test EU category		
Predicted EU category	R41	R36	NI
R41	5	3	0
R36	1	6	3
NI	1	0	9

71% of the chemicals are correctly classified into three classes with a sensitivity of 94%, a specificity of 75%, a positive predictive value of 83% and a negative predictive value of 90% (irritants versus non-irritants).

3.6.2.5 Relevance of the modified assay based on 12 additional chemicals

Additionally, the relevance of the modified assay was assessed by means of 12 reference chemicals that were not used for the development of the prediction model. Table 3.12 summarizes the mucus production, score for tissue damage, and EU eye irritation category based on both the Slug Mucosal Irritation test and the Draize eye irritation test for each of the chemicals.

Table 3.12 Irritation potential of additional reference chemicals as assessed with the Draize eye test and the Slug Mucosal Irritation test

Test substance	Draize eye test		Slug Mucosal Irritation test		
	EU class	MMAS	Mucus [a] (%)	Score [a]	Predicted EU class [b]
Potassium tetrafluoroborate	NI	0.0	2.8 ± 0.8	18 ± 4	NI
Tetra-aminopyrimidine sulphate	NI	10.3	2.6 ± 1.1	22 ± 18	NI
Methyl amyl ketone	NI	10.5	8.3 ± 7.3	187 ± 133	R41
Ethyl acetate	NI	15.0	-0.8 ± 0.7	22 ± 14	NI
Methyl acetate	R36	39.5	-0.6 ± 0.5	29 ± 39	NI
Isopropanol	R36	30.5	0.0 ± 0.6	84 ± 53	NI
γ-Butyrolactone	R36	43.0	0.3 ± 0.8	98 ± 118	NI
1-Butanol	R36	60.8	0.0 ± 1.1	35 ± 12	NI
Pyridine	R41	48.0	6.5 ± 2.3	245 ± 100	R41
Promethazine hydrochloride	R41	71.7	22.2 ± 6.8	2812 ± 656	R41
Chlorhexidine	R41	82.3	16.5 ± 4.5	998 ± 575	R41
Trichloroacetic acid	R41	106	7.0 ± 5.5	230 ± 157	R41

MMAS, modified maximum average score (ECETOC, 1998); NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes.

[a] Values are the mean ± standard deviation of 5 slugs.

[b] EU eye irritation categories were assigned using the classification prediction model described in Figure 3.1.

Using the prediction model presented in Figure 3.1, methyl amyl ketone, pyridine, promethazine hydrochloride, chlorhexidine, and trichloroacetic acid were classified as R41 based on the mucus production of the slugs. Potassium tetrafluoroborate, tetra-aminopyrimidine sulphate, ethyl acetate, methyl acetate, isopropanol, γ-butyrolactone, and 1-butanol were classified as NI based on the score for tissue damage. However, it has to be noted that the scores obtained for isopropanol and especially γ-butyrolactone were close to the NI-R36 score cut-off value.

When the EU categories predicted by the Slug Mucosal Irritation test were compared with the EU categories assigned on the basis of the rabbit eye irritation test, a concordance, sensitivity and specificity of 58%, 50% and 75% was obtained. Methyl acetate, isopropanol, γ-butyrolactone, and 1-butanol were falsely predicted non-irritant by the Slug Mucosal Irritation test. Methyl amyl ketone was falsely predicted R41.

3.6.2.6 Test validity

The validity of a test can be evaluated by means of negative and positive controls. A positive control has been defined by Balls *et al.* (1999) as a substance which is known to give a positive response in a particular assay and which is used to confirm the correct conduct of the assay. The data of 30 repeated experiments (150 negative and 150 positive control slugs), presented in Table 3.13, were analysed for this purpose. Figure 3.3 shows the average mucus production of slugs treated during 60 minutes with the negative control (PBS) or the positive control (1% (w/v) benzalkonium chloride). The average mucus productions of the negative control slugs varied from -2.1 to 1.5%, whereas those of the positive control slugs varied from 14.0 to 29.7%. Figure 3.4 presents the average score for tissue damage of both controls. The average scores of the negative control slugs varied from 3 to 89, whereas those of the positive control slugs varied from 1055 to 5967.

For the negative control, ANOVA testing revealed no significant difference in mucus production and score for tissue damage between the repeated experiments ($p > 0.05$). In each of the 30 repeated experiments, PBS was classified as NI. For the positive control, no significant differences in mucus productions on the one hand and scores on the other hand were detected between the repeated experiments ($p > 0.05$). Moreover, benzalkonium chloride was classified as R41 in each of the repeated experiments.

Table 3.13 Intra- and inter-experiment variability for the mucus production and score for tissue damage of the negative and positive control slugs

Experiment	Negative control		Positive control	
	Mucus (%) *	Score *	Mucus (%) *	Score *
1	-0.5 ± 1.4	89 ± 101	17.4 ± 7.3	2879 ± 1286
2	-2.0 ± 1.4	40 ± 36	14.0 ± 10.4	2489 ± 1512
3	-2.1 ± 1.0	25 ± 18	18.3 ± 4.4	2474 ± 250
4	-1.1 ± 0.5	24 ± 17	21.5 ± 13.9	3048 ± 1650
5	-0.7 ± 0.6	10 ± 6	29.7 ± 6.2	2970 ± 729
6	-0.2 ± 0.6	77 ± 87	15.4 ± 3.6	3389 ± 1296
7	-0.1 ± 0.5	13 ± 8	15.8 ± 3.6	1159 ± 396
8	0.7 ± 0.6	29 ± 17	17.8 ± 4.3	1356 ± 79
9	-0.3 ± 0.6	16 ± 5	16.4 ± 4.9	1833 ± 495
10	0.8 ± 1.2	14 ± 14	16.0 ± 4.9	1818 ± 357
11	-1.4 ± 0.3	17 ± 11	25.0 ± 8.6	1889 ± 687
12	-1.3 ± 0.4	40 ± 30	16.6 ± 6.2	1522 ± 684
13	-1.0 ± 0.7	23 ± 25	21.2 ± 6.0	3296 ± 1722
14	-1.1 ± 0.8	25 ± 16	18.1 ± 6.3	5967 ± 1121
15	-1.9 ± 0.8	3 ± 3	27.1 ± 9.9	4422 ± 2132
16	-1.4 ± 0.6	4 ± 4	25.6 ± 8.5	2636 ± 507
17	-1.8 ± 0.5	34 ± 13	16.7 ± 6.4	3446 ± 738
18	1.5 ± 1.1	27 ± 37	18.2 ± 6.2	1410 ± 632
19	1.2 ± 0.8	40 ± 43	20.7 ± 6.0	1872 ± 303
20	0.9 ± 2.4	21 ± 12	24.3 ± 3.8	1294 ± 704
21	1.1 ± 0.8	50 ± 24	14.6 ± 5.1	1432 ± 317
22	0.1 ± 0.8	44 ± 46	17.6 ± 3.8	2208 ± 1103
23	0.2 ± 1.2	41 ± 61	18.9 ± 4.0	1851 ± 631
24	0.5 ± 0.8	30 ± 44	22.3 ± 1.8	1577 ± 404
25	0.7 ± 1.1	11 ± 2	24.9 ± 6.5	1535 ± 863
26	0.3 ± 1.0	30 ± 20	24.8 ± 2.8	1310 ± 360
27	0.4 ± 2.5	12 ± 11	27.0 ± 5.0	1597 ± 285
28	0.5 ± 1.6	8 ± 4	18.7 ± 1.3	1055 ± 257
29	0.5 ± 0.7	18 ± 33	19.5 ± 2.7	1330 ± 388
30	0.4 ± 0.5	19 ± 6	18.6 ± 3.5	1443 ± 814
Inter-experiment mean	**-0.3 ± 1.0**	**28 ± 19**	**20.1 ± 4.2**	**2217 ± 1098**

Values are the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 30 experiments.

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

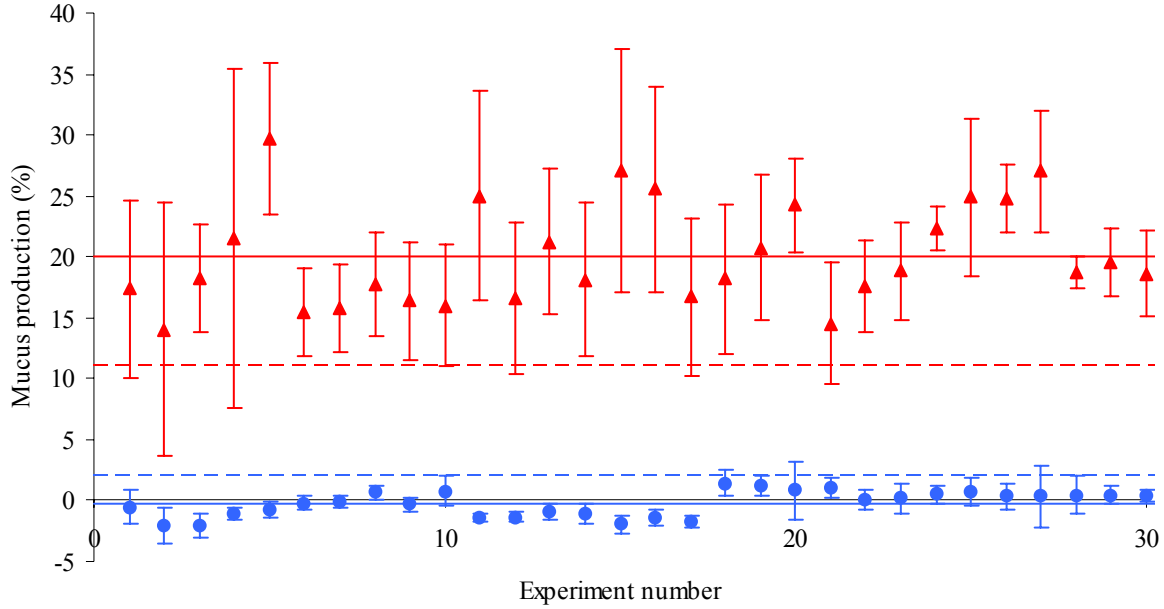

Figure 3.3 Mucus production of slugs treated during 60 minutes with the negative control PBS (●) or the positive control 1% (w/v) benzalkonium chloride (▲). Data points are presented as the mean values (n = 5) and standard deviation bars are indicated. Solid lines represent the mean mucus production of 150 slugs, whereas blue and red dashed lines respectively represent the acceptance criteria for the mean mucus production of 5 negative and positive control slugs.

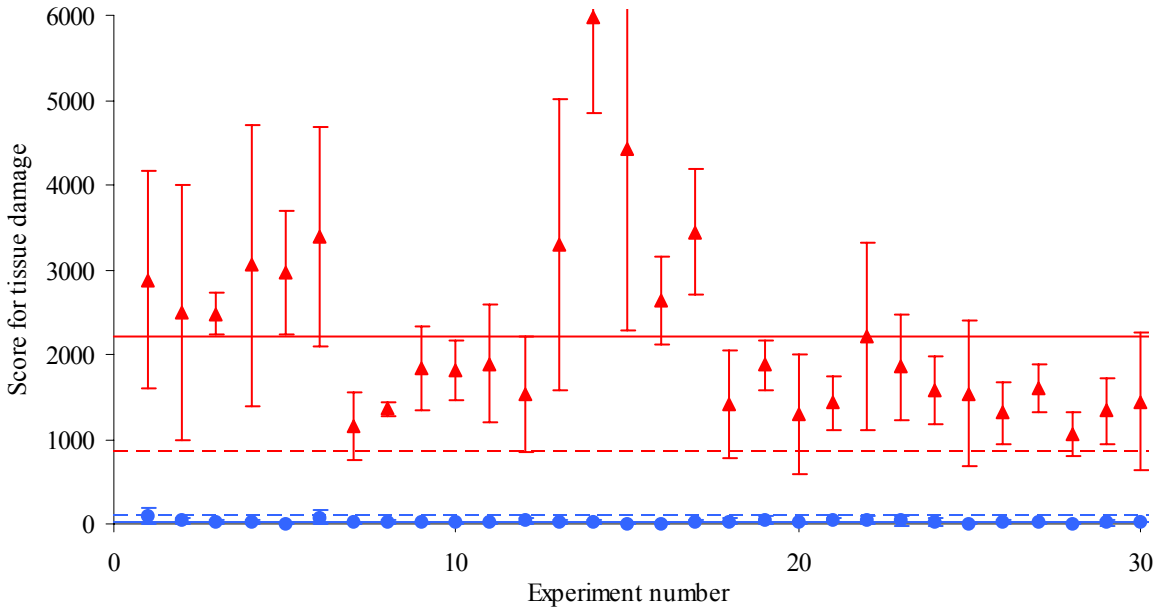

Figure 3.4 Score for tissue damage of slugs treated with PBS (●) or benzalkonium chloride (▲). Data points are presented as the mean values (n = 5) and standard deviation bars are indicated. Solid lines represent the mean score of 150 slugs, whereas blue and red dashed lines respectively represent the acceptance criteria for the mean score of 5 negative and positive control slugs.

Based on the data of 30 repeated experiments, acceptance criteria were established to provide criteria for test validity. The acceptance criteria were calculated as described by Hahn and Meeker (1991). Acceptance criteria were established based on the 95% prediction intervals to contain a future observation. The results of the tested reference chemicals were only accepted if the following conditions were satisfied. For the negative control slugs, the mean mucus production (n = 5) during the first contact period and the mean score for tissue damage (n = 5) should be less than 2.0% and 100, respectively. For the positive control slugs, both values should be more than 11.0% and 850, respectively.

3.7 DISCUSSION

Mucosal tissue and certain tissues of the eye resemble histologically. The conjunctiva is namely a mucous membrane that lines the inside of the eyelids and the visible part of the sclera (Marieb, 1995; Curren and Harbell, 1998). The HET-CAM is used as an alternative test for predicting the eye irritation potential of chemicals and cosmetics (Gilleron *et al.*, 1996; Gilleron *et al.*, 1997; Steiling *et al.*, 1999) as well as for predicting the irritation potential of dental products (Schendel *et al.*, 1994; Dahl, 1999). Previous research showed that the Slug Mucosal Irritation test can be used as an alternative for screening the eye irritation potential of chemicals (Adriaens and Remon, 2002). The proposed test protocol was a two-day test procedure and this can be a limiting factor for high throughput screening. Therefore, the objective of this study was to reduce the eye irritation/damage test procedure of the Slug Mucosal Irritation test to one day. Because several *in vivo* irritants can be detected based on the amount of mucus produced during a 60-minute contact period with a 1% (w/v) dilution of the chemical, this step of the test procedure was maintained. To select an appropriate concentration for the second contact period, the concentration response effect of several *in vivo* irritants that were underestimated by the mucus production, and a few *in vivo* non-irritants was investigated.

PEG 400, propylene glycol, glycerol, Tween 20®, toluene, and ethanol resulted in no adverse effects when applied undiluted to rabbit eyes (ECETOC, 1998). PEG 400 concentrations up to 10% (w/v) did not affect the end points of the Slug Mucosal Irritation test. Increasing concentrations of propylene glycol, glycerol, and Tween 20® affected the mucus production or protein release slightly indicating that they induced only mild irritation when tested at concentrations up to 10% (w/v). The response detected with the Slug Mucosal Irritation test was in agreement with clinical data reported for propylene glycol and glycerol.

Upon instillation, those chemicals may cause a stinging and burning sensation, but no obvious eye injury was noted (Grant, 1986). Rinsing of human eyes with aqueous solutions of PEG 400 (1/1) caused only a slight burning sensation without causing injury (Grant, 1974). Although toluene and ethanol were non-irritant in the Draize eye test, these chemicals induced severe tissue damage of the body wall of slugs when tested at a 2.5% and a 10% (w/v) concentration, respectively. A splash of ethanol on human eyes caused immediate burning and stinging discomfort and toluene vapours may cause irritation to the eye (Grant, 1986). 1-Octanol, 2-ethyl-1-hexanol, 1-hexanol, acetone, lauric acid, and cyclohexanol resulted all in irritation of the rabbit eye (ECETOC, 1998). When those chemicals were tested at a 1% (w/v) concentration, they did not affect the end points of the Slug Mucosal Irritation test. Lauric acid concentrations up to 10% (w/v) did not induce a positive response, while 2.5% (w/v) or higher concentrations of 1-octanol, 2-ethyl-1-hexanol, 1-hexanol, acetone, and cyclohexanol resulted in tissue damage. The results of the concentration response experiments showed that increasing the test concentration resulted in a faster onset of tissue damage. Consequently, chemicals that were underestimated when tested at a 1% (w/v) concentration can be predicted correctly when tested at a higher concentration. Therefore, the concentration of the second contact period was set at 3.5% (w/v).

The repeatability and relevance of the modified test procedure were evaluated using 28 eye reference chemicals (ECETOC, 1998). A single 60-minute exposure to especially irritating surfactants and inorganic chemicals induced a highly increased mucus production and tissue damage; those chemicals were consequently correctly predicted based on the mucus produced during the first contact period. It is known from several *in vitro* assays that surfactants have a fast onset of tissue damage and the mechanism is thought to involve membrane disruption (Harbell *et al.*, 1997). For chemicals that did not affect the mucus secretion, an additional contact period with a 3.5% (w/v) concentration was introduced. The protein and LDH release from the slug body wall after both contact periods was combined into a score for tissue damage. Based on the results, a classification prediction model was developed. The prediction model classifies the test substances into the EU eye irritation categories (EC, 2001) based on both end points (mucus production and score for tissue damage). A classification prediction model simplifies the interpretation of the data, but has also some disadvantages. A classification prediction model namely reduces the information about the severity of the irritation or tissue damage (Curren and Harbell, 1998). So especially for substances with a borderline irritation/damage potential, a careful analysis of the data is still recommended.

In order to investigate the repeatability of the modified test procedure and prediction model, all chemicals were tested independently on five separate occasions. For 16 out of the 28 chemicals, significantly different mucus productions were detected in the five runs. It was striking that seven of these 16 chemicals were alcohols. However, only for three out of these 16 chemicals, the differences in mucus production were not only statistically significant, but resulted also in different eye irritation categories. Furthermore, significantly different scores were detected in the five runs for seven out of the 28 chemicals. Three of these seven chemicals were alcohols. Only for three out of these seven chemicals, the statistically different scores obtained in the different runs resulted in different eye irritation categories. Furthermore, analysis of the repeatability of the data obtained for the end points revealed that the mucus production was the more reproducible end point. This is in agreement with the study of Adriaens and Remon (2002). The score for tissue damage was calculated using protein and LDH values after each contact period, which resulted probably in a higher variation. The mean κ-value for the agreement between the predicted irritation categories of the repeated trials was 0.74, indicating substantial reproducibility. For ten out of the 28 chemicals, different predicted EU categories were obtained throughout at least one of the five runs. Of interest is the fact that five of these ten chemicals belonged to the chemical class of the alcohols. It is important to note that four of these ten chemicals (1-octanol, 2-ethyl-1-hexanol, 1-hexanol, and cyclohexanol) were also underestimated or overestimated by at least one of the rabbits in the rabbit eye irritation test (ECETOC, 1998; EC, 2001) and by some experiments with the BCOP test (ICCVAM and NICEATM, 2004). Especially for the R36 labelled chemicals, differences between the EU eye irritation categories predicted by the Slug Mucosal Irritation test were observed. High variability in the rabbit eye irritation test data was also observed for mildly or moderately irritating chemicals, whereas a good reproducibility was reported for non-irritating and severely irritating chemicals (Balls *et al.*, 1995).

Next, the EU eye irritation categories predicted by the Slug Mucosal Irritation test were compared with the irritation categories assigned on the basis of the rabbit eye irritation data (Table 3.14). 75% of the 12 rabbit non-irritants were correctly predicted by the Slug Mucosal Irritation test. Three false positive results were obtained. Methyl isobutyl ketone, toluene, and 2-methyl-1-pentanol induced tissue damage, while they were classified as non-irritant in the rabbit eye irritation test. However, several references cited in the Hazard Substances Data bank mentioned the eye irritating potential of these chemicals (TOXNET®, 2005). Exposure to methyl isobutyl ketone irritated the conjunctiva and mucous membranes of the nose and throat of men (Hjelm *et al.*, 1990). The HET-CAM (Gilleron *et al.*, 1997) and

the HCE model (Van Goethem *et al.*, 2005) scored methyl isobutyl ketone as irritant. Methyl isobutyl ketone was also labelled R36 in the material safety data sheet provided by the chemical supplier. Furthermore, toluene was classified as moderate irritant by means of the BCOP test (Vanparys *et al.*, 1993) and as irritant by the HET-CAM (Gilleron *et al.*, 1997) and the HCE model (Van Goethem *et al.*, 2005). Additionally, 2-methyl-1-pentanol was rated 8 when tested on rabbit eye, whereas the most severe injuries were rated 10 (Grant, 1986). 2-Methyl-1-pentanol is also classified as eye irritant by the human corneal epithelial model (Van Goethem *et al.*, 2005) and as severe irritant by the BCOP test (personal communication F. Van Goethem and Ph. Vanparys, J & J, Beerse, Belgium, 2004). 2-Methyl-1-pentanol was classified as mild irritant (category 2B) on the basis of the GHS criteria (ECETOC, 1998; United Nations, 2003). These data indicate that the EU classification based on rabbit eye irritation data probably underestimated the eye irritation potential of those chemicals.

67% of the nine chemicals labelled R36 were correctly predicted by the Slug Mucosal Irritation test. The irritation potential of sodium lauryl sulphate, 1-hexanol, and Triton X-100® was overestimated. The discrepancy between the slug and rabbit classifications of sodium lauryl sulphate and Triton X-100® may be due to the fact that they were the only R36 labelled chemicals that were diluted before application to the rabbit eye, whereas the other R36 labelled chemicals were applied undiluted to the rabbit eyes. Sodium lauryl sulphate, 1-hexanol, and Triton X-100® were predicted as (severe) irritants with the HET-CAM test (Gilleron *et al.*, 1997; Steiling *et al.*, 1999) and the authors suggested that the test predicted the irritation potential of surfactants in a much more realistic way than the *in vivo* data (Steiling *et al.*, 1999). Treatment of bovine cornea's with sodium lauryl sulphate induced destruction of the corneal epithelium (Gautheron *et al.*, 1992). This chemical was also classified as category 1 (irreversible effects on eyes) on the basis of the GHS criteria (ECETOC, 1998; United Nations, 2003). Furthermore, 1-hexanol and Triton X-100® were classified as severe irritants with the BCOP test (Vanparys *et al.*, 1993). Moreover, Triton X-100® is labelled R41 in the material safety data sheet of the supplier.

71% of the seven chemicals classified as R41 based on the rabbit eye irritation data were correctly classified by the Slug Mucosal Irritation test. Cyclohexanol was underestimated one irritation category. For lauric acid, a false negative result was obtained. Lauric acid is a solid compound and in the Draize eye test solids are placed in the conjunctival sac of the rabbit eye where they can remain for up to 24 h (York and Steiling, 1998; Balls *et al.*, 1999). Such a high and prolonged exposure does not occur in case of accidental human exposure and can not be predicted by many alternatives (Balls *et al.*, 1999).

Furthermore, exposure of the rabbit eye to insoluble solids may provoke blinking (York and Steiling, 1998) and mechanical injury (Balls *et al.*, 1999). However, mechanical injury can not be measured in the Slug Mucosal Irritation test. Furthermore, it is worth noting that lauric acid is labelled R36 in the material safety data sheet of the supplier.

The overall performance of the assay was found satisfactory, 71% of the 28 chemicals were correctly classified into three EU categories. It is interesting to note that about half of the 29% incorrectly classified chemicals belonged to the chemical class of the alcohols. Several references cited in the Hazard Substances Data bank mentioned anaesthetizing properties of these under- or overestimated alcohols (TOXNET®, 2005). Anaesthesia of the slugs is probably responsible for an alteration of the mucus production (Adriaens and Remon, 2002), whereas tissue fixation by the alcohols can probably explain the modified protein and enzyme release. When the 28 chemicals were divided into non-irritants or irritants, a sensitivity and specificity of 94% and 75% were respectively obtained.

In the current study, the sensitivity of the test was increased in comparison with the two-day test procedure (Adriaens and Remon, 2002). Only one false negative was obtained, whereas lauric acid and acetone were predicted non-irritant with the two-day test procedure. The predictive ability of the middle category (R36) was also improved. 67% of the R36 labelled chemicals were correctly predicted with the one-day procedure, against 44% with the two-day procedure. However, the specificity of the test was decreased. In the previous study, only one false positive was obtained (methyl isobutyl ketone), whereas the one-day procedure resulted in three false positives (methyl isobutyl ketone, toluene, and 2-methyl-1-pentanol). Because several data from the literature mentioned the irritation potential of those chemicals, we suppose that the modified test procedure and prediction model of the Slug Mucosal Irritation test predicted the irritation potential more accurately.

The concordance obtained in the current study was comparable to that obtained in a post hoc data analysis of the results of 59 ECETOC reference chemicals tested in the international EC/HO validation study. 72.5% of the chemicals were namely correctly classified into three classes using a prediction model based on the end points of the neutral red uptake test (lgNRU) and the BCOP test (lgBCOPo5) (Moldenhauer, 2003). Twenty-two of those chemicals were also evaluated with the Slug Mucosal Irritation test and 16 out of the 22 substances were predicted the same.

The results of the Slug Mucosal Irritation test were also compared with available data of three other alternative eye irritation tests, namely BCOP test, HET-CAM, and HCE model (Table 3.14).

Table 3.14 Comparison of the irritation potential of the chemicals as assessed with the Draize eye test, Slug Mucosal Irritation (SMI) test, BCOP test, HET-CAM, and HCE model

Test substance	Draize test		SMI test	BCOP test [a]			HET-CAM [b]		HCE model [c]	
	EU class	GHS class	EU class	Class	Conc (%)	Source	Class	Conc (%)	Class	Conc (%)
3,3-Dimethylpentane	NI	NI	NI	NI	100	PC			NI	100
PEG 400	NI	NI	NI	NI	100	PC	I	100	NI	100
3-Methoxy-1,2-propanediol	NI	NI	NI	NI	100	PC			NI	100
Propylene glycol	NI	NI	NI	NI	100	V				
Glycerol	NI	NI	NI	NI	100	PC	I	100	NI	100
PEG 600	NI	NI	NI	NI	100	C				
Methylcyclopentane	NI	NI	NI	NI	100	PC	I	100	I	100
Tween 20®	NI	NI	NI	NI	100	V	I	100	NI	100
Methyl isobutyl ketone	NI	NI	R36	NI	100	G	I	100	I	100
Toluene	NI	NI	R36	I (Mod)	100	V	I	100	I	100
2-Methyl-1-pentanol	NI	Cat 2B	R36	I (Sev)	100	PC			I	100
Ethanol	NI	Cat 2B	NI	I (Sev)	100	V	I	100	I	100
Ammonium nitrate	R36	Cat 2B	R36	NI	20	PC	I	100		
1-Octanol	R36	Cat 2B	R36	I (Mod)	100	G	I	100	I	100
4-Carboxybenzaldehyde	R36	Cat 2A	R36	I (Mod)	20	PC	NI	100		
2-Ethyl-1-hexanol	R36	Cat 2B	R36	I (Mod)	100	PC	I	100	I	100
Sodium lauryl sulphate	R36	Cat 1	R41	I (Mod)	15	PC	I	15		
1-Hexanol	R36	Cat 2A	R41	I (Sev)	100	V	I	100	I	100
Acetone	R36	Cat 2A	R36	I (Sev)	100	V	I	100	I	100
Triton X-100®	R36	Cat 2A	R41	I (Sev)	100	V	I	10	I	5
p-Fluoroaniline	R36	Cat 2	R36	I (Mod)	100	PC	I	100		
Lauric acid	R41	Cat 1	NI							
Imidazole	R41	Cat 1	R41	I (Sev)	20	G	I	100		
Sodium oxalate	R41	Cat 1	R41	NI	20	G	I	100		
Cyclohexanol	R41	Cat 1	R36	I (Sev)	100	V	I	100	I	100
Cetylpyridinium bromide	R41	Cat 1	R41	I (Mod)	6	PC	I	10	I	6
Sodium hydroxide	R41	Cat 1	R41	I (Sev)	1	PC	I	10	I	1
Benzalkonium chloride	R41	Cat 1	R41	I (Sev)	20	V	I	10	I	10

Cat 1, irreversible effects on eyes; Cat 2, reversible effects on eyes; Cat 2A, irritating to eyes; Cat 2B, mildly irritating to eyes; Conc, concentration; I, irritant; Mod, moderate; NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes; Sev, severe.

[a] BCOP *in vitro* scores and classifications were taken from different sources in the literature: (C) Casterton *et al.*, 1996, (G) Gautheron *et al.*, 1994, (PC) personal communication with F. Van Goethem and Ph. Vanparys, J & J, Beerse, Belgium, 2004 and (V) Vanparys *et al.*, 1993.

[b] HET-CAM classifications were taken from Gilleron *et al.*, 1997.

[c] HCE classifications were taken from Van Goethem *et al.*, 2005.

SMI test	Draize test	
	I	NI
I	15	3
NI	1	9
Concordance	86%	
Sensitivity	94%	
Specificity	75%	

BCOP test	Draize test	
	I	NI
I	13	3
NI	2	9
Concordance	81%	
Sensitivity	87%	
Specificity	75%	

HET-CAM	Draize test	
	I	NI
I	14	7
NI	1	0
Concordance	64%	
Sensitivity	93%	
Specificity	0%	

HCE model	Draize test	
	I	NI
I	9	5
NI	0	5
Concordance	74%	
Sensitivity	100%	
Specificity	50%	

Figure 3.5 Agreement of the eye irritation classification obtained with the Draize eye test on the one hand and the Slug Mucosal Irritation (SMI) test, BCOP test, HET-CAM, and HCE model on the other hand (based on the data presented in Table 3.14)

When considering two irritation classes (irritants and non-irritants), 23 out of the 27 substances tested both with the BCOP test and the Slug Mucosal Irritation test were classified the same in both tests. Figure 3.5 shows that for the tested compounds the concordance, sensitivity, and specificity of the Slug Mucosal Irritation test (86%, 94% and 75%, respectively) were comparable to those of the BCOP test (81%, 87% and 75%, respectively). The concordance, sensitivity, and specificity of the BCOP test are in agreement with published data. When 52 compounds were classified as irritant or non-irritant based on an inter-laboratory study of the BCOP test, a concordance, sensitivity, and specificity of 73%, 93% and 66% was obtained with the EU classification based on the Draize eye test data, respectively (Gautheron *et al.*, 1994).

Only 16 out of the 22 chemicals tested with both the HET-CAM and the Slug Mucosal Irritation test were classified into the same category (namely irritant) by means of both tests. For the chemicals tested in this study, the concordance and specificity of the Slug Mucosal Irritation test were higher than those of the HET-CAM (concordance and specificity of 64% and 0%, respectively); only the sensitivities of both alternative tests were comparable. Gilleron *et al.* (1997) also reported the low specificity of the HET-CAM. An evaluation of the HET-CAM with 60 chemicals resulted in a concordance, sensitivity, and specificity of 80%,

96% and 23%, respectively. Especially liquids and surfactants were overestimated; when only those compounds were considered the specificity was reduced to 0%. For the solids on the other hand, the specificity was 100% (Gilleron *et al.*, 1997). The non-irritants tested in the current study were all liquids or surfactants.

Comparison of the Slug Mucosal Irritation test results with the data of the HCE model showed that 17 out of the 19 substances evaluated with both tests were classified the same. For the chemicals tested in this study, the concordance and specificity of the Slug Mucosal Irritation test were higher than those of the HCE model (concordance and specificity of 74% and 50%, respectively); only the sensitivities of both alternative tests were comparable. Comparison of the HCE classification of 20 test substances in a multi-center prevalidation with the NI/I classification based on the Draize eye test data resulted in a concordance, sensitivity, and specificity of 70%, 100% and 45%, respectively (Van Goethem *et al.*, 2005).

Finally, the relevance of the modified assay was evaluated by using 12 chemicals that were not used for the development of the prediction model (Table 3.15). 75% of the four rabbit non-irritants were correctly predicted by the Slug Mucosal Irritation test. The correctly classified non-irritants (potassium tetrafluoroborate, tetra-aminopyrimidine sulphate, and ethyl acetate) were an inorganic chemical, a heterocyclic hydrocarbon or an ester. The overall classification of inorganic chemicals and hydrocarbons tested in the repeatability study was also in agreement with the classification based on the rabbit eye irritation test and/or alternative eye irritation tests (Table 3.14). Only methyl amyl ketone induced an increased mucus production and tissue damage and was consequently falsely predicted as irritant, while it was classified as non-irritant in the Draize eye test. Methyl amyl ketone was also classified as moderate eye irritant by means of the BCOP test (personal communication F. Van Goethem and Ph. Vanparys, J & J, Beerse, Belgium, 2004) and as irritant by means of the HCE model (Van Goethem *et al.*, 2005). Moreover, methyl amyl ketone is labelled “irritating to eyes” in the material safety data sheet of the supplier. It is striking that methyl amyl ketone and methyl isobutyl ketone – the two tested ketones labelled as non-irritant based on the rabbit eye irritation test – were not only overestimated in the experiments with the Slug Mucosal Irritation test, but also by the HCE model and the BCOP test or HET-CAM (Tables 3.14 and 3.15). Although anaesthetizing properties of these ketones are reported in the Hazard Substances Data bank (TOXNET®, 2005), the experiments showed that exposure of the slugs to these ketones induced a slower onset of anaesthesia than exposure of the slugs to the tested alcohols.

Table 3.15 Comparison of the irritation potential of the chemicals as assessed with the Draize eye test, Slug Mucosal Irritation (SMI) test, BCOP test, HET-CAM, and HCE model

Test substance	Draize test		SMI test	BCOP test [a]			HET-CAM [b]		HCE model [c]	
	EU class	GHS class	EU class	Class	Conc (%)	Source	Class	Conc (%)	Class	Conc (%)
Potassium tetrafluoroborate	NI	NI	NI						NI	100
Tetra-aminopyrimidine sulph.	NI	NI	NI	NI	20	G	NI	100	I	100
Methyl amyl ketone	NI	NI	R41	I (Mod)	100	PC			I	100
Ethyl acetate	NI	NI	NI	I (Mod)	100	B	I	100		
Methyl acetate	R36	Cat 2B	NI	I (Mod)	100	C	I	100		
Isopropanol	R36	Cat 2B	NI	I (Sev)	100	V	I	100		
γ-Butyrolactone	R36	Cat 2B	NI	I (Mod)	100	G	I	100		
1-Butanol	R36	Cat 2B	NI	I (Sev)	100	V				
Pyridine	R41	Cat 1	R41	I (Sev)	100	G	I	100		
Promethazine hydrochloride	R41	Cat 1	R41	I (Sev)	20	G	I	100		
Chlorhexidine	R41	Cat 1	R41	I (Sev)	20	B	I	100		
Trichloroacetic acid	R41	Cat 1	R41	I (Sev)	30	C	I	30		

Cat 1, irreversible effects on eyes; Cat 2B, mildly irritating to eyes; Conc, concentration; I, irritant; Mod, moderate; NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes; Sev, severe.

[a] BCOP *in vitro* scores and classifications were taken from different sources in the literature: (B) Balls *et al.*, 1995, (C) Casterton *et al.*, 1996, (G) Gautheron *et al.*, 1994, (PC) personal communication with F. Van Goethem and Ph. Vanparys, J & J, Beerse, Belgium, 2004 and (V) Vanparys *et al.*, 1993.

[b] HET-CAM classifications were taken from Gilleron *et al.*, 1997.

[c] HCE classifications of potassium tetrafluoroborate and tetra-aminopyrimide sulphate were obtained via personal communication with F. Van Goethem, J & J, Beerse, Belgium, 2004, whereas HCE classification of methyl amyl ketone was taken from Van Goethem *et al.*, 2005.

None of the four chemicals labelled as R36 on the basis of the rabbit eye irritation data were correctly predicted by the Slug Mucosal Irritation test. Methyl acetate, isopropanol, γ-butyrolactone, and 1-butanol were falsely predicted non-irritant based on the score for tissue damage. However, it has to be noted that the scores obtained for isopropanol and especially γ-butyrolactone were close to the NI-R36 score cut-off value. Of interest is the fact that not only the alcohols isopropanol and 1-butanol, but also the esters methyl acetate and γ-butyrolactone have anaesthetizing properties (ACGIH, 1991; Gill *et al.*, 1995; Kapp *et al.*, 1996; TOXNET®, 2005). Balls *et al.* (1999) stated that developers of an alternative test intended as a screening test in a stepwise strategy have to worry less about the generation of false negatives than about the generation of false positives, because chemicals falsely predicted as non-

irritant will normally be detected by the rabbit eye irritation test which is performed as last step of the stepwise testing strategy.

The four severe irritants (R41) were correctly predicted by the Slug Mucosal Irritation test. Two of these severe irritants (pyridine and promethazine hydrochloride) were heterocyclic hydrocarbons. As mentioned above, the overall classification of hydrocarbons tested in the repeatability study was also in agreement with the classification based on the rabbit eye irritation test and/or alternative eye irritation tests.

The results of this study demonstrate that by increasing the test concentration of the second contact period to 3.5% (w/v), the Slug Mucosal Irritation test procedure for the evaluation of the eye irritating and damaging properties of chemicals can be reduced to one day. The end points – mucus production and score for tissue damage – are measured objectively and both are complementary for a better prediction. The one-day test procedure seems to be a reliable method that can accurately classify chemicals other than alcohols and other than substances with anaesthetizing properties into three eye irritation categories corresponding with the EU label.

Because the results of the study are sufficiently promising, the transferability of the test and the inter-laboratory reproducibility will be assessed. Several benchmark chemicals will be tested by the participating laboratories using standard operation procedures. Any necessary refinements to the test protocol and the prediction model will be made in order to maximise the inter- and intra-laboratory reproducibility. Hartung *et al.* (2004) reported that this step contributes to the evaluation of the practicability of the test, the assessment of the extent of training needed to properly transfer the test to another laboratory, and the evaluation of the intra-laboratory and inter-laboratory variability of the test.

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CHAPTER 4: SLUG SPECIES- AND POPULATION-SPECIFIC EFFECTS ON THE TEST END POINTS

Based on Dhondt, M.M.M., Adriaens, E., Pinceel, J., Jordaens, K., Backeljau, T. and Remon, J.P. (accepted September 2005).

4.1 INTRODUCTION

In previous studies, the terrestrial slug *A. lusitanicus* collected in Belgium was always used as test organism. In order to evaluate the robustness of the Slug Mucosal Irritation test, it is important to investigate the possible effects of the used slug population and species on the end points of the test. Information about the robustness of the test is useful in case research laboratories or companies located outside Belgium appeal to the Slug Mucosal Irritation test or in case of problems with the availability of Belgian *A. lusitanicus*. Therefore, the present study investigated the effects of one Belgian and two Swiss *A. lusitanicus* populations and the effects of *Limax* species on both the end points of the Slug Mucosal Irritation test and the predicted eye irritation classification. It was opted to compare the Belgian *A. lusitanicus* with two conspecific populations from Switzerland in order to make sure that both geographical distance and ecological differentiation were as large as possible, without risking that different species were sampled. Furthermore, it was opted to compare *A. lusitanicus* with a slug species which is at least as big as *A. lusitanicus*, because the use of small slugs is not recommended from a practical viewpoint. Additionally, it was preferred that the selected species is widely distributed throughout the world. Based on these criteria, the medium-sized and widespread slug species *L. flavus* and *L. maximus* were selected. Furthermore, life spans of several years are recorded for *Limax* species, whereas *Arion* species have a life span of one year (South, 1992). It was opted to choose two species belonging to the same family to investigate if they affect the end points of the Slug Mucosal Irritation test and the eye irritation classification differently or not.

Because 28 eye reference chemicals were extensively tested in previous studies with the Slug Mucosal Irritation test (Adriaens and Remon, 2002; Adriaens *et al.*, 2005), the effects of the slug population and slug species on the test end points and on the eye irritation

classification were assessed using the same eye reference chemicals and the eye irritation/damage test procedure. The mucus productions and the scores for tissue damage of Swiss *A. lusitanicus* and Belgian *L. flavus* and *L. maximus* slugs were compared with those of Belgian *A. lusitanicus* slugs. Furthermore, the effects of the slug population and species on the predicted EU eye irritation classifications were investigated.

4.2 MATERIALS AND METHODS

4.2.1. Chemicals

1% and 3.5% (w/v) dilutions of the chemicals presented in Table 3.1 were prepared in PBS (pH 7.4). The negative control was PBS, whereas benzalkonium chloride was used as a positive control.

4.2.2 Eye irritation/damage test procedure of the Slug Mucosal Irritation test

Belgian *A. lusitanicus* and *L. flavus* were collected in local gardens along Varsenare and Aalter, whereas Belgian *L. maximus* slugs were collected in gardens along Aalter and Nazareth. The Swiss *A. lusitanicus* slugs were collected in Nuglar and Court (located 60 km from each other). The housing and feeding conditions were identical to the ones described in Chapter 3.

Each experiment contained five negative control slugs (PBS), five positive control slugs (benzalkonium chloride), and six series of five slugs each treated with the test chemicals. At the beginning of the experiment, the slugs on the one hand and the Petri dishes containing a membrane filter (cellulose acetate 0.45 μm, 90 mm; Sartorius AG, Goettingen, Germany) moistened with 2 ml of a 1% (w/v) dilution of the chemical on the other hand were weighed. Subsequently, the slugs were placed individually during 60 minutes in a Petri dish on the test medium. After this first contact period, the amount of mucus produced was measured by reweighing the Petri dishes containing the test medium (without the slugs). The mucus production was expressed as percentage (w/w) of the body weight. Next, the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. One hour later, the PBS samples were collected with a micropipette. Two hours after the end of the first contact period, the slugs were placed for 60 minutes in a Petri dish on a membrane filter moistened with 2 ml of a 3.5% (w/v) dilution of the chemical. After this second contact period, the slugs

were transferred to a fresh Petri dish and 1 ml PBS was added. The PBS samples were collected after 60 minutes. The samples were analysed immediately for the presence of proteins and LDH released from the slug body wall.

4.2.3 Analytical procedures

The protein and LDH determinations were performed according to the analytical procedures described in Chapter 3.

4.2.4 Data analysis

For each slug, the mucus production during the first contact period and the score for tissue damage were calculated. The score for tissue damage combines the protein release after the first and second contact period (P1 and P2) and the LDH release after the second contact period (LDH2) and was calculated as follows: P1 + P2 + 30 • LDH2. These values were used for the statistical analyses using the computer program SPSS (version 12.0; SPSS, Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

A two-way ANOVA was performed to investigate the population effect and species effect on the mucus production and the score for tissue damage. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If variances were unequal, the data were log-transformed. If no significant interaction was present, the main effects were interpreted separately from the interaction and a multiple comparison among pairs of means was performed using a Scheffé post hoc test. If a significant interaction between both factors was present, for each test substance the population or species effects were investigated with a Bonferroni post hoc test.

For both *L. flavus* and *L. maximus*, a classification prediction model for the determination of the EU eye irritation category of chemicals was developed using linear discriminant analysis. Various random subsets of the Slug Mucosal Irritation test data were used to calculate cut-off values to discriminate between the eye irritation categories. On the basis of these results and in-house experience, cut-off values were established and the chemicals were classified into one of the corresponding EU categories.

The agreement between the EU categories obtained with the different slug species was evaluated using the κ-statistics. A κ-value of 0.75 or higher suggests strong agreement above chance (Fleiss, 1981). The relevance of the assay using different slug populations and species

was evaluated by assessing the agreement between the eye irritation categories predicted by the Slug Mucosal Irritation test and the categories assigned on the basis of the Draize eye test. Concordance, sensitivity, and specificity were calculated as defined by Cooper *et al.* (1979). The chemicals were divided into three eye irritation categories for the determination of the concordance of the test, whereas they were divided into two classes, non-irritant (NI) and irritant (R36 and R41), for the determination of the sensitivity and specificity.

4.3 RESULTS

4.3.1 Slug population-specific effects on the test end points

The effect of the *A. lusitanicus* population on the test end points and on the eye irritation classification was investigated by comparing the data of two Swiss slug populations with the data of the Belgian slug population. For this purpose, the negative control and seven chemicals (including the positive control) covering the whole irritancy range were used.

The Belgian and the Swiss populations were put on 1% (w/v) dilutions of the chemicals during one hour and the mucus production was measured (Table 4.1). For the mucus production, two-way ANOVA testing revealed no significant interaction between the population and the chemical ($p = 0.346$). The chemical had a statistically significant effect on the mucus production ($p < 0.001$). The population had no statistically significant effect on the mucus production ($p = 0.750$). The average mucus productions of the Belgian population (4.2 ± 6.4%), the Swiss population from Nuglar (4.5 ± 7.8%), and the Swiss population from Court (5.5 ± 9.5%) were consequently not significantly different. Therefore, the Swiss populations can be used as test organism without modifying the mucus production cut-off values determined in Chapter 3 for Belgian *A. lusitanicus*.

Furthermore, all chemicals were tested with each of the three slug populations during a second contact period. The protein and LDH values after the two contact periods were used to calculate the scores for tissue damage (Table 4.2). The high score for tissue damage of Swiss *A. lusitanicus* from Court treated with 2-ethyl-1-hexanol was mainly due to the high release of LDH by these slugs after the second contact period (33 U/l.g which was six times higher than in both other populations). For the Swiss slugs from Nuglar treated with benzalkonium chloride, the LDH release after the second contact period (81 U/l.g) was twofold higher than the LDH release of the other slug populations. This resulted also in a high score for tissue damage.

Table 4.1 Effects of *A. lusitanicus* population and test substance on the mucus production

Test substance	Mucus production (%)		
	Belgian population	Swiss population from Nuglar	Swiss population from Court
PBS	0.2 ± 1.2	0.7 ± 0.7	0.0 ± 0.5
PEG 600	0.5 ± 0.7	0.2 ± 0.6	1.4 ± 1.1
Tween 20®	-0.3 ± 1.2	-0.5 ± 0.2	-0.1 ± 0.7
Acetone	1.4 ± 0.3	1.4 ± 0.5	-0.1 ± 1.6
2-Ethyl-1-hexanol	2.2 ± 1.0	1.5 ± 1.3	2.5 ± 2.4
4-Carboxybenzaldehyde	6.1 ± 3.6	4.2 ± 0.6	5.5 ± 2.7
Imidazole	5.6 ± 2.2	6.4 ± 3.4	8.5 ± 3.6
Benzalkonium chloride	18.1 ± 7.0	22.5 ± 8.8	26.1 ± 12.8
Average *	**4.2 ± 6.4**	**4.5 ± 7.8**	**5.5 ± 9.5**

Values are the mean ± standard deviation of 5 slugs; bold values are the mean ± standard deviation of 40 slugs.

Two-way ANOVA testing revealed no significant interaction and no significant population effect ($p > 0.05$), but a significant chemical effect ($p < 0.001$).

* There are no significant differences between the mucus productions of the three populations ($p > 0.05$, Two-way ANOVA).

Table 4.2 Effects of *A. lusitanicus* population and test substance on the score for tissue damage

Test substance	Score for tissue damage		
	Belgian slug population	Swiss slug population from Nuglar	Swiss slug population from Court
PBS*	41 ± 61	42 ± 52	58 ± 77
PEG 600*	10 ± 6	16 ± 11	38 ± 26
Tween 20®*	78 ± 14	67 ± 38	67 ± 25
Acetone*	102 ± 117	143 ± 123	145 ± 119
2-Ethyl-1-hexanol	407 ± 286 [a]	335 ± 244 [a]	1327 ± 768 [b]
4-Carboxybenzaldehyde*	80 ± 82	137 ± 91	179 ± 65
Imidazole*	848 ± 254	790 ± 186	616 ± 170
Benzalkonium chloride	1758 ± 1259 [a]	3159 ± 996 [b]	1912 ± 883 [a]
Average	**416 ± 718**	**586 ± 1068**	**543 ± 771**

Values are the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 40 slugs.

Two-way ANOVA testing revealed a significant interaction and a significant chemical effect ($p < 0.05$), but no significant population effect ($p > 0.05$).

* For this test substance, there are no significant differences between the scores of the three populations ($p > 0.05$, Bonferroni test).

[a, b] Scores of the slugs treated with this test substance marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

For the score for tissue damage, two-way ANOVA testing revealed a significant interaction between the population and the test substance ($p < 0.001$). The chemical had a statistically significant effect on the score ($p < 0.001$), whereas the population had no statistically significant effect on the score ($p = 0.178$). Only for 2-ethyl-1-hexanol and benzalkonium chloride, the score for tissue damage of one of the Swiss populations was significantly higher than the score of the Belgian population ($p < 0.05$, Bonferroni test). Consequently, the Swiss *A. lusitanicus* populations can be used as test organism without changing the score cut-off values determined in Chapter 3.

By means of the prediction model presented in Figure 3.1, the chemicals were classified into the EU eye irritation categories (Table 4.3). PBS and four of the seven chemicals (PEG 600, Tween 20®, acetone, and benzalkonium chloride) were classified into the same category by using each of the three slug populations. Comparison of the assigned categories predicted by the Swiss populations with those predicted by the Belgian population showed that 2-ethyl-1-hexanol, 4-carboxybenzaldehyde, and imidazole were classified in different categories by at least one population. For 4-carboxybenzaldehyde and imidazole, differences in EU classification were based on differences in mucus production. 2-Ethyl-1-hexanol was classified in different categories because of differences in the scores for tissue damage.

Table 4.3 Effects of *A. lusitanicus* population on the eye irritation classification

Test substance	Slug Mucosal Irritation test [a]			Draize eye test
	Belgian slug population	Swiss slug population from Nuglar	Swiss slug population from Court	Rabbit
PBS	NI	NI	NI	
PEG 600	NI	NI	NI	NI
Tween 20®	NI	NI	NI	NI
Acetone	R36	R36	R36	R36
2-Ethyl-1-hexanol	R36	R36	R41	R36
4-Carboxybenzaldehyde	R41	R36	R36	R36
Imidazole	R36	R41	R41	R41
Benzalkonium chloride	R41	R41	R41	R41

[a] EU eye irritation categories (NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes) were assigned using the data presented in Table 4.1 and Table 4.2 and the prediction model presented in Figure 3.1.

4.3.2 Slug species-specific effects on the test end points

The effects of the slug species on the end points of the Slug Mucosal Irritation test and on the eye irritation classification were investigated by comparing data of Belgian *L. flavus* and *L. maximus* with data of Belgian *A. lusitanicus*. For this purpose, the negative control and 28 eye reference chemicals (including the positive control) covering the entire irritancy range were used.

A. lusitanicus, *L. flavus*, and *L. maximus* were put on 1% (w/v) dilutions of the chemicals during one hour and the mucus production was measured (Table 4.4). For the mucus production, a two-way ANOVA revealed a statistically significant interaction between the chemical and the slug species ($p < 0.001$). Moreover, both the chemical and the species had a significant effect on the mucus production ($p < 0.001$). The mucus productions of *L. flavus* treated with PBS and 20 out of the 28 chemicals were higher than those of *A. lusitanicus* treated with the same substances. However, only for glycerol, PEG 600, Tween 20®, methyl isobutyl ketone, 2-methyl-1-pentanol, 1-hexanol, imidazole, cyclohexanol, and cetylpyridinium bromide, the mucus production of *L. flavus* was significantly higher than that of *A. lusitanicus* ($p < 0.05$, Bonferroni test). The mucus production of *L. flavus* was on average 1.4 ± 2.8% higher than that of *A. lusitanicus*. Based on linear discriminant analyses and on the fact that *L. flavus* treated with NI and R36 chemicals respectively produced 1.5 ± 1.6% and 0.9 ± 2.2% more mucus than *A. lusitanicus* treated with the same chemicals, the NI-R36 mucus production cut-off value was elevated to 4% for *L. flavus*. Treatment of *L. flavus* with R41 chemicals resulted in mucus productions that were on average 1.9 ± 5.0% higher in comparison with *A. lusitanicus*. Based on this observation and on linear discriminant analyses, the R36-R41 mucus production cut-off value was elevated to 8% for *L. flavus*.

The mucus productions of *L. maximus* treated with PBS and 23 out of the 28 chemicals were higher than those of *A. lusitanicus* treated with the same substances. For PBS, 3,3-dimethylpentane, Tween 20®, methyl isobutyl ketone, 2-methyl-1-pentanol, 1-octanol, 2-ethyl-1-hexanol, sodium lauryl sulphate, 1-hexanol, acetone, Triton X-100®, imidazole, cyclohexanol, and cetylpyridinium bromide, the mucus production of *L. maximus* was significantly higher than that of *A. lusitanicus* ($p < 0.05$, Bonferroni test). *L. maximus* produced significantly less mucus than *A. lusitanicus* slugs, when they were put on a 1% (w/v) dilution of 4-carboxybenzaldehyde ($p < 0.05$, Bonferroni test). The mucus production of *L. maximus* was on average 3.9 ± 5.6% higher than that of *A. lusitanicus*. *L. maximus* treated with NI and R36 chemicals respectively produced 1.7 ± 2.2% and 4.6 ± 6.7% more mucus

than *A. lusitanicus* treated with the same chemicals. Based on linear discriminant analyses, the NI-R36 mucus production cut-off value was elevated to 5% for *L. maximus*. Treatment of *L. maximus* with R41 chemicals resulted in mucus productions that were on average 7.3 ± 7.4% higher in comparison with *A. lusitanicus*. Based on linear discriminant analyses, the R36-R41 mucus production cut-off value was elevated to 10% for *L. maximus*.

All chemicals were also tested with each of the three slug species during a second contact period. Table 4.5 shows the scores for tissue damage. The high scores for tissue damage of *L. flavus* treated with Triton X-100® and benzalkonium chloride were due to high release of LDH after the second contact period (> 29 U/l.g), which was four times higher than the LDH release of *A. lusitanicus*. For *A. lusitanicus* treated with imidazole, the release of proteins after the second contact period (584 µg/ml.g) was twofold higher than the protein release of *L. flavus* and resulted in a high score for tissue damage. The higher score of *L. flavus* treated with cyclohexanol was due to high protein release after the second contact period (141 µg/l.g for *L. flavus* versus 23 µg/l.g for *A. lusitanicus*). The high scores for tissue damage of *L. maximus* treated with 2-methyl-1-pentanol, 1-octanol, sodium lauryl sulphate, 1-hexanol, Triton X-100®, cetylpyridinium bromide, and benzalkonium chloride were related to high release of LDH after the second contact period (> 8 U/l.g), which was at least three times higher than the LDH release of *A. lusitanicus*.

For the score for tissue damage, a two-way ANOVA revealed a statistically significant interaction between the chemical and the slug species ($p < 0.001$). The chemical and the species had a statistically significant effect on the score ($p < 0.001$). For Triton X-100® and benzalkonium chloride, the score of *L. flavus* was significantly higher than the score of *A. lusitanicus* ($p < 0.05$, Bonferroni test). For both chemicals, significantly different scores were also obtained throughout the five runs of the repeatability study with Belgian *A. lusitanicus* (Chapter 3). Therefore, the score cut-off values determined for *A. lusitanicus* were not modified for *L. flavus*.

For 2-methyl-1-pentanol, sodium lauryl sulphate, 1-hexanol, Triton X-100®, cetylpyridinium bromide, and benzalkonium chloride, the score of *L. maximus* was significantly higher than the score of *A. lusitanicus* ($p < 0.05$, Bonferroni test). For four out of these six chemicals (1-hexanol, Triton X-100®, cetylpyridinium bromide, and benzalkonium chloride), the scores for tissue damage obtained with *A. lusitanicus* during the repeatability study were also significantly different (Chapter 3). Consequently, the score cut-off values determined for *A. lusitanicus* were not modified for *L. maximus*.

Table 4.4 Effects of slug species and test substance on the mucus production

Test substance	Mucus production (%)		
	A. lusitanicus	*L. flavus*	*L. maximus*
PBS	0.3 ± 1.0 [a]	1.4 ± 0.8 [a, b]	2.4 ± 1.2 [b]
3,3-Dimethylpentane	0.0 ± 1.3 [a]	0.9 ± 0.9 [a, b]	1.9 ± 1.1 [b]
PEG 400*	1.3 ± 0.7	2.3 ± 0.7	1.5 ± 0.9
3-Methoxy-1,2-propanediol*	1.6 ± 0.7	2.1 ± 1.2	2.0 ± 0.8
Propylene glycol*	1.6 ± 0.5	1.9 ± 0.6	0.2 ± 0.6
Glycerol	1.4 ± 0.7 [a]	3.7 ± 1.7 [b]	2.4 ± 2.2 [a, b]
PEG 600	0.7 ± 1.5 [a]	3.2 ± 1.1 [b]	1.7 ± 1.9 [a, b]
Methylcyclopentane	0.7 ± 1.0 [a, b]	0.2 ± 0.9 [a]	2.2 ± 1.1 [b]
Tween 20®	-0.3 ± 1.2 [a]	2.4 ± 1.6 [b]	1.6 ± 1.1 [b]
Methyl isobutyl ketone	1.0 ± 0.9 [a]	5.9 ± 1.8 [b]	7.8 ± 2.4 [b]
Toluene*	2.5 ± 1.3	3.8 ± 1.3	4.1 ± 1.0
2-Methyl-1-pentanol	1.0 ± 0.8 [a]	3.8 ± 1.3 [b]	6.1 ± 2.4 [b]
Ethanol*	0.9 ± 0.9	0.7 ± 0.3	1.0 ± 0.8
Ammonium nitrate*	5.0 ± 1.7	5.0 ± 1.2	7.5 ± 1.5
1-Octanol	2.3 ± 0.9 [a]	4.4 ± 1.2 [a, b]	6.0 ± 2.3 [b]
4-Carboxybenzaldehyde	5.4 ± 1.8 [a]	3.7 ± 1.2 [a, b]	2.1 ± 0.5 [b]
2-Ethyl-1-hexanol	2.2 ± 1.0 [a]	2.6 ± 1.3 [a, b]	4.9 ± 1.3 [b]
Sodium lauryl sulphate	16.9 ± 3.7 [a]	17.6 ± 7.8 [a]	34.2 ± 3.5 [b]
1-Hexanol	1.6 ± 0.9 [a]	7.6 ± 1.9 [b]	12.3 ± 4.9 [c]
Acetone	1.4 ± 0.3 [a]	1.9 ± 0.8 [a]	5.1 ± 2.1 [b]
Triton X-100®	9.2 ± 2.5 [a]	11.7 ± 3.4 [a]	18.7 ± 3.0 [b]
p-Fluoroaniline*	4.1 ± 1.1	4.6 ± 2.3	3.6 ± 2.6
Lauric acid*	1.1 ± 0.8	0.9 ± 1.3	1.7 ± 0.6
Imidazole	5.6 ± 2.2 [a]	11.4 ± 2.4 [b]	13.4 ± 6.1 [b]
Sodium oxalate*	12.0 ± 2.6	11.2 ± 3.9	10.5 ± 5.2
Cyclohexanol	1.8 ± 0.6 [a]	7.4 ± 2.2 [b]	12.0 ± 3.4 [c]
Cetylpyridinium bromide	8.4 ± 0.7 [a]	16.7 ± 7.0 [b]	29.8 ± 5.0 [c]
Sodium hydroxide*	14.9 ± 3.5	10.4 ± 2.3	14.3 ± 2.4
Benzalkonium chloride	24.8 ± 2.8 [a, b]	20.6 ± 4.5 [a]	32.5 ± 2.8 [b]
Average	**4.5 ± 6.0**	**5.9 ± 5.9**	**8.4 ± 9.6**

Values are the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 145 slugs.

Two-way ANOVA testing revealed a significant interaction, a significant chemical effect and a significant species effect ($p < 0.001$).

* For this test substance, there are no significant differences between the mucus productions of the three species ($p > 0.05$, Bonferroni test).

[a, b, c] Mucus productions of the slugs treated with this test substance marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

Table 4.5 Effects of slug species and test substance on the score for tissue damage

Test substance	Score for tissue damage		
	A. lusitanicus	*L. flavus*	*L. maximus*
PBS*	30 ± 20	9 ± 4	23 ± 14
3,3-Dimethylpentane*	15 ± 13	11 ± 4	16 ± 6
PEG 400*	25 ± 21	11 ± 5	16 ± 6
3-Methoxy-1,2-propanediol*	11 ± 3	24 ± 10	12 ± 3
Propylene glycol*	9 ± 5	9 ± 3	21 ± 15
Glycerol*	23 ± 23	11 ± 3	17 ± 7
PEG 600*	18 ± 7	11 ± 11	24 ± 24
Methylcyclopentane*	28 ± 17	16 ± 9	25 ± 6
Tween 20®*	78 ± 14	27 ± 13	56 ± 27
Methyl isobutyl ketone*	493 ± 365	348 ± 362	217 ± 137
Toluene*	115 ± 86	108 ± 50	421 ± 220
2-Methyl-1-pentanol	312 ± 195 [a]	432 ± 149 [a, b]	935 ± 749 [b]
Ethanol*	20 ± 10	27 ± 13	25 ± 12
Ammonium nitrate*	218 ± 108	128 ± 149	88 ± 153
1-Octanol	409 ± 166 [a, b]	307 ± 145 [a]	914 ± 530 [b]
4-Carboxybenzaldehyde*	35 ± 33	44 ± 54	25 ± 10
2-Ethyl-1-hexanol*	408 ± 285	430 ± 353	845 ± 379
Sodium lauryl sulphate	310 ± 220 [a]	504 ± 176 [a]	1241 ± 532 [b]
1-Hexanol	185 ± 123 [a]	406 ± 189 [a]	2192 ± 1807 [b]
Acetone*	102 ± 117	124 ± 61	28 ± 14
Triton X-100®	451 ± 369 [a]	1375 ± 850 [b]	980 ± 517 [b]
p-Fluoroaniline*	103 ± 33	209 ± 96	440 ± 162
Lauric acid*	34 ± 22	13 ± 4	15 ± 5
Imidazole*	848 ± 254	492 ± 167	576 ± 368
Sodium oxalate*	446 ± 134	330 ± 94	408 ± 118
Cyclohexanol*	43 ± 35	214 ± 127	66 ± 48
Cetylpyridinium bromide	188 ± 214 [a]	475 ± 235 [a, b]	920 ± 395 [b]
Sodium hydroxide*	342 ± 317	251 ± 136	324 ± 66
Benzalkonium chloride	1310 ± 360 [a]	2869 ± 645 [b]	3940 ± 1579 [c]
Average	**228 ± 327**	**318 ± 599**	**511 ± 946**

Values are the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 145 slugs.

Two-way ANOVA testing revealed a significant interaction, a significant chemical effect and a significant species effect ($p < 0.001$).

* For this test substance, there are no significant differences between the scores for tissue damage of the three species ($p > 0.05$, Bonferroni test).

[a, b, c] Scores of the slugs treated with this test substance marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

Classification of the chemicals tested with *L. flavus* into the EU eye irritation categories by using the classification prediction model determined for *A. lusitanicus* (Figure 3.1) resulted in overestimation of the eye irritation potential of rabbit non-irritants (Table 4.6). Overestimation of the eye irritating properties of glycerol and PEG 600 was due to the fact that the mucus production of *L. flavus* was higher than 3% (NI-R36 mucus cut-off value for *A. lusitanicus*). The κ-value for the agreement between the classifications (of the 29 test substances) predicted by *A. lusitanicus* and *L. flavus* was 0.74 ± 0.10. When the chemicals tested with *L. flavus* were classified into the eye irritation categories by using the *L. flavus* cut-off values presented in Figure 4.1, there was a strong agreement between the classifications of both species (κ-value of 0.79 ± 0.10). PBS and 25 out of the 28 chemicals were classified into the same irritation category by the two species. *L. flavus* classified 4-carboxybenzaldehyde, imidazole, and cyclohexanol into different categories compared to *A. lusitanicus*. 4-Carboxybenzaldehyde and imidazole were classified in different categories because of differences in the mucus production. For cyclohexanol, differences in EU classification were based on differences in mucus production and score for tissue damage.

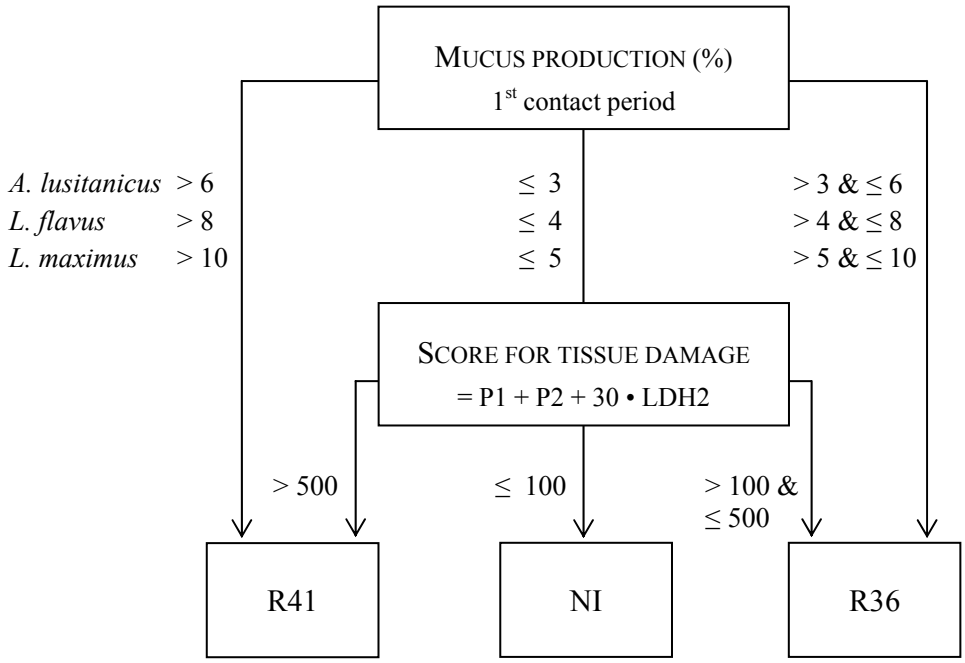

Figure 4.1 Prediction model of the Slug Mucosal Irritation test for the determination of the EU eye irritation category of chemicals using *A. lusitanicus*, *L. flavus* or *L. maximus* as test organism (P1 and P2, protein release after 1st and 2nd contact period; LDH2, LDH release after 2nd contact period; NI, non-irritant; R36, irritating to eyes; R41, risk of serious damage to eyes).

Table 4.6 Effects of test organism and test substance on the eye irritation classification

Test substance	Slug Mucosal Irritation test			Draize eye test
	A. lusitanicus	*L. flavus*	*L. maximus*	Rabbit
PBS	NI [a]	NI [a, b]	NI [a, c]	
3,3-Dimethylpentane	NI [a]	NI [a, b]	NI [a, c]	NI
PEG 400	NI [a]	NI [a, b]	NI [a, c]	NI
3-Methoxy-1,2-propanediol	NI [a]	NI [a, b]	NI [a, c]	NI
Propylene glycol	NI [a]	NI [a, b]	NI [a, c]	NI
Glycerol	NI [a]	R36 [a] / NI [b]	NI [a, c]	NI
PEG 600	NI [a]	R36 [a] / NI [b]	NI [a, c]	NI
Methylcyclopentane	NI [a]	NI [a, b]	NI [a, c]	NI
Tween 20®	NI [a]	NI [a, b]	NI [a, c]	NI
Methyl isobutyl ketone	R36 [a]	R36 [a, b]	R41 [a] / R36 [c]	NI
Toluene	R36 [a]	R36 [a, b]	R36 [a, c]	NI
2-Methyl-1-pentanol	R36 [a]	R36 [a, b]	R41 [a] / R36 [c]	NI
Ethanol	NI [a]	NI [a, b]	NI [a, c]	NI
Ammonium nitrate	R36 [a]	R36 [a, b]	R41 [a] / R36 [c]	R36
1-Octanol	R36 [a]	R36 [a, b]	R36 [a, c]	R36
4-Carboxybenzaldehyde	R36 [a]	R36 [a] / NI [b]	NI [a, c]	R36
2-Ethyl-1-hexanol	R36 [a]	R36 [a, b]	R36 [a] / R41 [c]	R36
Sodium lauryl sulphate	R41 [a]	R41 [a, b]	R41 [a, c]	R36
1-Hexanol	R36 [a]	R41 [a] / R36 [b]	R41 [a, c]	R36
Acetone	R36 [a]	R36 [a, b]	R36 [a, c]	R36
Triton X-100®	R41 [a]	R41 [a, b]	R41 [a, c]	R36
p-Fluoroaniline	R36 [a]	R36 [a, b]	R36 [a, c]	R36
Lauric acid	NI [a]	NI [a, b]	NI [a, c]	R41
Imidazole	R36 [a]	R41 [a, b]	R41 [a, c]	R41
Sodium oxalate	R41 [a]	R41 [a, b]	R41 [a, c]	R41
Cyclohexanol	NI [a]	R41 [a] / R36 [b]	R41 [a, c]	R41
Cetylpyridinium bromide	R41 [a]	R41 [a, b]	R41 [a, c]	R41
Sodium hydroxide	R41 [a]	R41 [a, b]	R41 [a, c]	R41
Benzalkonium chloride	R41 [a]	R41 [a, b]	R41 [a, c]	R41

[a] The *A. lusitanicus* cut-off values presented in Figure 3.1 were used to assign EU eye irritation categories based on the data presented in Table 4.4 and Table 4.5.

[b] The *L. flavus* cut-off values presented in Figure 4.1 were used to assign EU eye irritation categories based on the data presented in Table 4.4 and Table 4.5.

[c] The *L. maximus* cut-off values presented in Figure 4.1 were used to assign EU eye irritation categories based on the data presented in Table 4.4 and Table 4.5.

When the chemicals tested with *L. maximus* were classified into the EU eye irritation categories using the prediction model presented in Figure 3.1, the irritation potential of non-irritants and chemicals labelled with EU risk phrase R36 based on the Draize eye irritation test was overestimated with *L. maximus* (Table 4.6). The κ-value for the agreement between the EU categories (of the 29 test substances) predicted by *A. lusitanicus* and those predicted by *L. maximus* was 0.64 ± 0.11. Classification of the chemicals tested with *L. maximus* into the EU eye irritation categories by using the *L. maximus* cut-off values presented in Figure 4.1 resulted in a good agreement between the classifications of both species (κ-value of 0.74 ± 0.10). PBS and 23 out of the 28 chemicals were classified into the same irritation category by the two species. *L. maximus* classified 4-carboxybenzaldehyde, 2-ethyl-1-hexanol, 1-hexanol, imidazole, and cyclohexanol in different categories in comparison with *A. lusitanicus*. For 4-carboxybenzaldehyde and imidazole, differences in EU classification were based on differences in mucus production. 2-Ethyl-1-hexanol was classified differently because of differences in the score for tissue damage. For 1-hexanol and cyclohexanol, differences in EU classification were based on differences in mucus production and score for tissue damage.

4.4 DISCUSSION

The slug *A. lusitanicus* was hitherto always used as test organism in the Slug Mucosal Irritation test. In this study, the effects of other slug populations and species on the test end points and on the predicted eye irritation classification were evaluated in order to get an idea of the robustness of the test. Based on the criteria mentioned in the introduction of this chapter, it was opted to compare the data of Belgian *A. lusitanicus* with two Swiss *A. lusitanicus* populations on the one hand and with Belgian *L. flavus* and *L. maximus* species on the other hand.

The effects of the *A. lusitanicus* population on the mucus production, the score for tissue damage, and the EU eye irritation classification were investigated by comparing the data of two Swiss *A. lusitanicus* populations with the data of the Belgian slug population. The population had no statistically significant effect on both the mucus production and score for tissue damage. Moreover, the mucus production and the score of the negative and positive control slugs of the three slug populations met the acceptance criteria determined for Belgian *A. lusitanicus*. The mucus production and score for tissue damage of each of the populations treated with PBS were less than 2.0% and 100, respectively. For the *A. lusitanicus* populations treated with benzalkonium chloride, the mucus production and score were more than 11.0%

and 850, respectively. When the test substances were classified into EU categories using the prediction model presented in Figure 3.1, three of the eight test substances (2-ethyl-1-hexanol, 4-carboxybenzaldehyde, and imidazole) were classified differently (R36 or R41) by one of the *A. lusitanicus* populations. However, for those chemicals the same switch between R36 and R41 was seen in the repeatability study with Belgian *A. lusitanicus* described in Chapter 3. Therefore, the Swiss *A. lusitanicus* populations can be used as test organism without changing the mucus production and score cut-off values determined in Chapter 3.

Comparison of the eye irritation categories based on the Slug Mucosal Irritation test with the categories assigned on the basis of the rabbit eye irritation data (ECETOC, 1998; EC, 2001) showed that the three rabbit non-irritants were predicted as NI with each of the three slug populations. Furthermore, the five chemicals labelled as irritant (R36 or R41) based on the rabbit eye test were also classified as irritant with the three slug populations. So the results indicate that the Swiss *A. lusitanicus* populations can be used for the evaluation of the eye irritating and damaging properties of chemicals.

The effect of the slug species on the end points of the Slug Mucosal Irritation test was investigated by comparing data of Belgian *L. flavus* and *L. maximus* with data of Belgian *A. lusitanicus*. Firstly, the effect of the slug species on the end point mucus production was investigated. For PBS and 20 out of the 28 chemicals, the mucus production of *L. flavus* was higher than the mucus production of *A. lusitanicus*. For nine out of the 28 chemicals (five rabbit non-irritants, one chemical labelled as R36 based on the rabbit eye irritation data, and three chemicals with EU risk phrase R41 among which five alcohols and ketones), the mucus production of *L. flavus* was also significantly higher than that of *A. lusitanicus*. When 3% was used as NI-R36 mucus production cut-off value (such as in the classification prediction model for *A. lusitanicus*), the irritation potential of non-irritants (e.g. glycerol, PEG 600) was overestimated with *L. flavus*. However, it is important that an alternative test does not generate too many false positives (Gilleron *et al.*, 1996; Gilleron *et al.*, 1997; Balls *et al.*, 1999). Interestingly, the mucus production of the negative control *L. flavus* slugs did not meet the acceptance criteria established for *A. lusitanicus* in two of the seven experiments, because *L. flavus* slugs treated with PBS produced more than 2% mucus (data not shown). Based on linear discriminant analyses and in-house experience, the NI-R36 and R36-R41 mucus production cut-off values for *L. flavus* were elevated to 4% and 8%, respectively.

For PBS and 23 out of the 28 chemicals, the mucus production of *L. maximus* was higher than the mucus production of *A. lusitanicus*. For PBS and 13 out of the 28 chemicals (four rabbit non-irritants, six chemicals labelled as R36 based on the rabbit eye irritation data,

and three chemicals with EU risk phrase R41 among which five alcohols), the mucus production of *L. maximus* was also significantly higher than that of *A. lusitanicus*. In one of the four experiments, the mucus production of the negative control *L. maximus* slugs did not meet the acceptance criteria established for the mucus production of negative control *A. lusitanicus* slugs. Based on statistical analyses and in-house experience, the NI-R36 and R36-R41 mucus production cut-off values for *L. maximus* were elevated to 5% and 10%, respectively.

For 18 out of the 28 chemicals, significant differences between the mucus productions of the three slug species were observed. It is interesting to note that for ten of these 18 chemicals, the mucus productions obtained with *A. lusitanicus* were also significantly different throughout the five runs of the repeatability study (Chapter 3). Furthermore, six of these 18 chemicals belonged to the chemical classes of the alcohols. The higher mucus production of *L. flavus* and *L. maximus* in comparison with *A. lusitanicus* is probably related to the fact that the *Limax* species are more active and move more than *A. lusitanicus*. Mucus secretions namely serve for the locomotion of the slugs (South, 1992; Deyrup-Olsen and Luchtel, 1998). This hypothesis is supported by the fact that in several experiments the mucus productions of the negative control *Limax* species did not meet the acceptance criteria established for negative control *A. lusitanicus* slugs.

Next, the effect of the slug species on the score for tissue damage was evaluated. The scores for tissue damage of *L. flavus* were not significantly different from those of *A. lusitanicus* for PBS and 26 out of the 28 chemicals. For each of the two chemicals that induced significantly different scores (Triton X-100® and benzalkonium chloride), the scores obtained with *A. lusitanicus* throughout the repeatability study described in Chapter 3 were also significantly different. The scores of *L. maximus* did not significantly differ from the scores of *A. lusitanicus* for PBS and 22 out of the 28 chemicals. Four of the six chemicals with significant differences between the scores of *L. maximus* and *A. lusitanicus* induced also significantly different scores in the repeatability study with *A. lusitanicus* (Chapter 3). When only those chemicals that induced a mucus production by the *Limax* species lower than their NI-R36 mucus production cut-off values were classified based on the score for tissue damage, none of the scores of *L. flavus* resulted in different predicted eye irritation classes in comparison with *A. lusitanicus*, whereas only the higher score of *L. maximus* treated with 2-ethyl-1-hexanol resulted in a different predicted EU category in comparison with *A. lusitanicus*. For 2-ethyl-1-hexanol, a similar switch between R36 and R41 was seen in the

repeatability study with *A. lusitanicus* as described in Chapter 3. Therefore, the score cut-off values determined for *A. lusitanicus* were not modified for *L. flavus* and *L. maximus*.

Further, the effect of the slug species on the eye irritation classification was investigated. Classification of the test substances tested with *A. lusitanicus*, *L. flavus*, and *L. maximus* into the EU categories by means of their corresponding prediction model (Figure 4.1) resulted in the same eye irritation categories by the three species for PBS and 23 of the 28 chemicals. 2-Ethyl-1-hexanol, 1-hexanol, and imidazole were classified as R36 or R41 dependent on the used slug species. However, for these chemicals, the EU categories obtained with both *Limax* species were comparable to those obtained with *A. lusitanicus* in the repeatability study described in Chapter 3. 4-Carboxybenzaldehyde was classified as NI or R36 dependent on the used slug species, whereas cyclohexanol was classified as NI, R36 or R41.

Comparison of the EU eye irritation categories predicted by the Slug Mucosal Irritation test with the EU categories assigned on the basis of the rabbit eye irritation data (ECETOC, 1998; EC, 2001) revealed that 71% of the 28 chemicals were correctly classified into three eye irritation categories by *A. lusitanicus* and *L. flavus*, whereas 68% of the 28 chemicals were correctly classified by *L. maximus*. For the three species, two of the 16 rabbit irritants were falsely predicted non-irritant by the Slug Mucosal Irritation test, which resulted in a sensitivity of 88%. *A. lusitanicus* predicted lauric acid and cyclohexanol falsely as non-irritants, whereas *L. flavus* and *L. maximus* underestimated lauric acid and 4-carboxybenzaldehyde. For lauric acid, false negative results were also obtained in five repeated experiments with *A. lusitanicus* (Chapter 3). 4-Carboxybenzaldehyde was classified as non-irritant by means of the HET-CAM (Gilleron *et al.*, 1997). Three of the 12 rabbit non-irritants - methyl isobutyl ketone, toluene, and 2-methyl-1-pentanol - were overestimated (false positives) by each of the three slug species resulting in a specificity of 75%. However, those chemicals were also overestimated in five repeated experiments of the repeatability study with *A. lusitanicus* (Chapter 3). As mentioned in Chapter 3, several studies reported the eye irritating potential of these chemicals (Grant, 1986; Hjelm *et al.*, 1990; Vanparys *et al.*, 1993; Gilleron *et al.*, 1997; Van Goethem *et al.*, 2005). Based on these results, it is concluded that *L. flavus* and *L. maximus* can be used for the evaluation of the eye irritation/damage potential of chemicals without considerable decrease of the concordance and specificity of the test.

It is interesting to note that in case another slug species is effectively used as test organism instead of *A. lusitanicus*, the repeatability of the assay with the selected slug species

has also to be evaluated. This study included no investigation of the effects of slug species on the repeatability of the assay, because the use of Belgian *A. lusitanicus* as test organism is preferred to the use of other species. Indeed, both an extensive list of data and test validity criteria are available for *A. lusitanicus*, so that future results obtained with *A. lusitanicus* can be compared directly with previous data.

The results of this study indicate that the geographic and ecological origins of the tested slug populations influence neither the end points of the Slug Mucosal Irritation test nor the eye irritation classification. So no matter which *A. lusitanicus* population can probably be used as test organism in the Slug Mucosal Irritation test without affecting the test end points. However, because this study demonstrates that the use of other slug species can influence the test end points and the eye irritation classification, it is important to evaluate the effects of the selected species. So if another slug species is used instead of *A. lusitanicus*, the test procedure and prediction model have to be optimised and validated by means of benchmark chemicals.

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CHAPTER 5: EVALUATION OF MUCOSAL TOLERANCE OF BUCCAL POWDER FORMULATIONS

Based on Adriaens, E., Ameye, D., Dhondt, M.M.M., Foreman, P. and Remon, J.P. (2003).

5.1 INTRODUCTION

The buccal mucosa offers an alternative route for the delivery of e.g. peptides. Several attempts have been made to increase the bioavailability of buccal administered drugs (Veuillez *et al.*, 2001). Buccal powder formulations frequently contain mucoadhesive polymers which allow close contact between the formulation and the mucosa and which enable high drug concentrations for prolonged periods (Jacques and Buri, 1998). Such formulations may be applied frequently over a period of months or years. Because irritation and damage of the buccal mucosa can break the protective barrier against micro-organisms and noxious substances, it is important to evaluate the mucosal tolerance of buccal bioadhesive powder formulations.

5.2 ORAL MUCOSA

The human oral cavity is lined with a stratified squamous epithelium (Marieb, 1995). The mucosae of the soft palate, the sublingual and buccal regions are non-keratinised (Harris and Robinson, 1992). The epithelium of the gingiva, hard palate, inner side of the lips, and dorsum of the tongue is slightly keratinised for extra protection against mechanical stress during eating (Marieb, 1995). Mucus is secreted by the submandibular, buccal and sublingual glands. Those glands consist not only of mucous cells, but also of serous cells (Marieb, 1995).

5.3 EVALUATION OF ORAL TOLERANCE OF FORMULATIONS

The evaluation of the local tolerance of newly developed medicinal products has to be performed in laboratory experiments prior to human exposure to the product (EC, 1990).

Buccal tolerance studies of pharmaceuticals are carried out in vertebrates in compliance with EU directives and guidelines (EC, 1965; EC, 1975a; EC, 1975b; EC, 1990). Buccal tolerance testing is required by regulatory authorities for medicinal products intended for buccal administration on the one hand and for products which are not envisaged for buccal delivery but can come accidentally into contact with the buccal mucosa on the other hand. Local tolerance testing is usually conducted with the definitive formulation consisting of the drug in its vehicle at the concentration intended for human use. The frequency and duration of administration to animals need to be determined by the proposed conditions of administration in clinical use (EC, 1990).

Several pre-clinical *in vivo* and *in vitro* models have been developed to study the impact of locally applied drugs, excipients, pharmaceutical formulations, personal care products or dental materials on the oral mucosa. Histological changes of the oral mucosa have been investigated *in vivo* using small animals such as rats and hamsters by macroscopic or microscopic examination (Cutright *et al.*, 1974; Piliero *et al.*, 1979; Lemons *et al.*, 1980; Roy and Wishe, 1986; Harsanyi *et al.*, 1987; Harsanyi *et al.*, 1991; Cummins *et al.*, 1996; Namiki *et al.*, 1998). The hamster cheek pouch test is recommended for biocompatibility testing of dental materials (Council on Dental Materials and Devices, 1972; ANSI/ADA, 1979). The test materials are retained in the hamster cheek pouch by closing the pouch by means of sutures (Council on Dental Materials and Devices, 1972), by sewing a perforated disk made of the test material directly onto the inside of the cheek pouch (ANSI/ADA, 1979) or by placing the disk of test material in a pouch created within the hamster cheek pouch (Harsanyi *et al.*, 1987; Harsanyi *et al.*, 1991). However, the use of the hamster cheek pouch as model of the human buccal mucosa is not ideal due to the keratinisation of the hamsters' cheek mucosa (Garren and Repta, 1989). Because of the histological similarities between the non-keratinised buccal mucosa of dogs and the human buccal mucosa, the effects of locally applied products on the oral mucosa have also been evaluated macroscopically or microscopically using dogs (Zhang *et al.*, 1994; Redlich *et al.*, 1996; Wirthlin *et al.*, 1997; Tiwari *et al.*, 1999).

The irritating and tissue damaging properties of products applied locally in the oral cavity have also been studied *in vitro*. The cell viability of immortalised human cell lines consisting of buccal cells (Burgalassi *et al.*, 1996) or gingival epithelial cells and gingival fibroblasts (Babich and Babich, 1997; Tipton *et al.*, 2003) after exposure to bioadhesive formulations or ingredients of orally applied products has been evaluated using various assays. The effects of exposure of reconstructed three dimensional human buccal and gingival tissue to the products of interest have been evaluated by means of histology and viability

assays (Kubilus *et al.*, 2004; SkinEthic, 2005). Furthermore, the HET-CAM is not only used as an alternative test for eye irritation (Gilleron *et al.*, 1996; Gilleron *et al.*, 1997; Steiling *et al.*, 1999) but also for predicting the irritation potential of dental materials (Schendel *et al.*, 1994; Schendel *et al.*, 1995; Dahl, 1999; Dahl and Polyzois, 2000; Syverud *et al.*, 2001).

5.4 OBJECTIVES

The availability of a reliable and relevant alternative method for repeated dose local tolerance testing would be very useful for academic and pharmaceutical research centres to screen pharmaceuticals intended for e.g. repeated buccal application. In the present study, it was investigated if the five-day (Slug Mucosal Irritation) test procedure – currently used for local tolerance testing of nasal bioadhesive powders at the start of this research work– was useful to evaluate the concentration and formulation effects of bioadhesive powder formulations intended for buccal administration. In analogy with the studies of Callens *et al.* (2001), Ceulemans *et al.* (2001) and Dhondt (2001), the slugs were put on 20 mg neat formulation for 30 minutes during five successive days. Untreated slugs and slugs treated with drum dried waxy maize starch/benzalkonium chloride (DDWM/BAC) 95/5 (w/w) were used as negative and positive control, respectively (Callens *et al.*, 2001; Ceulemans *et al.*, 2001; Dhondt, 2001). A lyophilised mixture of DDWM/BAC 95/5 (w/v) was used as positive control in these studies, because a 12-day nasal administration of this powder to rabbits induced ulcerations and an increased protein and LDH release from the nasal mucosa (Callens *et al.*, 2001). Moreover, this mixture induced severe irritation and damage of the slug mucosa as was demonstrated by a high mucus production, a high protein release and an increased enzyme release (Callens *et al.*, 2001; Ceulemans *et al.*, 2001; Dhondt, 2001).

For Amioca® starch/polyacrylic acid and Amioca® starch/Carbopol® 974P co-spray dried powders, the influence of the concentration of linear polyacrylic acid and cross-linked polyacrylic acid on the mucosal tolerance was evaluated. Additionally, the effect of heat treatment, the influence of dilution of co-spray dried Amioca®/polyacrylic acid powder mixtures, and the effect of the mixture process of Amioca®/Carbopol® 974P powders were investigated. In order to investigate the repeatability of the test procedure, the negative and positive control and some powders were tested independently on two or more separate occasions. The results of the Slug Mucosal Irritation test were compared with available *in vivo* data on the local tolerance of the tested powders. Based on the results of this study and data previously obtained with the Slug Mucosal Irritation test on the one hand and on pre-clinical

and clinical data on the other hand, a classification prediction model was developed for the determination of the irritation and tissue damage potential of formulations.

5.5 MATERIALS AND METHODS

5.5.1 Chemicals

Drum-dried waxy maize starch (DDWM), containing 99.9% amylopectin, was obtained from Eridania-Béghin Say Cerestar (Vilvoorde, Belgium). Carbopol® 974P (Carb) was supplied by B.F. Goodrich (Cleveland, OH, USA). Benzalkonium chloride (BAC) and linear polyacrylic acid (PAA, MW 250 000) were purchased from Sigma (St. Louis, MO, USA). Amioca® starch, a pregelatinised amylopectin corn starch, was obtained from National Starch and Chemical Company (Bridgewater, NJ, USA).

5.5.2 Powder formulations

The positive control powder DDWM/BAC 95/5 (w/w) was prepared by dispersing DDWM in a benzalkonium chloride solution. After neutralisation of the dispersion with sodium hydroxide 0.2 N, the mixture was lyophilised in vials using an Amsco-Finn Aqua GT4 freeze-dryer (Hürth, Germany) and passed through a 63-µm sieve. The sieved powder fraction was stored in a desiccator at 4-8°C until use.

The Amioca®/polyacrylic acid and the Amioca®/Carbopol® 974P co-spray dried mixtures were prepared by National Starch and Chemical Company. To obtain the co-spray dried Amioca®/polyacrylic acid mixtures, an aqueous mixture of Amioca® starch and a linear polyacrylic acid was co-spray dried. The used ratios of Amioca®/PAA were 50/50 and 25/75 (w/w). After co-spray drying, a part of the yielded powder was additionally heat-treated (HT) in an oven at 120°C for 15 minutes. The dilutions of the co-spray dried Amioca®/PAA 50/50 (w/w) with the non-irritating DDWM were prepared by physical blending of the two powders. The used DDWM concentrations were 10%, 20%, 40%, 60% and 80% (w/w).

The co-spray dried Amioca®/Carbopol® 974P mixtures were prepared by co-spray drying an aqueous mixture of Amioca® starch and Carbopol® 974P. The tested ratios of Amioca®/Carbopol® 974P were 95/5, 90/10, 85/15, 80/20, 75/25, 60/40 and 50/50 (w/w). The Amioca®/Carbopol® 974P physical mixtures were prepared by physical blending Amioca® and Carbopol® 974P in a mortar with a pestle. The tested ratios were 95/5, 90/10 and 75/25 (w/w).

5.5.3 Local tolerance test procedure of the Slug Mucosal Irritation test

Belgian *A. lusitanicus* slugs were used as test organisms. The housing and feeding conditions of the slugs were identical to the ones described in Chapter 3.

After weighing of the slugs on the one hand and the Petri dishes with the neat test substance on the other hand, the slugs were placed individually in the Petri dish on the neat test substance for 30 minutes. Each experiment contained five untreated negative control slugs (blank), five positive control slugs, and six series of five slugs each treated with the test formulations. After the contact period, the amount of mucus produced during the 30-minute contact period was measured by reweighing the Petri dishes containing the test substance (without the slugs). The mucus production was expressed as a percentage (w/w) of the body weight. Additionally, the reduction in body weight caused by the treatment was measured and was expressed as a percentage (w/w) of the initial body weight. Immediately after the contact period, the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. After 30 minutes, the PBS samples were collected with a micropipette. Then, the slugs were placed in a fresh Petri dish and again 1 ml PBS was added. After one hour, the PBS samples were again collected. Subsequently, the slugs were placed in a fresh Petri dish and again 1 ml PBS was added for one hour. The PBS samples were analysed immediately for the presence of proteins, LDH, and ALP released from the body wall of the slugs. The slugs were placed in a Petri dish on a membrane filter (cellulose acetate 0.45 µm, 90 mm; Sartorius AG, Goettingen, Germany) moistened with 2 ml PBS until the next contact period and were fed with commercial dog food. This procedure was repeated during five successive days.

5.5.4 Analytical procedures

The protein, LDH, and ALP determinations were performed according to the analytical procedures described in Chapter 3.

5.5.5 Data analysis

For each slug, the total mucus production was calculated by adding up the amounts of mucus produced during each contact period. Because all the treatments resulted in a high protein release on the first day of treatment, the mean protein concentrations were calculated without using the data of the samples collected on the first day (Callens *et al.*, 2001), which

allowed a more defined distinction between non-irritating and irritating formulations. Furthermore for each slug, the mean LDH release and the mean ALP release were calculated using the data of all samples collected on the five days. These values were used for the statistical analyses using the computer program SPSS (version 12.0; SPSS, Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Statistically significant differences between repeated experiments or different treatments were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal, the data were transformed to their logarithm. To further compare the effects of the different treatments, a multiple comparison among pairs of means was performed using a Scheffé test.

A two-way ANOVA was performed to investigate the effect of Amioca®/Carbopol® 974P ratio and the effect of mixing process on the end points. The normal distribution of the data and the homogeneity of variances were tested with the tests mentioned above. Because no significant interaction was present, the main effects were interpreted separately from the interaction and a multiple comparison among pairs of means was performed using a Scheffé post hoc test.

5.6 RESULTS

5.6.1 Repeatability of the test procedure

In order to investigate the repeatability of the test results obtained with the test procedure for local tolerance testing of powder formulations, the negative control (blank) and the positive control (DDWM/BAC 95/5) were tested independently on seven separate occasions. Table 5.1 shows the total mucus production, mean protein and LDH release of the negative control slugs and the number of slugs for which ALP release was detected for each of the repeated experiments and the mean of the seven experiments. All the blank slugs survived a repeated treatment on five successive days. For the negative control, ANOVA testing suggested no significant difference in total mucus production and in mean protein release between the repeated experiments ($p > 0.05$) (Table 5.1).

A daily treatment with DDWM/BAC 95/5 led to 20%, 20%, 0%, 60%, 80%, 0% and 20% mortality by day 5 (before the 5th treatment) in the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th experiment, respectively. For the positive control, ANOVA testing suggested no significant

difference in total mucus production, in mean protein release, and in mean LDH release between the repeated experiments ($p > 0.05$) (Table 5.2).

Table 5.1 Intra- and inter-experiment variability for the total mucus production and mean protein release of the negative control blank slugs

Experiment	Total mucus production (%) *	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Experiment 1	1.1 ± 0.4	9 ± 3	–	0	5
Experiment 2	-0.8 ± 0.7	18 ± 12	–	0	5
Experiment 3	-0.6 ± 0.7	8 ± 5	–	0	5
Experiment 4	0.9 ± 0.8	9 ± 5	–	0	5
Experiment 5	0.8 ± 1.0	5 ± 2	–	0	5
Experiment 6	-0.8 ± 1.0	16 ± 14	–	0	5
Experiment 7	-0.8 ± 1.1	12 ± 4	–	0	5
Inter-experiment mean	**0.0 ± 0.9**	**11 ± 5**	–		**7**

Total mucus production and mean protein release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 7 experiments; -, below the detection limit.

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

Table 5.2 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the positive control slugs treated with 20 mg DDWM/BAC 95/5 on 5 successive days

Experiment	Total mucus production (%) *	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g) *	Number of slugs with ALP release	n
Experiment 1	18.8 ± 2.6	165 ± 72	10.8 ± 5.5	4	5
Experiment 2	17.9 ± 5.2	223 ± 122	10.7 ± 6.5	1	5
Experiment 3	17.9 ± 4.1	109 ± 62	2.8 ± 2.3	3	5
Experiment 4	20.0 ± 7.2	111 ± 61	1.8 ± 0.9	4	5
Experiment 5	12.3 ± 8.1	241 ± 93	2.4 ± 2.7	1	5
Experiment 6	17.5 ± 6.7	134 ± 77	5.8 ± 4.6	2	5
Experiment 7	20.0 ± 3.5	126 ± 71	6.2 ± 3.6	3	5
Inter-experiment mean	**17.8 ± 2.6**	**151 ± 54**	**5.8 ± 3.8**		**7**

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 7 experiments.

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

5.6.2 Evaluation of local tolerance of buccal powder formulations

5.6.2.1 Local tolerance of Amioca®/PAA co-spray dried mixtures

The mucosal tolerance of Amioca®/PAA co-spray dried powders intended to prepare formulations for buccal administration was evaluated by placing the slugs on 20 mg powder for 30 minutes during five successive days. All untreated slugs and all slugs exposed to each of the heat-treated or not heat-treated Amioca®/PAA mixtures survived the five-day experiment. Only a repeated treatment with DDWM/BAC 95/5 caused 20%, 0%, 60%, 80% and 20% mortality by day 5 in the 1st, 2nd, 3rd, 4th and 5th experiment, respectively.

The effects of a repeated treatment on the total mucus production, mean protein and enzyme release are summarised in Table 5.3. All the co-spray dried mixtures produced significantly more mucus than the untreated slugs ($p < 0.001$, Scheffé test). The total amount of mucus production increased with an increasing PAA concentration. Heat treatment after co-spray drying had no effect on the mucus production. The protein release data of the slugs treated with the different Amioca®/PAA mixtures were comparable with the negative control slugs ($p > 0.05$, Scheffé test). Only the positive control slugs exhibited a significantly increased protein release ($p < 0.001$, Scheffé test) and the protein release increased with a repeated contact period. An increased release of the cytosolic enzyme LDH and the membrane-bound enzyme ALP from the mucosa of the slugs is an indication of severe membrane damage. Enzyme release was only detected for the positive control slugs.

Table 5.3 Effect of a repeated treatment for 5 successive days with 20 mg of co-spray dried Amioca®/PAA mixtures on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Blank	0.8 ± 1.1 [a]	7 ± 4 [a]	–	0	25
Amioca®/PAA 50/50	9.2 ± 3.7	9 ± 5 [a]	–	0	5
Amioca®/PAA 50/50 HT	9.7 ± 3.5	7 ± 5 [a]	–	0	5
Amioca®/PAA 25/75	14.1 ± 2.5 [b]	11 ± 2 [a]	–	0	5
Amioca®/PAA 25/75 HT	12.7 ± 2.0 [b]	13 ± 3 [a]	–	0	5
DDWM/BAC 95/5	17.4 ± 6.0 [b]	160 ± 92 [b]	5.7 ± 5.7	13	25

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; HT, heat-treated; -, below the detection limit.

[a] For this end point, data are not significantly different from blank ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from DDWM/BAC 95/5 ($p > 0.05$, Scheffé test).

Because all tested Amioca®/PAA co-spray dried mixtures resulted in irritation of the slug mucosa, the influence of diluting the co-spray dried Amioca®/PAA 50/50 with a non-irritating starch DDWM was investigated. All the slugs survived a repeated treatment with DDWM and each of the Amioca®/PAA/DDWM mixtures. The total mucus production of the slugs treated with the co-spray dried Amioca®/PAA 50/50 mixture and with its dilutions containing 60% or less DDWM fluctuated between 9.3 and 11.5% indicating mild irritation of the slug mucosa (Table 5.4). Only a dilution with 80% DDWM resulted in a decrease of the total amount of mucus production compared with the co-spray dried Amioca®/PAA 50/50 mixture. A repeated treatment with all Amioca®/PAA/DDWM mixtures resulted in a protein release comparable to that of the untreated slugs and the DDWM treated slugs ($p > 0.05$, Scheffé test). Moreover, the release of LDH and ALP by slugs treated with these mixtures was under the detection limit. Enzyme release was detected only after treatment with DDWM/BAC 95/5.

Table 5.4 Effect of a repeated treatment for 5 successive days with 20 mg of co-spray dried Amioca®/PAA/DDWM mixtures on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Blank	0.8 ± 1.1 [a, b]	7 ± 4 [a, b]	–	0	25
Amioca®/PAA/DDWM					
0/0/100	3.0 ± 0.7 [a, b]	13 ± 10 [a, b]	–	0	5
10/10/80	6.4 ± 1.5 [b]	12 ± 5 [a, b]	–	0	5
20/20/60	9.6 ± 3.0	12 ± 5 [a, b]	–	0	5
30/30/40	9.7 ± 1.2	3 ± 1 [a, b]	–	0	5
40/40/20	11.5 ± 3.6 [c]	11 ± 2 [a, b]	–	0	5
45/45/10	9.3 ± 1.4	8 ± 8 [a, b]	–	0	5
50/50/0	10.2 ± 2.5	5 ± 1 [a, b]	–	0	5
DDWM/BAC 95/5	17.4 ± 6.0 [c]	160 ± 92 [c]	5.7 ± 5.7	13	25

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a] For this end point, data are not significantly different from blank ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from DDWM ($p > 0.05$, Scheffé test).

[c] For this end point, data are not significantly different from DDWM/BAC 95/5 ($p > 0.05$, Scheffé test).

5.6.2.2 Local tolerance of Amioca®/Carbopol® 974P co-spray dried mixtures

Next, the influence of the concentration of cross-linked polyacrylic acid – Carbopol® 974 – on the slug mucosa was evaluated. The mucosal tolerance of Amioca®/Carbopol® 974P co-spray dried powders intended for buccal administration was evaluated by placing the slugs on 20 mg powder for 30 minutes during five successive days. All the untreated slugs and the slugs treated with DDWM or the Amioca®/Carbopol® 974P mixtures survived the five-day experiment. Only a repeated treatment with DDWM/BAC 95/5 caused 20%, 20%, 0%, 60%, 0% and 20% mortality by day 5 in the 1st, 2nd, 3rd, 4th, 5th and 6th experiment, respectively.

Amioca®/Carbopol® 974P powders in ratios 90/10, 80/20 and 75/25 were tested independently on two or more separate occasions. ANOVA testing resulted in no significant differences in the total mucus production and in the mean protein release for the repeated experiments ($p > 0.05$). There was only one exception for the repeated experiments with Amioca®/Carbopol® 974P 90/10 where the total mucus production of the first experiment (2.9 ± 1.6, n = 5) was significantly lower compared to the second (6.8 ± 1.4, n = 5) and the third experiment (7.4 ± 1.4, n = 5).

The effect of the Amioca®/Carbopol® 974P co-spray dried mixtures with increasing Carbopol® 974P concentrations on the end points of the Slug Mucosal Irritation test is shown in Table 5.5. All the Amioca®/Carbopol® 974P co-spray dried mixtures induced a significantly higher total mucus production compared to the untreated slugs ($p < 0.05$, Scheffé test). However, the total mucus production induced after treatment with mixtures containing up to 20% Carbopol® 974P was similar to the mucus production of the slugs treated with DDWM ($p > 0.05$, Scheffé test). The mucus secretion induced by the mixtures containing 25% Carbopol® 974P was significantly different from the mucus production of the DDWM treated slugs and the positive control slugs ($p < 0.05$, Scheffé test). Slugs treated with a mixture containing 40% or more Carbopol® 974P produced a total amount of mucus comparable to that of the positive control slugs ($p > 0.05$, Scheffé test).

Table 5.5 Effect of a repeated treatment for 5 successive days with 20 mg of co-spray dried Amioca®/Carbopol® 974P mixtures on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Blank	-0.3 ± 1.4 [a]	12 ± 10 [a, b]	–	0	30
DDWM	3.6 ± 1.3 [b]	11 ± 7 [a, b]	–	0	15
Amioca®/Carbopol® 974P					
95/5	3.7 ± 0.8 [b]	5 ± 4 [a, b]	–	0	5
90/10	5.7 ± 2.5 [b]	11 ± 12 [a, b]	–	0	15
85/15	5.9 ± 1.7 [b]	13 ± 8 [a, b]	–	0	5
80/20	7.7 ± 2.5 [b]	17 ± 14 [a, b]	–	0	10
75/25	9.2 ± 3.7	15 ± 12 [a, b]	–	0	15
60/40	17.0 ± 2.2 [c]	28 ± 30 [a, b]	0.7 ± 0.8 [c]	0	5
50/50	18.5 ± 8.3 [c]	27 ± 20 [a, b]	1.2 ± 1.1 [c]	0	5
0/100	21.4 ± 3.7 [c]	55 ± 30 [c]	0.4 ± 0.3	0	5
DDWM/BAC 95/5	18.7 ± 4.8 [c]	145 ± 83 [c]	6.4 ± 5.3 [c]	17	30

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a] For this end point, data are not significantly different from blank ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from DDWM ($p > 0.05$, Scheffé test).

[c] For this end point, data are not significantly different from DDWM/BAC 95/5 ($p > 0.05$, Scheffé test).

Co-spray dried mixtures containing up to 25% Carbopol® 974P had no additional effect on the protein release in comparison with the untreated slugs and no enzyme release was detected. A repeated treatment with co-spray dried powders containing 40% or 50% Carbopol® 974P resulted in a (not-significantly) higher protein release in comparison to the untreated slugs. Furthermore, the latter two powders induced release of cytosolic LDH which was comparable to the positive control slugs ($p > 0.05$, Scheffé test), but induced no detectable ALP release. Repeated treatment with pure Carbopol® 974P resulted in a significantly higher protein release compared with the untreated slugs ($p < 0.05$, Scheffé test) and in the release of LDH.

The effect of co-spray drying of the Amioca®/Carbopol® 974P mixtures on the mucosal tolerance was investigated by comparing co-spray dried mixtures containing up to 25% Carbopol® 974P with their equivalent physical mixtures (Table 5.6). Because a Carbopol® 974P concentration up to 25% affected only the total mucus production, only these data are shown. Two-way ANOVA testing revealed no significant interaction between the

Amioca®/Carbopol® 974P ratio and the mixing process (p = 0.081). However, there was a significant main effect of the Carbopol® 974P concentration (p < 0.001). A Scheffé post hoc analysis revealed that the total mucus production induced by the Amioca®/Carbopol® 974P 75/25 mixtures was significantly higher than both the 95/5 and 90/10 mixtures. The mixing process had also a significant effect (p = 0.029) on the total mucus production, co-spray dried mixtures induced a higher mucus production than the physical mixtures.

Table 5.6 Influence of the mixing process and the Amioca®/Carbopol® 974P ratio on the total amount of mucus produced by slugs during a repeated treatment on 5 successive days

Amioca®/ Carbopol® ratio	Total mucus production (%)		
	Co-spray drying	Physical blending	**Average Amioca®/ Carbopol® ratio**
Amioca®/Carbopol® 974P			
95/5	3.7 ± 0.8	2.7 ± 1.5	**3.2 ± 1.3** [a]
90/10	6.8 ± 1.4	2.8 ± 1.0	**4.8 ± 2.4** [a]
75/25	8.3 ± 4.1	8.3 ± 1.0	**8.3 ± 2.8** [b]
Average mixing process	**6.3 ± 3.1** [c]	**4.6 ± 2.9** [d]	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 10 and 15 slugs, respectively.

Two-way ANOVA testing revealed no significant interaction effect (p > 0.05), but a significant effect of Amioca®/Carbopol® 974P ratio and a significant effect of mixing process (p < 0.05).

[a, b] Same letter indicates no significant difference between the Amioca®/Carbopol® ratios (p > 0.05, Scheffé test).

[c, d] Same letter indicates no significant difference between the mixing processes (Two-way ANOVA).

5.6.3 Development of prediction model

Based on the results of this study and on Slug Mucosal Irritation test data obtained in the previous six years on the one hand and on pre-clinical and clinical safety data on the other hand, a classification prediction model was developed that distinguishes into irritation and tissue damage. Generally, formulations that cause neither irritation nor tissue damage induce a low mucus production, a low protein release and no enzyme release, whereas irritating and damaging formulations result in an increased mucus production and an increased protein and enzyme release (Adriaens and Remon, 1999; Adriaens *et al.*, 2001a; Callens *et al.*, 2001; Ceulemans *et al.*, 2001; Adriaens *et al.*, 2003; Dhondt *et al.*, 2004; Weyenberg *et al.*, 2004; Dhondt *et al.*, 2005). Cut-off values were established based on in-house experience. Statistical methods such as linear discriminant analysis could namely not be used to determine cut-off

values, because the human and animal data against which the relevance of the Slug Mucosal Irritation test was assessed were obtained in studies which differed with respect to their purpose, administration frequency, administration duration, sample sizes, means and end points of assessing safety outcomes.

Based on this study and the studies of Callens *et al.* (2001), Ceulemans *et al.* (2001), Dhondt (2001), Adriaens *et al.* (2003), and Weyenberg *et al.* (2004), a classification prediction model was developed for the determination of the irritation category of bioadhesive powders. Powder formulations that induce a low total mucus production (< 7%) are classified as non-irritating. Powders that cause a mucus production between 7% and 12% are predicted as mildly irritating formulations. Powder formulations that result in 12% to 20% mucus production are classified as moderately irritating powders, whereas powders that cause ≥ 20% mucus production are classified as severely irritating (Figure 5.1) (Adriaens *et al.*, 2004).

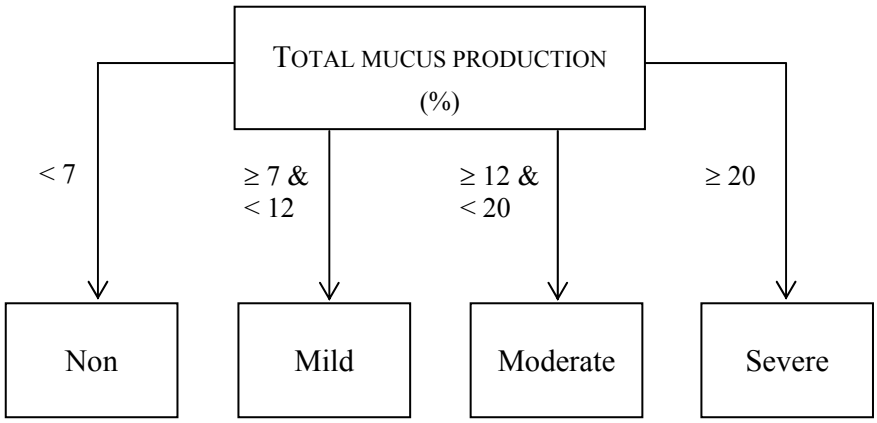

Figure 5.1 Prediction model for the determination of the irritation category of powders. The total mucus production is calculated after a repeated 30-minute treatment of *A. lusitanicus* with the powder on five successive days. (Based on Adriaens *et al.*, 2004)

Furthermore, based on the studies of Adriaens and Remon (1999), Adriaens *et al.* (2001a), Callens *et al.* (2001), Ceulemans *et al.* (2001), Adriaens *et al.* (2003), Dhondt *et al.* (2004), Weyenberg *et al.* (2004), and Dhondt *et al.* (2005), a classification prediction model was developed for the determination of the tissue damage category of formulations. The formulations are classified into four tissue damage classes based on the protein and enzyme release as presented in Figure 5.2 (Adriaens *et al.*, 2004).

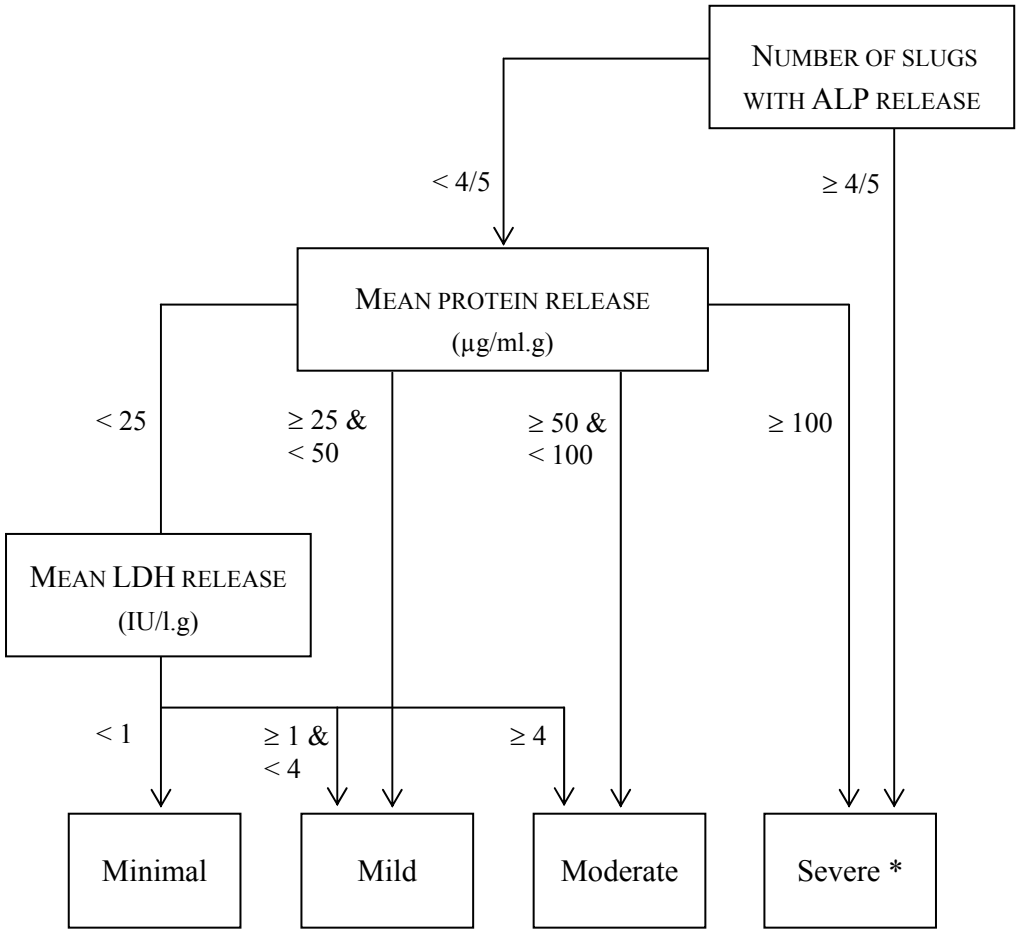

Figure 5.2 Classification prediction model for the determination of the tissue damage category of formulations. The mean protein release and mean LDH release are calculated after a repeated 30-minute treatment of *A. lusitanicus* on five successive days. * A formulation is considered severely toxic in case of mortality. (Based on Adriaens *et al.*, 2004)

5.7 DISCUSSION

A reliable and relevant alternative method for repeated dose mucosal tolerance testing would be very useful for academic and pharmaceutical researchers to screen pharmaceuticals intended for e.g. buccal application. The objective of this study was to investigate if the five-day procedure of the Slug Mucosal Irritation test was useful to evaluate the concentration and formulation effects of Amioca® starch/polyacrylic acid and Amioca® starch/Carbopol® 974P bioadhesive powders intended for buccal administration. The results of the Slug Mucosal Irritation test were compared with available pre-clinical and clinical *in vivo* data on the safety of the tested powders.

Based on the results of this study and data previously obtained with the Slug Mucosal Irritation test on the one hand and on (pre-)clinical data on the other hand, a classification prediction model was developed for the determination of the irritation and tissue damage potential of formulations. The mucus production is used to classify the formulations into four irritation classes (no, mild, moderate and severe irritation), whereas the release of proteins and enzymes is a measure of tissue damage (minimal, mild, moderate and severe tissue damage). A classification prediction model simplifies the interpretation of the data. A disadvantage of a classification model is that it reduces the information about the severity of the irritating and damaging properties of test substances (Curren and Harbell, 1998). So especially for formulations with a borderline irritation/damage potential, a careful analysis of the data is still recommended. It is important to note that the proposed prediction model is susceptible to change due to the absence of reference formulations intended for screening the mucosal tolerance. The formulations used to develop the classification prediction model are namely tested in studies that differed with respect to their purpose, administration frequency, administration duration, sample sizes, means and end points of assessing safety outcomes.

The results of the study indicate that the used PAA concentrations were mildly to moderately irritating to the slug mucosal tissue, because a repeated treatment with all tested co-spray dried Amioca®/PAA mixtures induced between 9 and 15% mucus production. The irritation potential increased with increasing PAA content; 50% PAA was mildly irritating to the slug mucosa, whereas 75% PAA was moderately irritating. However, no increased protein release and no enzyme release were detected, indicating that no tissue damage occurred. These results are in agreement with the outcome of a study in dogs. Amioca®/PAA buccal bioadhesive tablets in ratio 50/50 resulted in irritation of the buccal mucosa of dogs after the sixth day of application, while the Amioca®/PAA buccal bioadhesive tablets in ratio 25/75 resulted in irritation after the fourth day of application. Irritation could be observed as disappearance of the black pigment of the upper lip (Adriaens *et al.*, 2001b).

Diluting the co-spray dried Amioca®/PAA 50/50 mixture with DDWM was only effective when the physical blend contained 80% DDWM. Some caution about the interpretation of the mucus production of the slugs treated with powder formulations should be made. Upon contact with the body wall of slugs, dry powder formulations can absorb water by dehydration of the mucosal tissue. This absorption of water by the powder is reported as an increased mucus production compared to the untreated slugs. Therefore, the effect of the different co-spray dried mixtures on the mucus production was also compared with DDWM treated slugs. The fact that DDWM does not irritate human buccal mucosa has been

previously reported. A single application of a buccal bioadhesive tablet consisting of pure DDWM did not result in mucosal irritation in each of the 18 human volunteers (Bottenberg *et al.*, 1991). DDWM induced no irritation of the slug mucosa, because the increased weight of the Petri dish after treatment (reported as mucus production) was probably mainly the result of hydration of the dry bioadhesive powder formulation.

When comparing linear polyacrylic acid (PAA) and cross-linked polyacrylic acid (Carbopol® 974P), the mucosal irritation and damage potential of the cross-linked polyacrylic acid was more pronounced. Co-spray dried mixtures containing up to 75% PAA induced only an increased mucus production, while no increased protein or enzyme release could be detected. However, the mixtures containing 40 or 50% Carbopol® 974P resulted in a higher mucus production, protein release and LDH release. Both co-spray dried powder mixtures induced moderate irritation and mild damage of the slug mucosal tissue. A repeated treatment with pure Carbopol® 974P resulted in severe irritation and moderate damage of the slug mucosa. The buccal (and nasal) irritation potential of high concentrations of Carbopol® is also reported in humans and rabbits. A single application of bioadhesive buccal tablets consisting of hydroxypropyl methylcellulose/Carbopol® 934P in ratios of 50/50 resulted in small mucosal lesions in 5% of 18 human volunteers (Bottenberg *et al.*, 1991). Pure Carbopol® 934P tablets caused small buccal lesions in 25% of 16 human volunteers and 44% of the volunteers had to remove the tablet due to irritation (Bottenberg *et al.*, 1991). Ugwoke *et al.* (2000) concluded that 85% and 100% Carbopol® 971P induced severe inflammation of the nasal mucosa of rabbits; however, no necrosis, squamous metaplasia or ciliary degeneration was observed even after a four-week twice daily treatment.

The increased mucus secretion of the slugs treated with mixtures containing 20 or 25% Carbopol® 974P indicated that repeated treatment with these powders induced mild mucosal irritation. However, both co-spray dried Amioca®/Carbopol® 974P mixtures induced minimal tissue damage as was demonstrated by a low protein release and no detectable enzyme release. A single application of a buccal mucoadhesive tablet containing 20 or 30% Carbopol® 974P was well accepted by 10 human volunteers and caused no irritation (Ceschel *et al.*, 2001).

Co-spray dried Amioca®/Carbopol® 974P mixtures containing up to 15% Carbopol® 974P induced no irritation of the mucosal tissue of slugs. The mixing process had only a minor effect on the irritation potential of the powders. Co-spray dried mixtures induced a slightly higher mucus production compared to the corresponding physical mixtures. Furthermore, all these powders induced minimal damage of the slug mucosa. Irritation studies

in other models also indicate that Carbopol® is well tolerated when it is used in small amounts (< 10%). Buccal tablets containing 5% Carbopol® together with non-irritating DDWM were well accepted by human volunteers (Bottenberg *et al.*, 1991; Bouckaert *et al.*, 1996). A buccal erodible tablet containing 7.5% Carbopol® 974P induced no irritation over a period of 6 hours in human volunteers (Khanna *et al.*, 1997). A single application of a buccal mucoadhesive tablet containing 10% Carbopol® 974P was well accepted by 10 human volunteers and caused no irritation (Ceschel *et al.*, 2001). Daily nasal administration of DDWM/Carbopol® 974P in ratio 90/10 to rabbits during 28 days led to an intact nasal epithelium and a slightly increased number of granulocytes, so that it was concluded that a repeated treatment with the powder did not irritate the rabbit nasal mucosa (Callens *et al.*, 2001).

The results of this study indicate that the Slug Mucosal Irritation test seems to be a promising alternative to evaluate the local tolerance of bioadhesive powder formulations intended for repeated buccal administration. The test enables to investigate the concentration effect of ingredients or drugs – intended to prepare mucosally applied formulations – on the mucosal tissue. These concentration response experiments can be a very helpful tool in prioritising concentrations of formulation compounds in the drug development phase. Furthermore, the test can classify the formulations into four irritation and tissue damage categories. Local tolerance data may be obtained accurately, quickly and economically. The Slug Mucosal Irritation test seems to be useful to screen new compounds and formulations intended for repeated administration early in the development process before pre-clinical studies in vertebrates and clinical studies.

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CHAPTER 6: EVALUATION OF LOCAL TOLERANCE OF OCULAR POWDER FORMULATIONS

6.1 INTRODUCTION

Various methods were investigated to increase the bioavailability of ophthalmic drugs. One approach was the prolongation of the contact time between drug and corneal-conjunctival epithelium using bioadhesive polymers (Hui and Robinson, 1985; Saettone and Salminen, 1995). Consequently, the use of mucoadhesive polymers may result locally in a high drug concentration. Certain ingredients of ophthalmic formulations (e.g. preservatives) may cause ocular irritation and damage (Gasset *et al.*, 1974; Collin and Carroll, 1986). Adverse effects may be more pronounced after frequent application over a long period. Because irritation and damage of the eye can have serious consequences, it is important to evaluate the local tolerance of ophthalmic formulations – including formulations based on bioadhesive powders – before clinical use.

6.2 EVALUATION OF OCULAR TOLERANCE OF FORMULATIONS

For medicinal products intended for ophthalmic administration, regulatory authorities require a single dose ocular tolerance test on the one hand and a repeated dose ocular tolerance test on the other hand. Single dose ocular tolerance testing is also necessary to investigate accidental exposure for products which are not envisaged for ocular delivery, but which can come into contact with the eyes during their normal clinical use (e.g. medicinal facial lotions, facial gels or shampoos). Ocular tolerance testing is usually conducted in rabbits with the definitive formulation consisting of the drug in its vehicle at the concentration intended for human use. In case of repeated dose ocular tolerance tests, the frequency and duration of administration to animals are determined based on the proposed administration frequency and duration in clinical use (EC, 1990).

Ocular irritation caused by single or repeated application of ophthalmic adhesive delivery systems has been evaluated in rabbits by scoring ocular effects such as conjunctival redness (Gurtler *et al.*, 1995; Gurtler *et al.*, 1996; Bayens *et al.*, 1998; Srividya *et al.*, 2001).

6.3 OBJECTIVES

The objective of the present study was to optimise and evaluate the five-day procedure of the Slug Mucosal Irritation test (as described in Chapter 5) for the evaluation of the local tolerance of powder formulations intended for repeated ocular administration. Because analysis of the data of Chapter 5 and of previously obtained data (Adriaens, 2000; Callens *et al.*, 2001; Ceulemans *et al.*, 2001) indicated that for irritating and damaging formulations the highest release of proteins and enzymes occurred during the first two hours after treatment of the slugs (analysis not shown) and that a better discrimination between minimally, mildly, moderately and severely damaging formulations could be made on the basis of two samples, it was decided to reduce the number of samples from three to two.

Firstly, it was determined if a repeated treatment on five successive days was necessary. Based on the results of Chapter 5 and on the fact that ocular minitablets containing 94% DDWM were well accepted by human volunteers (Ceulemans *et al.*, 2001), DDWM was used as negative control. Because the preparation of the lyophilised positive control powder DDWM/BAC 95/5 (used in Chapter 5) is laborious, it was decided to look for another positive control powder. Sodium lauryl sulphate was selected for the preparation of the novel positive control powder, because it induces ocular irritation in several models (Gilleron *et al.*, 1997; ECETOC, 1998; personal communication with F. Van Goethem and Ph. Vanparys, J & J, Beerse, Belgium, 2004; Adriaens *et al.*, 2005) and because treatment of the slugs with sodium lauryl sulphate causes a mucus production comparable to the mucus production of slugs treated with benzalkonium chloride as was demonstrated in Chapter 3.

Furthermore, the influence of the amount of powder on the end points of the test was evaluated. In previous studies, the slugs were put on 20 mg powder formulation (Adriaens, 2000; Callens *et al.*, 2001; Ceulemans *et al.*, 2001; Dhondt, 2001). However, preliminary studies had shown that the amount of formulation influences especially the mucus production of the slugs and the end points of the Slug Mucosal Irritation test in general. Next, the repeatability of the optimised test procedure and prediction model (described in Chapter 5) was assessed. For this purpose, the negative control and the positive control were tested on four separate occasions. Both the intra-laboratory reproducibility of the test results and the

intra-laboratory reproducibility of the classifications obtained with the Slug Mucosal Irritation test were evaluated. Finally, the optimised test procedure was used to evaluate the local tolerance of ingredients and bioadhesive powder mixtures intended to prepare ocular minitablets.

6.4 MATERIALS AND METHODS

6.4.1 Chemicals

DDWM was obtained from Eridania-Béghin Say, Cerestar (Vilvoorde, Belgium). Benzalkonium chloride (BAC), sodium lauryl sulphate (SLS), gentamycin sulphate, and vancomycin hydrochloride were purchased from Sigma (St. Louis, MO, USA). Carbopol® 974P (Carb) was obtained from B.F. Goodrich (Cleveland, OH, USA). Sodium stearyl fumarate (NaSF) and ciprofloxacin hydrochloride were a gift of Edward Mendell Co. Inc. (New York, NY, USA) and Dr. Reddy's Laboratories (Hyderabad, India). Amioca® starch was obtained from National Starch and Chemical Company (Bridgewater, NJ, USA). All other reagents used were of analytical grade.

6.4.2 Powder formulations

The lyophilised powder DDWM/BAC 95/5 (w/w) was prepared as described in Chapter 5. The powders containing DDWM/SLS in ratios 95/5, 90/10, 80/20 and 50/50 (w/w) were prepared by homogeneously mixing the different compounds with a pestle in a mortar.

The non-sterilised and sterilised powder mixtures DDWM/Carb/NaSF/ciprofloxacin HCl and Amioca®/Carb/NaSF/gentamycin sulphate/vancomycin HCl were prepared by the Laboratory of Pharmaceutical Technology and Biopharmacy (University of Antwerp, Antwerp, Belgium). The DDWM/Carb/NaSF/ciprofloxacin HCl powders contained DDWM, 5% (w/w) Carbopol® 974P, 1% (w/w) sodium stearyl fumarate, and 0 to 10% (w/w) ciprofloxacin HCl. The Amioca®/Carb/NaSF/gentamycin sulphate/vancomycin HCl powder mixtures consisted of 1% (w/w) sodium stearyl fumarate, 5% (w/w) gentamycin sulphate, and 5% (w/w) vancomycin HCl on the one hand and 89% (w/w) co-spray dried Amioca®/Carbopol® 974P 95/5 (w/w) or 85/15 (w/w) (National Starch and Chemical Company) on the other hand. A part of some powder mixtures was sterilised by γ-irradiation at 25kGy (Gammir-I-Sulzer irradiator unicell; Sterigenics, IBA-Mediris, Fleurus, Belgium).

6.4.3 Local tolerance test procedure of the Slug Mucosal Irritation test

Belgian *A. lusitanicus* slugs were used as test organisms. The housing and feeding conditions of the slugs were identical to the ones described in Chapter 3.

The local tolerance of both bioadhesive powder formulations intended for repeated ocular administration and their ingredients was assessed according to a slightly modified version of the procedure described in Chapter 5. During five successive days, the slugs were placed on the neat test substance for 30 minutes and the mucus production of the slugs was measured. After each of the five contact periods, however, only two samples were collected instead of three samples. The first samples were collected one hour after each contact period, whereas the second samples were collected two hours after each contact period. The samples were analysed immediately for the presence of proteins, LDH, and ALP released from the body wall of the slugs.

6.4.4 Analytical procedures

The protein, LDH, and ALP determinations were performed according to the analytical procedures described in Chapter 3.

6.4.5 Data analysis

For each slug, the total mucus production, mean protein release, mean LDH release and mean ALP release were calculated as described in the data analysis section of Chapter 5. These values were used for the statistical analyses using the computer program SPSS (version 12.0; SPSS, Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Statistically significant differences between different treatments or repeated experiments were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal, the data were transformed to their logarithm or inverse. To compare further the effects of the different treatments or experiments, a multiple comparison among pairs of means was performed with a Scheffé test.

A two-way ANOVA was performed to investigate the test amount effect and formulation effect on the total mucus production and mean protein release. The normal distribution of the data and the homogeneity of variances were tested with the tests mentioned

above. Because a significant interaction between both factors was present for the total mucus production, the test amount effect for each formulation and the formulation effect for each test amount were investigated with a Bonferroni post hoc test. Because transformation of the mean protein release data did not result in equal variances and no significant interaction was present, a multiple comparison among pairs of means was performed using a Dunnett T3 post hoc test.

Using the cut-off values presented in Figures 5.1 and 5.2, the powders were classified into one of the irritation and tissue damage categories.

6.5 RESULTS

6.5.1 Optimisation of the test procedure

Firstly, it was investigated if a repeated treatment on five successive days was necessary. For this purpose, DDWM, DDWM/BAC 95/5 and powders containing DDWM and SLS in different ratios were tested. All the slugs treated with 20 mg DDWM, DDWM/SLS 95/5, DDWM/SLS 90/10 or DDWM/BAC 95/5 survived a repeated treatment on five successive days. A daily treatment with DDWM/SLS 80/20 led to 40% mortality by day 4 (before the 4^{th} treatment) and to 80% mortality by day 5. Two out of the five slugs treated with DDWM/SLS 50/50 were dead by day 3 and finally all slugs were dead by day 4.

In order to enable discrimination between minimally, mildly, moderately and severely irritating and damaging formulations, it is important that for the different end points the difference between non-irritating/damaging formulations on the one hand and irritating/damaging formulations on the other hand is as large as possible. Figure 6.1 illustrates that discrimination between the mucus productions of the slugs treated with the different powders became more pronounced after a repeated treatment and the best discrimination could be made after five treatments.

Table 6.1 shows that the total mucus production of the slugs treated for five successive days with DDWM/SLS 90/10, DDWM/SLS 80/20, DDWM/SLS 50/50 or DDWM/BAC 95/5 were significantly higher than the total mucus production of the negative control slugs. Because DDWM/SLS 50/50 caused high mortality, its use as positive control was not recommended. Consequently, DDWM/SLS in ratios 90/10 and 80/20 were considered possible positive control powders and were investigated in more detail.

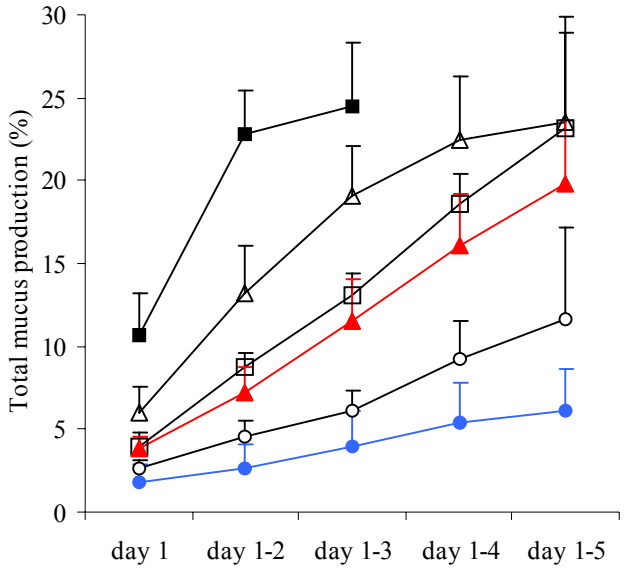

Figure 6.1 Total mucus production of slugs treated with 20 mg DDWM (●), DDWM/SLS 95/5 (○), DDWM/SLS 90/10 (□), DDWM/SLS 80/20 (Δ), DDWM/SLS 50/50 (■) or DDWM/BAC 95/5 (▲) for 5 successive days. Data points are presented as the mean values (n = 5) and standard deviation bars are indicated.

Table 6.1 Effect of a repeated treatment for 5 successive days with the negative control DDWM and several positive controls on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
DDWM	6.2 ± 2.5 [a]	17 ± 10 [a]	–	0	5
DDWM/SLS 95/5	11.6 ± 5.6 [a, b]	46 ± 38 [a]	0.06 ± 0.12 [a]	0	5
DDWM/SLS 90/10	23.2 ± 6.7 [c]	74 ± 38 [a, b]	1.02 ± 0.94 [a]	0	5
DDWM/SLS 80/20	23.6 ± 5.3 [c]	172 ± 45 [b]	1.65 ± 0.48 [a]	3	5
DDWM/SLS 50/50	24.4 ± 3.9 [c]	335 ± 59 [c]	2.22 ± 2.14 [a]	4	5
DDWM/BAC 95/5	19.8 ± 3.9 [b, c]	99 ± 81 [a, b]	3.10 ± 3.14 [a]	1	5

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a, b, c] For this end point, the values marked with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

Figure 6.2 shows that all the powders induced a high protein release after the first treatment. However, the negative control powder DDWM resulted in low protein release levels comparable to the ones of untreated slugs (described in Chapter 5) from the second day on. The protein release by the slugs treated with DDWM/SLS 90/10, DDWM/SLS 80/20 and

DDWM/BAC 95/5 increased with a repeated treatment. For the end point protein release, the best discrimination between the different powders could also be made after five successive treatments. Table 6.1 shows that the protein release of the slugs treated with DDWM/SLS 80/20 was significantly higher than that of the negative control slugs ($p < 0.05$, Scheffé test).

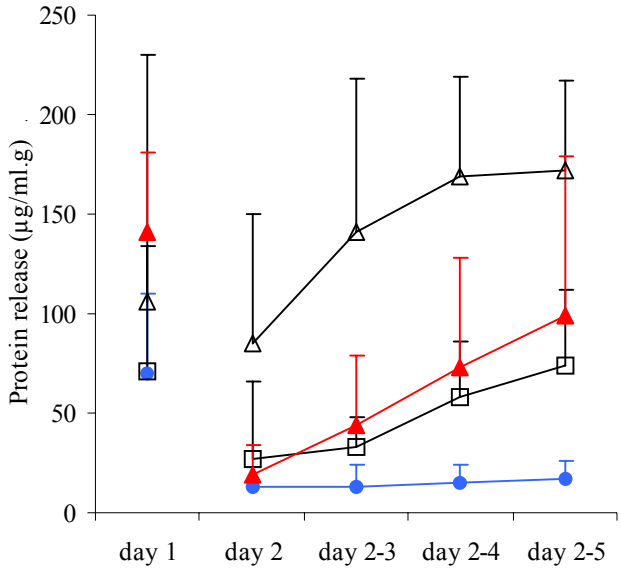

Figure 6.2 Mean protein release of slugs treated with 20 mg DDWM (●), DDWM/SLS 90/10 (□), DDWM/SLS 80/20 (Δ) or DDWM/BAC 95/5 (▲) for 5 successive days. Data points are presented as the mean values (n = 5) and standard deviation bars are indicated.

An increased release of the cytosolic enzyme LDH and the membrane-bound ALP from the slug mucosa is an indication of tissue damage. Increased LDH release was detected for slugs treated with DDWM/SLS in ratios 90/10 and 80/20 and DDWM/BAC 95/5. LDH release was induced after the 2nd treatment with DDWM/SLS 80/20 or DDWM/BAC 95/5 and after the 4th treatment with DDWM/SLS 90/10. LDH release increased with a repeated treatment (left graph of Figure 6.3). ALP release occurred at least one day after LDH release and increased with a repeated treatment (right graph of Figure 6.3).

The results indicated that with respect to the irritation and tissue damage potential, the best discrimination between the different powders can be made after a repeated treatment on five successive days. Prolongation of the experiment was not useful, because the mortality of the slugs treated with severely irritating formulations would increase so that the end point range between non-irritating and irritating formulations would not further increase. Furthermore, DDWM/SLS 80/20 seemed to be a good replacement powder for DDWM/BAC 95/5 and was therefore selected as positive control powder for future experiments.

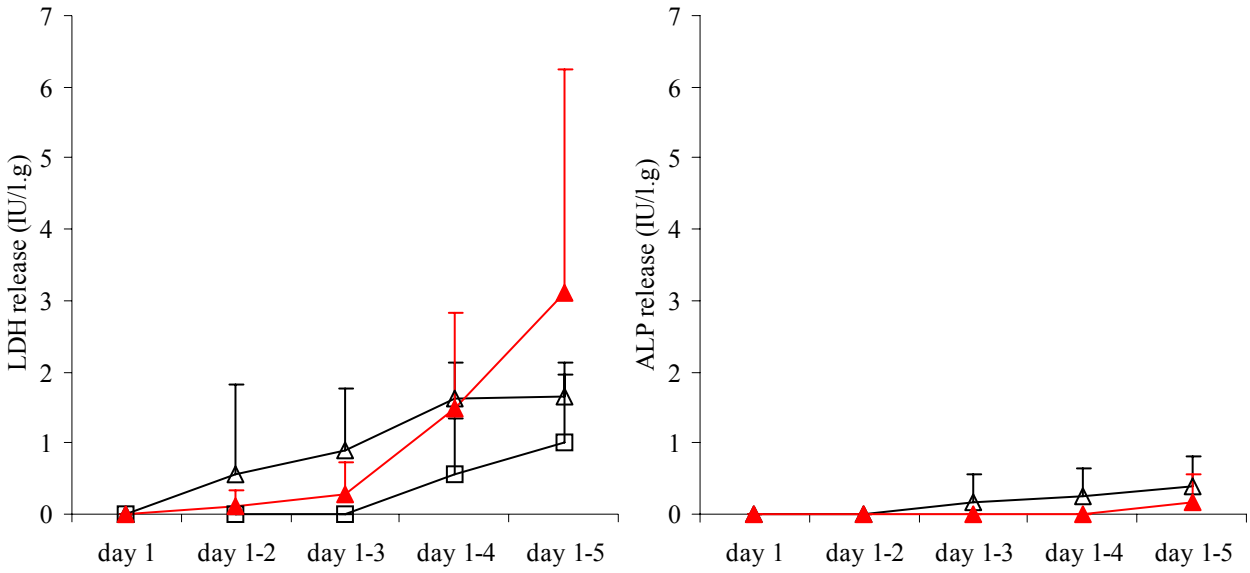

Figure 6.3 Mean LDH release and ALP release of slugs treated with 20 mg DDWM (●), DDWM/SLS 90/10 (□), DDWM/SLS 80/20 (Δ) or DDWM/BAC 95/5 (▲) for 5 successive days. Data points are presented as the mean values (n = 5) and standard deviation bars are indicated.

Next, the influence of the amount of powder on the end points of the Slug Mucosal Irritation test was investigated. Several amounts (20, 50, 100 and 200 mg) of DDWM and DDWM/SLS 80/20 were tested on five successive days. All the slugs treated with the different amounts of DDWM survived a repeated treatment on five successive days. A repeated treatment with 20 mg DDWM/SLS 80/20 caused 20% mortality by day 5. A repeated treatment with 50 mg DDWM/SLS 80/20 led to 40% mortality by day 4 and to 80% mortality by day 5. At least 40% of the slugs treated with 100 mg or 200 mg DDWM/SLS 80/20 were dead by day 3 and finally all the slugs were dead by day 5.

The effect of the amount of powder was investigated by comparing the total mucus production and mean protein release data of the negative and positive control powders. For the total mucus production, two-way ANOVA testing revealed a significant interaction between the test amount and the powder formulation ($p = 0.001$) (Table 6.2). Subsequently, a Bonferroni post hoc analysis was performed to investigate the test amount effect for each formulation. Bonferroni post hoc testing revealed that the total mucus production induced by 20 mg and 50 mg of DDWM was significantly lower than the total mucus production induced by 200 mg of DDWM. Although the total amount of mucus secreted by the slugs treated with DDWM/SLS 80/20 decreased with increasing amount of powder, Bonferroni testing revealed

no significant differences between the mucus productions induced by the four test amounts of DDWM/SLS 80/20 ($p > 0.05$). This decrease in mucus production can be explained by the earlier onset of mortality of the slugs treated with larger amounts of the positive control powder. Next, a Bonferroni post hoc analysis was performed to investigate the formulation effect for each test amount. Bonferroni post hoc testing revealed that the total mucus production of slugs treated with each of the four amounts of DDWM was significantly lower than the total mucus productions of the slugs treated with the corresponding amount of DDWM/SLS 80/20. The largest difference in mucus production between the negative and the positive control was obtained after a repeated treatment with 20 mg powder formulation on five successive days.

Table 6.2 Influence of the test amount and the powder formulation on the total amount of mucus produced by slugs during a repeated treatment on 5 successive days

Formulation	Total mucus production (%)				
	20 mg	50 mg	100 mg	200 mg	**Average formulation**
DDWM	6.0 ± 3.3 [a, c]	7.9 ± 2.8 [a, c]	13.1 ± 2.8 [a, b, c]	20.3 ± 6.2 [b, c]	**11.8 ± 6.8**
DDWM/SLS 80/20	30.9 ± 4.9 [a, d]	29.3 ± 3.4 [a, d]	28.5 ± 4.0 [a, d]	27.7 ± 4.4 [a, d]	**29.1 ± 4.1**
Average test amount	**18.5 ± 13.7**	**18.6 ± 11.7**	**20.8 ± 8.8**	**24.0 ± 6.4**	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 20 and 10 slugs, respectively.

Two-way ANOVA testing revealed a significant interaction and a significant formulation effect ($p \leq 0.001$).

[a, b] For this formulation, total mucus productions marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

[c, d] For this test amount, total mucus productions marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

For the mean protein release, two-way ANOVA testing revealed no significant interaction between the test amount and the powder formulation ($p = 0.099$) (Table 6.3). There was a significant main effect of the formulation ($p < 0.001$). The mean protein release of the slugs treated with DDWM was significantly lower than that of the slugs treated with DDWM/SLS 80/20. Table 6.3 shows that treatment of the slugs with the different amounts of the negative control powder resulted in low protein concentrations. The mean protein release of the positive control slugs increased with increasing amount of powder. However, Dunnett T3 post hoc testing revealed no significant differences between the mean protein release data induced by the four test amounts ($p > 0.05$).

Table 6.3 Influence of the test amount and the powder formulation on the mean protein release induced by a repeated treatment of the slugs on 5 successive days

Formulation	Mean protein release (µg/ml.g)				
	20 mg	50 mg	100 mg	200 mg	**Average formulation**
DDWM	6 ± 3	8 ± 4	9 ± 6	15 ± 9	**10 ± 6** [a]
DDWM/SLS 80/20	82 ± 65	162 ± 35	176 ± 69	219 ± 66	**160 ± 75** [b]
Average test amount *	**41 ± 49**	**69 ± 64**	**75 ± 80**	**112 ± 107**	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 20 and 10 slugs, respectively.

Two-way ANOVA testing revealed no significant interaction ($p = 0.099$), but a significant formulation effect ($p < 0.05$).

[a, b] Same letter indicates no significant difference between the powder formulations (Two-way ANOVA).

* There are no significant differences between the mean protein release data induced by the four test amounts ($p > 0.05$, Dunnett T3 test).

For the slugs treated with 20 and 50 mg DDWM, the LDH release was under the detection limit. Repeated treatment with 100 and 200 mg DDWM caused comparable mean LDH release data ($p > 0.05$, One-way ANOVA). Treatment of the slugs with different amounts of DDWM/SLS 80/20 resulted in comparable LDH release data ($p > 0.05$, One-way ANOVA). ALP release was detected only for the positive control slugs. Different amounts of DDWM/SLS 80/20 caused comparable mean ALP release data ($p > 0.05$, One-way ANOVA). Three, two, two, and five out of the five slugs treated with 20, 50, 100 and 200 mg DDWM/SLS 80/20, respectively, released ALP.

Based on the fact that the largest difference between the mucus productions of the slugs treated with DDWM and DDWM/SLS 80/20 was obtained when the slugs were placed on 20 mg powder and on the fact that the statistical analyses revealed no significant differences between the protein release data induced by the four test amounts on the one hand and taking into account the low availability of certain test compounds on the other hand, 20 mg was selected as test amount to evaluate the local tolerance of powder formulations.

6.5.2 Repeatability of the test procedure and prediction model

In order to investigate the repeatability of the test results obtained with the optimised test procedure for local tolerance testing of powder formulations, the negative control (DDWM) and the positive control (DDWM/SLS 80/20) were tested independently on four separate occasions. Table 6.4 shows the total mucus production and mean protein and enzyme release of the negative control slugs measured in each of the repeated experiments and the mean of the four experiments. All the slugs treated with DDWM survived a repeated treatment on five successive days. For the negative control, Scheffé post hoc testing suggested two homogenous subsets for the total mucus production. One subset comprised only the total mucus production of the fourth experiment, whereas another subset contained the total mucus productions of the first, second and third experiment. However, no significant difference in mean protein release was detected between the repeated experiments ($p > 0.05$, One-way ANOVA). Based on the prediction models presented in Figures 5.1 and 5.2, DDWM was classified as a non-irritating and minimally damaging formulation in each of the repeated experiments.

Table 6.4 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the negative control slugs treated with 20 mg DDWM on 5 successive days

Experiment	Total mucus production (%)	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Experiment 1	6.2 ± 2.5 [a]	17 ± 10	–	0	5
Experiment 2	5.9 ± 0.8 [a]	15 ± 4	–	0	5
Experiment 3	6.0 ± 0.7 [a]	18 ± 20	–	0	5
Experiment 4	2.7 ± 1.7 [b]	4 ± 3	–	0	5
Inter-experiment mean	**5.2 ± 1.7**	**13 ± 6**	–		**4**

Total mucus production and mean protein release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 4 experiments; -, below the detection limit.

[a, b] For this end point, the values marked with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

A daily treatment with DDWM/SLS 80/20 led to 80%, 100%, 80% and 20% mortality by day 5 in the 1st, 2nd, 3rd and 4th experiment, respectively. For the positive control, ANOVA testing revealed no significant difference in total mucus production, in mean protein release and in mean LDH release between the repeated experiments ($p > 0.05$) (Table 6.5). ALP release was detected for three, two, three, and three out of the five slugs in the 1st, 2nd, 3rd and 4th experiment, respectively. Based on the data presented in Table 6.5 and on the mortality induced, DDWM/SLS 80/20 was classified as a severely irritating and severely damaging formulation in each of the four experiments.

Table 6.5 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the positive control slugs treated with 20 mg DDWM/SLS 80/20 on 5 successive days

Experiment	Total mucus production (%) *	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g) *	Number of slugs with ALP release	n
Experiment 1	23.6 ± 5.3	172 ± 45	1.65 ± 0.48	3	5
Experiment 2	23.0 ± 4.1	164 ± 58	1.86 ± 1.41	2	5
Experiment 3	25.2 ± 1.7	141 ± 65	3.54 ± 1.80	3	5
Experiment 4	30.9 ± 4.9	82 ± 65	1.24 ± 1.43	3	5
Inter-experiment mean	**25.6 ± 3.6**	**140 ± 41**	**2.07 ± 1.01**		**4**

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 4 experiments.

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

6.5.3 Evaluation of local tolerance of ocular powder formulations

6.5.3.1 Local tolerance of ingredients intended to prepare ocular minitablets

The local tolerance of ingredients envisaged to prepare ocular minitablets was evaluated by placing the slugs on 20 mg powder for 30 minutes on five successive days. All the slugs survived a repeated treatment with DDWM, sodium stearyl fumarate, Amioca®, ciprofloxacin HCl, gentamycin sulphate, vancomycin HCl, and Carbopol® 974P. Only a repeated treatment with DDWM/SLS 80/20 caused 40% mortality by day 4 and 80% mortality by day 5.

The effects of a repeated treatment on the total mucus production, mean protein and enzyme release are presented in Table 6.6. Slugs treated with sodium stearyl fumarate or Amioca® produced a smaller or comparable amount of mucus compared to the negative control slugs. Based on the prediction model presented in Figure 5.1, sodium stearyl fumarate and Amioca® were classified as non-irritating compounds. Ciprofloxacin HCl and gentamycin sulphate induced a mucus production that was significantly higher than the mucus production of the negative control slugs, but significantly lower than the positive control slugs ($p < 0.05$, Scheffé test). These ingredients were classified as mildly irritating. Vancomycin HCl and Carbopol® 974P induced a mucus production that was comparable to the mucus production of the positive control slugs ($p > 0.05$, Scheffé test). Vancomycin HCl and Carbopol® 974P were classified as severely irritating ingredients.

Table 6.6 Effect of a repeated treatment for 5 successive days with 20 mg of ingredients intended to prepare ocular minitablets on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
DDWM	3.3 ± 1.5 [a]	7 ± 4 [a]	–	0	5
Sodium stearyl fumarate	0.1 ± 0.8	17 ± 9 [a]	–	0	5
Amioca®	6.2 ± 1.4 [a]	23 ± 12 [a]	–	0	5
Ciprofloxacin HCl	10.2 ± 3.4	24 ± 36 [a]	–	0	5
Gentamycin sulphate	10.1 ± 0.6	not interpretable	–	0	3
Vancomycin HCl	27.2 ± 5.0 [b]	16 ± 7 [a]	–	0	5
Carbopol® 974P	21.4 ± 3.7 [b]	61 ± 44 [b]	0.55 ± 0.46	0	5
DDWM/SLS 80/20	23.6 ± 5.3 [b]	172 ± 45 [b]	1.65 ± 0.48 [b]	3	5

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a] For this end point, data are not significantly different from DDWM ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from DDWM/SLS 80/20 ($p > 0.05$, Scheffé test).

The protein release of the slugs treated with sodium stearyl fumarate, Amioca®, ciprofloxacin HCl, and vancomycin HCl was comparable to protein release of the negative control slugs ($p > 0.05$, Scheffé test). Based on the prediction model presented in Figure 5.2, these ingredients were classified as minimally damaging compounds. Because gentamycin sulphate interfered with the NanoOrange® protein determination assay, the end point protein release could not be interpreted for this ingredient. However, because no enzyme release was

detected and the body weight of the slugs after a five-day treatment with gentamycin sulphate was 80% of the initial body weight which was comparable to the reduction in body weight of the negative control slugs (whereas the body weight of positive control slugs was reduced to 50% after the fifth treatment), tissue damage was very unlikely and gentamycin sulphate was classified as a minimally damaging ingredient. The protein release of the slugs treated with Carbopol® 974P was comparable to the protein release of the positive control slugs ($p > 0.05$, Scheffé test). Furthermore, LDH release was detected for the slugs treated with Carbopol® 974P. Based on the protein and enzyme release data, Carbopol® 974P was classified as a moderately damaging ingredient.

6.5.3.2 Local tolerance of powder mixtures intended to prepare ocular minitablets

Taking into account these test results, powder mixtures intended to prepare ocular minitablets were made. The local tolerance of DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl mixtures containing different ratios of ciprofloxacin and Amioca®/Carbopol® 974P/ NaSF/gentamycin sulphate/vancomycin HCl mixtures containing different ratios of Carbopol® 974P was evaluated using the optimised test procedure (Table 6.7). All the slugs survived a repeated treatment on five successive days with DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl powders and Amioca®/Carbopol® 974P/NaSF/gentamycin sulphate/vancomycin HCl mixtures. Only a repeated treatment with DDWM/SLS 80/20 caused 20% mortality by day 5.

The total mucus production of the slugs treated with each of the different DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl mixtures was comparable to that of the negative control slugs ($p > 0.05$, Scheffé test). No statistically significant differences were detected between the non-sterilised and the sterilised powder mixtures. The different DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl mixtures were classified as non-irritating powders. Slugs treated with the two Amioca®/Carbopol® 974P/NaSF/gentamycin sulphate/vancomycin HCl powders produced significantly more mucus than the negative control slugs and significantly less mucus than the positive control slugs ($p \leq 0.001$, Scheffé test). These two powder mixtures were classified as mildly irritating formulations.

A repeated treatment with all DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl and Amioca®/Carbopol® 974P/NaSF/gentamycin sulphate/vancomycin HCl mixtures resulted in a protein release comparable to that of the negative control slugs ($p > 0.05$, Scheffé test). Moreover, the LDH and ALP release by the slugs treated with these mixtures was under the detection limit. Each of the DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl and

Amioca®/Carbopol® 974P/NaSF/gentamycin sulphate/vancomycin HCl mixtures was classified as a minimally damaging powder formulation.

Table 6.7 Effect of a repeated treatment for 5 successive days with 20 mg of ocular powder mixtures on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
DDWM	2.7 ± 1.7 [a]	4 ± 3 [a]	–	0	5
DDWM/Carb/NaSF/ciprofloxacin HCl					
94/5/1/0 NS	3.9 ± 1.0 [a]	11 ± 4 [a]	–	0	5
94/5/1/0 S	4.7 ± 1.3 [a]	11 ± 9 [a]	–	0	5
92/5/1/2 NS	4.7 ± 1.8 [a]	6 ± 2 [a]	–	0	5
90.5/5/1/3.5 NS	3.9 ± 0.9 [a]	4 ± 2 [a]	–	0	5
90.5/5/1/3.5 S	2.5 ± 0.7 [a]	8 ± 7 [a]	–	0	4
89/5/1/5 NS	5.3 ± 0.9 [a]	4 ± 1 [a]	–	0	5
84/5/1/10 NS	5.5 ± 2.0 [a]	7 ± 3 [a]	–	0	5
Amioca®/Carb/NaSF/gentamycin sulphate/vancomycin HCl					
84.55/4.45/1/5/5 S	8.5 ± 1.9	7 ± 2 [a]	–	0	5
75.65/13.35/1/5/5 S	9.3 ± 1.8	10 ± 3 [a]	–	0	5
DDWM/SLS 80/20	30.9 ± 4.9 [b]	82 ± 65 [b]	1.24 ± 1.43	3	5

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; Carb, Carbopol® 974P; NaSF, sodium stearyl fumarate; NS, not sterilised; S, sterilised; -, below the detection limit.

[a] For this end point, data are not significantly different from DDWM ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from DDWM/SLS 80/20 ($p > 0.05$, Scheffé test).

6.6 DISCUSSION

Irritation and damage of the eye can have serious consequences. Therefore, the evaluation of the local tolerance of formulations intended for repeated ocular administration is performed by academic, pharmaceutical and cosmetic research centres. The objective of this study was to optimise and evaluate the five-day procedure of the Slug Mucosal Irritation test (described in Chapter 5) for local tolerance testing of bioadhesive powder formulations intended for repeated ocular administration.

It was investigated if a repeated treatment on five successive days was necessary by testing a negative control powder (DDWM), a positive control powder DDWM/BAC 95/5 and powders containing DDWM and SLS in different ratios. The results demonstrated that a

repeated treatment on five successive days was necessary to obtain a good discrimination between the mucus productions and protein and enzyme release data of the negative and positive control slugs. Prolongation of the experiment was not useful, because the mortality of the slugs treated with severely irritating formulations would increase so that the end point range between non-irritating and irritating formulations would not further increase. Furthermore, the powder DDWM/SLS 80/20 was selected as replacement for DDWM/BAC 95/5.

Next, the influence of the amount of powder on the end points of the Slug Mucosal Irritation test was investigated in order to select the most suitable test amount. The results indicated that after treatment with 20 mg powder the largest difference between the negative and positive control was achieved for the mucus production. Based on this observation and on statistical analyses, 20 mg was selected as test amount to evaluate the local tolerance of powder formulations. It is interesting to note that 20 mg was also the amount that was used to evaluate the mucosal tolerance of nasal and buccal bioadhesive powder formulations in Chapter 5 and in previous studies (Adriaens, 2000; Callens *et al.*, 2001).

The optimised test procedure was used to evaluate the local tolerance of ingredients intended to prepare ocular minitablets. By means of the classification prediction model described in Chapter 5, DDWM, sodium stearyl fumarate, and Amioca® were classified as non-irritating ingredients because of the low total mucus production. Furthermore, low protein release and no enzyme release by the slugs treated with DDWM, sodium stearyl fumarate, and Amioca® indicated minimal tissue damage. For DDWM, the results obtained with the Slug Mucosal Irritation test were in agreement with clinical data. Ocular minitablets containing 94% DDWM were well accepted by human volunteers (Ceulemans *et al.*, 2001).

Based on the mucus production, ciprofloxacin HCl and gentamycin sulphate were classified as mildly irritating compounds. Furthermore, these ingredients induced minimal tissue damage as was indicated by low protein release and enzyme release below the detection limit. Little is known about the eye irritating properties of pure ciprofloxacin HCl and gentamicin sulphate, but ophthalmic preparations containing ciprofloxacin or gentamicin resulted only occasionally in eye irritation (see below).

Vancomycin HCl and Carbopol® 974P induced a total mucus production comparable to the positive control slugs which is an indication of severe irritation. Furthermore, the increased protein release and LDH release of the slugs with Carbopol® 974P indicated that this compound causes moderate tissue damage. The irritation potential of vancomycin HCl and Carbopol® is also reported in animals and humans. Animal studies indicated that

vancomycin is irritating to eyes (Eli Lilly, 2004). Furthermore, an oral solution containing 95% vancomycin HCl was identified as eye irritant (Eli Lilly, 2004). Carbopol® is often considered to be irritating for the eye (B.F. Goodrich, 1986). Furthermore, a single application of bioadhesive buccal tablets consisting of pure Carbopol® 934P caused small buccal lesions in 25% of 16 human volunteers; 44% of the volunteers had to remove the tablet due to irritation (Bottenberg *et al.*, 1991).

Taking into account these results, powder mixtures intended to prepare ocular minitablets were made. The local tolerance of DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl mixtures containing different ratios of ciprofloxacin and the local tolerance of Amioca®/Carbopol® 974P/NaSF/gentamycin sulphate/vancomycin HCl mixtures containing different ratios of Carbopol® 974P were evaluated using the optimised test procedure of the Slug Mucosal Irritation test. Powders containing DDWM, 5% Carbopol® 974P, 1% NaSF and up to 10% ciprofloxacin HCl were classified as non-irritating formulations based on the low mucus production of the slugs. Furthermore, enzyme release below the detection limit and low protein release induced by these powders indicated that treatment of the slugs with these powders caused minimal damage of the mucosa. No significant differences were detected between the non-sterilised and the sterilised powder mixtures. Ophthalmic formulations containing DDWM, 5% Carbopol®, 1% NaSF and/or 3.5% or more ciprofloxacin HCl were also described to be non-irritating to human and rabbit eyes. When ocular minitablets made of DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl 94/5/1/0 were applied three times to each of eight healthy volunteers, the general irritation score was low, indicating that the minitablet was well accepted by the volunteers (Ceulemans *et al.*, 2001). A single application of an ocular insert containing 5% Carbopol® to rabbit eyes did not cause eye irritation (Gurtler *et al.*, 1995). A single administration of an ocular minitablet made of DDWM/Carbopol® 974P/NaSF/ciprofloxacin HCl 90.5/5/1/3.5 to six healthy volunteers demonstrated that the minitablets were well tolerated as was indicated by a low general irritation score (Weyenberg *et al.*, 2004). A single application of a bioadhesive formulation containing 32.2% ciprofloxacin HCl resulted in minimal irritation of rabbit eyes. Moderate lacrimation observed during the study was ascribed to the nature of the formulation (Bhadra *et al.*, 2004).

A repeated treatment of the slugs with powder mixtures containing Amioca®, 1% NaSF, 5% gentamycin sulphate, 5% vancomycin HCl and 4.45% or 13.35% Carbopol® 974P resulted in mild irritation of the slug mucosa. The low protein release and enzyme release below the detection limit indicated that minimal damage of the slug mucosa occured. Because a single application of an ocular insert containing 25% gentamycin sulphate and 2.2%

Carbopol® 934P in the rabbit eye caused minimal irritation (Baeyens *et al.*, 1998) on the one hand and co-spray dried Amioca®/Carbopol® 974P mixtures containing up to 15% Carbopol® 974P induced no irritation of the slug mucosal tissue (Adriaens *et al.*, 2003) on the other hand, the mild irritation potential of these powder mixtures is probably due to the presence of vancomycin HCl.

The results of this study indicate that the Slug Mucosal Irritation test seems to be a promising alternative to evaluate the local tolerance of both ingredients of bioadhesive powder formulations and powder formulations intended for repeated ocular administration.

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CHAPTER 7: EVALUATION OF MUCOSAL TOLERANCE OF SUPPOSITORIES

7.1 INTRODUCTION

The rectal route is not only interesting for achieving local action of drugs, but it is also a useful alternative administration route for systemic delivery of drugs when oral or invasive administration of medication is impractical or impossible (van Hoogdalem et al., 1991). Because administration of suppositories can cause rectal irritation on the one hand and irritation and damage of the rectal mucosa can break the protective barrier against micro-organisms and noxious substances on the other hand, it is important that the local tolerance of suppositories is evaluated pre-clinically.

7.2 RECTAL MUCOSA

The human rectal mucosa is a simple columnar epithelium with goblet cells (van Hoogdalem *et al.*, 1991; Marieb, 1995). The lubricating mucus produced by the goblet cells eases the passage of faeces to the end of the digestive tract and protects the intestinal wall from irritating acids and gases released by bacteria in the colon (Marieb, 1995). The mucosa of the anal canal consists of a stratified squamous epithelium (van Hoogdalem *et al.*, 1991; Marieb, 1995). The mucus produced in the anal canal aids in emptying the anal canal (Marieb, 1995).

7.3 EVALUATION OF RECTAL TOLERANCE OF FORMULATIONS

The regulatory authorities require that the mucosal tolerance of medicinal products intended for rectal administration is evaluated by a repeated dose rectal tolerance test. Local tolerance testing is usually conducted in rabbits or dogs once or twice daily for at least seven

days. For this purpose, the definitive formulation consisting of the drug in its vehicle at the concentration intended for human use is administrated. During the treatment period, the animals are examined for clinical signs of irritation or pain and for anal discharge. After administration, the animals are sacrificed and the rectal mucosa is examined macroscopically and eventually microscopically (EC, 1990).

Several pre-clinical *in vivo* and *in vitro* models have been used to study the local tolerance of rectal formulations or their ingredients. Histological changes of the rectal mucosa induced by repeated or single administration of suppositories or rectal gels have been investigated *in vivo* in rabbits (De Muynck *et al.*, 1991; De Muynck *et al.*, 1993; De Muynck *et al.*, 1994; Stei *et al.*, 1996; Watanabe *et al.*, 1996; Yagahi *et al.*, 2000; Berryman *et al.*, 2002; Peeters *et al.*, 2004) or dogs (Berryman *et al.*, 2002) by light microscopy. Rectal irritation and damage of rectally applied formulations or their ingredients have been investigated in mice and rats by light microscopy (Reid *et al.*, 1987; Thomas *et al.*, 1988; Oberle *et al.*, 1995; Kinouchi *et al.*, 1996; Phillips and Zacharapoulos, 1998; Miyake *et al.*, 2004) and/or the *in vivo* or *in situ* release of mucus, proteins and LDH from the rectal mucosa (Oberle *et al.*, 1995; Miyake *et al.*, 2004). The disruption of the rectal tissues induced by rectal administration of gels has been investigated in monkeys by microscopic examination of rectal lavage 15 minutes after the rectal application (Patton *et al.*, 2002). Furthermore, the cytotoxicity of suppositories and their ingredients has been evaluated *in vitro* using Caco-2 cells (Dash *et al.*, 1999).

7.4 OBJECTIVES

The objective of the present study was to optimise and evaluate the five-day procedure of the Slug Mucosal Irritation test for the evaluation of the local tolerance of suppositories intended for repeated administration. The irritation and tissue damaging properties of the suppositories were evaluated after grinding the suppositories into powder with a pestle in a mortar. The influence of the amount of grinded suppository on the test end points was evaluated. For the selection of the test amounts, the contact surface between the slug mucosa and the grinded suppository was taken into account. Because 20 mg of grinded suppository takes up a smaller surface than 20 mg bioadhesive powder formulation, the contact surface between the slug mucosa and 20 mg grinded suppository would be too small. Consequently, it was opted to put the slugs on larger amounts of suppository (50 and 75 mg) than in case of bioadhesive powders. Furthermore, Novata® B was used as negative control. Several ratios of

Novata® B/SLS were tested as positive control. It was opted to use SLS to prepare the positive control suppositories, because SLS was also used for the preparation of the positive control powders in Chapter 6.

Next, the repeatability of the optimised assay was assessed. For this purpose, the negative control and the positive control were tested on three separate occasions. Finally, the optimised test procedure was used to evaluate the local tolerance of suppositories. The classification prediction model developed for the determination of the irritation and tissue damage category of bioadhesive powders (described in Chapter 5) was used to classify the suppositories.

7.5 MATERIALS AND METHODS

7.5.1 Suppositories

Novata® B suppositories consisted of pure Novata® B (Cognis, Dusseldorf, Germany), a glyceride base mainly consisting of C12-C14 glycerides (78.6% (w/w) triglycerides, 19.5% (w/w) diglycerides, and 1.9% (w/w) monoglycerides). Suppocire® AM suppositories were made of pure Suppocire® AM (Gattefossé, Saint-Priest, France), a glyceride base mainly consisting of C12-C14 glycerides (96.5% (w/w) triglycerides, 3.0% (w/w) diglycerides, and 0.5% (w/w) monoglycerides). The hydrophilic PEG 1500/PEG 4000 3/7 (w/w) suppositories were prepared using 30% (w/w) polyethylene glycol 1500 (Sigma, St. Louis, MO, USA) and 70% (w/w) polyethylene glycol 4000 (Sigma). Novata® B/SLS suppositories in ratios 95/5, 90/10 and 80/20 (w/w) were prepared using Novata® B (Cognis) and sodium lauryl sulphate (SLS, Sigma).

Colitofalk® 500 suppositories (n.v. Codali, Brussels, Belgium) contain 500 mg (or 23% (w/w)) mesalazine and a fatty suppository base. Pentasa® suppositories (Ferring, Aalst, Belgium) are composed of 1 g (or 63% (w/w)) mesalazine, povidone, magnesium stearate, and PEG 6000.

7.5.2 Local tolerance test procedure of the Slug Mucosal Irritation test

The local tolerance of the suppositories was assessed according to the procedure described in Chapter 6. During five successive days, the slugs were placed on the (neat) grinded suppository for 30 minutes and the mucus production of the slugs was measured. One

and two hours after each of the five contact periods, the samples were collected. The samples were analysed immediately for the presence of proteins, LDH, and ALP released from the body wall of the slugs.

7.5.3 Analytical procedures

The protein, LDH, and ALP determinations were performed according to the analytical procedures described in Chapter 3.

7.5.4 Data analysis

For each slug, the total mucus production, mean protein release, mean LDH release and mean ALP release were calculated as described in the data analysis section of Chapter 5. These values were used for the statistical analyses using the computer program SPSS (version 12.0; SPSS, Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

A two-way ANOVA was performed to investigate the test amount effect and formulation effect on the total mucus production and mean protein release. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If variances were unequal, the data were log-transformed. Because no significant interaction was present for both end points, the main effects were interpreted separately from the interaction and a multiple comparison among pairs of means was performed using a Scheffé post hoc test.

Statistically significant differences between repeated experiments or different treatments were determined using a one-way ANOVA. The normal distribution of the data and the homogeneity of variances were tested with the tests mentioned above. If the variances were found to be not equal, the data were transformed to their logarithm. To further compare the effects of the different experiments or treatments, a multiple comparison among pairs of means was performed using a Scheffé test.

Using the cut-off values presented in Figures 5.1 and 5.2, the formulations were classified into one of the irritation and tissue damage categories.

7.6 RESULTS

7.6.1 Optimisation of the test procedure

The effect of the amount of suppository on the test end points was evaluated in order to select the most suitable amount. For this purpose, 50 and 75 mg of grinded suppositories consisting of neat Novata® B on the one hand and grinded Novata® B/SLS suppositories in ratios 95/5, 90/10 and 80/20 on the other hand were tested. All the slugs treated with the different amounts of Novata® B survived a repeated treatment on 5 successive days. A daily treatment with 50 mg of Novata® B/SLS 95/5 and 90/10 led to 20% and 60% mortality on day 5, respectively. 40% of the slugs treated with 50 mg Novata® B/SLS 80/20 were dead on day 3 and all the slugs were finally dead on day 4. Repeated treatment with 75 mg Novata® B/SLS 95/5 and 90/10 resulted respectively in 40% and 100% mortality on day 5. All the slugs treated with 75 mg Novata® B/SLS 80/20 were already dead on day 3.

For the total mucus production, two-way ANOVA testing revealed no significant interaction between the test amount and the suppository formulation ($p = 0.252$) (Table 7.1). The test amount had no significant effect on the total mucus production ($p = 0.625$). However, there was a significant main effect of the formulation ($p < 0.001$). Scheffé post hoc testing revealed that the total mucus production of slugs treated with Novata® B was significantly lower than the total mucus productions of the slugs treated with Novata® B/SLS ratios.

Table 7.1 Influence of the test amount and the suppository formulation on the total amount of mucus produced by slugs during a repeated treatment on 5 successive days

Formulation	Total mucus production (%)		
	50 mg	75 mg	**Average formulation**
Novata® B	0.8 ± 1.4	2.4 ± 2.4	**1.6 ± 2.0** [a]
Novata® B/SLS 95/5	29.3 ± 4.0	28.4 ± 2.7	**28.8 ± 3.2** [b]
Novata® B/SLS 90/10	31.1 ± 6.0	34.2 ± 6.2	**32.6 ± 6.0** [b]
Novata® B/SLS 80/20	33.5 ± 3.8	29.6 ± 3.3	**31.5 ± 4.0** [b]
Average test amount *	**23.7 ± 14.1**	**23.6 ± 13.3**	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 10 and 20 slugs, respectively.

Two-way ANOVA testing revealed no significant interaction and no significant test amount effect ($p > 0.05$), but a significant formulation effect ($p < 0.05$).

[a, b] Same letter indicates no significant difference between the suppository formulations ($p > 0.05$, Scheffé test).

* There are no significant differences between the mucus productions induced by the two test amounts ($p > 0.05$, Two-way ANOVA).

For the mean protein release, two-way ANOVA testing revealed no significant interaction between the test amount and the suppository formulations ($p = 0.971$) (Table 7.2). The test amount had no significant effect on the mean protein release ($p = 0.080$). However, there was a significant main effect of the formulation ($p < 0.001$). Scheffé post hoc testing suggested the following three homogeneous subsets for the mean protein release: (1) Novata® B, (2) Novata® B/SLS 95/5 and Novata® B/SLS 90/10, and (3) Novata® B/SLS 90/10 and Novata® B/SLS 80/20.

Table 7.2 Influence of the test amount and the suppository formulation on the mean protein release induced by a repeated treatment of the slugs on 5 successive days

Formulation	Mean protein release (µg/ml.g)		
	50 mg	75 mg	**Average formulation**
Novata® B	10 ± 3	13 ± 7	**12 ± 5** [a]
Novata® B/SLS 95/5	76 ± 38	135 ± 86	**105 ± 70** [b]
Novata® B/SLS 90/10	153 ± 102	187 ± 68	**170 ± 83** [b, c]
Novata® B/SLS 80/20	223 ± 117	320 ± 148	**272 ± 136** [c]
Average test amount *	**116 ± 110**	**164 ± 141**	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 10 and 20 slugs, respectively.

Two-way ANOVA testing revealed no significant interaction and no significant test amount effect ($p > 0.05$), but a significant formulation effect ($p < 0.05$).

[a, b, c] Same letter indicates no significant difference between the suppository formulations ($p > 0.05$, Scheffé test).

* There are no significant differences between the mean protein release data induced by the two test amounts ($p > 0.05$, Two-way ANOVA).

For the slugs treated with 50 mg or 75 mg Novata® B, the LDH release was under the detection limit. For the mean LDH release of the slugs treated with the different amounts of Novata® B/SLS in different ratios, two-way ANOVA testing revealed also no significant interaction between the test amount and the suppository formulations ($p = 0.899$). The test amount ($p = 0.377$) and the formulation ($p = 0.310$) had no significant effect on the mean LDH release (data not shown). LDH release was detected after the 1st treatment with 50 or 75 mg Novata® B/SLS 95/5, 90/10 or 80/20. There were only two exceptions: (1) for treatment with 50 mg of Novata® B/SLS 95/5 where LDH release was detected after the 3rd treatment and (2) for treatment with 75 mg of Novata® B/SLS 90/10 where LDH release was detected after the 2nd treatment. LDH release increased with a repeated treatment. ALP release was only detected for one slug treated with 50 mg Novata® B/SLS 95/5, 50 mg Novata® B/SLS

80/20, 75 mg Novata® B/SLS 95/5 or 75 mg Novata® B/SLS 90/10 and for three slugs treated with 75 mg Novata® B/SLS 80/20. ALP release occurred on the same day of LDH release or one day after LDH release.

Because statistical analyses revealed no significant differences between the mucus productions and mean protein release data induced by 50 mg and 75 mg of the tested suppositories on the one hand and treatment with 75 mg of severely irritating and damaging suppositories resulted in a higher mortality, 50 mg was selected as test amount to evaluate the local tolerance of suppositories. Furthermore, Novata® B was a good negative control, because the suppository base induced a low mucus production and protein release, and no enzyme release was detected. The Novata® B/SLS 95/5, 90/10 and 80/20 suppositories induced a high mucus production, an increased protein release, and the release of enzymes. However, the protein release after a repeated treatment with 50 mg Novata® B/SLS 95/5 was lower than protein release after treatment with 50 mg Novata® B/SLS in ratios 90/10 and 80/20. Furthermore, a repeated treatment with 50 mg Novata® B/SLS 80/20 resulted in an early onset of mortality. Consequently, Novata® B/SLS 90/10 was chosen as positive control.

7.6.2 Repeatability of the test procedure and prediction model

In order to investigate the repeatability of the test results obtained with the optimised test procedure for local tolerance testing of suppository formulations, the negative and positive control were tested independently on three separate occasions. Table 7.3 shows the total mucus production and mean protein and enzyme release of the negative control slugs measured in each of the repeated experiments and the mean of the three experiments. All the slugs treated with Novata® B survived a repeated treatment on five successive days. For the negative control, Scheffé post hoc testing suggested two homogenous subsets for the total mucus production. One subset comprised only the total mucus production of the first experiment, whereas another subset contained the total mucus productions of the second and third experiment. However, no significant difference in mean protein release was detected between the repeated experiments ($p > 0.05$, One-way ANOVA). Based on the prediction models presented in Figures 5.1 and 5.2, Novata® B was classified as a non-irritating and minimally damaging formulation in each of the repeated experiments.

A daily treatment with Novata® B/SLS 90/10 led to 60%, 100% and 0% mortality by day 5 in the first, second and third experiment, respectively. For the positive control, ANOVA testing revealed no significant difference in total mucus production, in mean protein release

and in mean LDH release between the repeated experiments ($p > 0.05$) (Table 7.4). Repeated treatment with Novata®/SLS 90/10 resulted in severe irritation and damage of the slug mucosa in each of the three experiments.

Table 7.3 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the negative control slugs treated with 50 mg Novata® B on 5 successive days

Experiment	Total mucus production (%)	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Experiment 1	0.8 ± 1.4^{a}	10 ± 3	–	0	5
Experiment 2	3.6 ± 1.4^{b}	22 ± 22	–	0	4
Experiment 3	3.9 ± 1.2^{b}	7 ± 2	–	0	5
Inter-experiment mean	**2.8 ± 1.7**	**13 ± 8**	–		**3**

Total mucus production and mean protein release data are presented as the mean ± standard deviation of 4-5 slugs; bold values represent the mean ± standard deviation of 3 experiments; -, below the detection limit.

[a, b] For this end point, the values marked with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

Table 7.4 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the positive control slugs treated with 50 mg Novata® /SLS 90/10 on 5 successive days

Experiment	Total mucus production (%) *	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g) *	Number of slugs with ALP release	n
Experiment 1	31.7 ± 6.0	153 ± 102	2.18 ± 1.92	0	5
Experiment 2	27.1 ± 5.7	238 ± 80	2.60 ± 2.05	0	5
Experiment 3	35.3 ± 4.3	110 ± 16	2.53 ± 0.80	4	5
Inter-experiment mean	**31.1 ± 4.1**	**167 ± 65**	**2.44 ± 0.22**		**3**

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 3 experiments.

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

7.6.3 Evaluation of local tolerance of suppositories

The local tolerance of four additional suppositories was evaluated by placing the slugs on 50 mg grinded suppository for 30 minutes on five successive days. All the slugs survived a repeated treatment with Novata® B, Suppocire® AM, Colitofalk®, Pentasa®, and PEG 1500/PEG 4000 3/7. A daily treatment with Novata® B/SLS 90/10 led to 60%, 100% and 0% mortality by day 5 in the first, second and third experiment, respectively.

The effects of a daily treatment on the total mucus production, mean protein and enzyme release are presented in Table 7.5. The total mucus production of the slugs treated with Suppocire® AM or Colitofalk® was comparable to that of the negative control slugs ($p > 0.05$, Scheffé test). Based on the prediction model presented in Figure 5.1, these suppositories were classified as non-irritating formulations. Slugs treated with Pentasa® or PEG 1500/PEG 4000 3/7 produced significantly more mucus than the negative control slugs and significantly less mucus than the positive control slugs ($p < 0.05$, Scheffé test). Pentasa® was classified as a mildly irritating formulation, whereas PEG 1500/PEG 4000 3/7 was classified as a severely irritating formulation.

Table 7.5 Effect of a repeated treatment for 5 successive days with 50 mg of different suppositories on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Novata® B	2.8 ± 2.0 [a]	13 ± 13 [a]	–	0	14
Suppocire® AM	3.8 ± 1.1 [a]	6 ± 5 [a]	–	0	5
Colitofalk®	4.6 ± 0.8 [a]	14 ± 8 [a]	–	0	5
Pentasa®	8.7 ± 2.9	9 ± 3 [a]	–	0	5
PEG 1500/PEG 4000 3/7	21.7 ± 2.7	11 ± 3 [a]	–	0	5
Novata® B/SLS 90/10	31.1 ± 6.1 [b]	167 ± 89 [b]	2.44 ± 1.57	4	15

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a] For this end point, data are not significantly different from Novata® B ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from Novata® B/SLS 90/10 ($p > 0.05$, Scheffé test).

A repeated treatment with Suppocire® AM, Colitofalk®, Pentasa® or PEG 1500/PEG 4000 3/7 resulted in a protein release comparable to that of the negative control slugs ($p > 0.05$, Scheffé test). Moreover, the release of LDH and ALP by slugs treated with these

suppositories was under the detection limit. Each of these suppositories was classified as a minimally damaging formulation.

7.7 DISCUSSION

A reliable and relevant alternative method for repeated dose rectal tolerance testing would be very useful to select non-irritating suppository formulations early in the development process. The objective of this study was to optimise and evaluate the five-day procedure of the Slug Mucosal Irritation test for local tolerance testing of suppositories intended for repeated administration.

The influence of the amount of grinded suppository on the end points of the Slug Mucosal Irritation test was investigated. The statistical analyses revealed that treatment with 50 mg and 75 mg of the tested suppositories did not result in statistical significant differences in the total mucus production and in the mean protein release. Because treatment with 75 mg of severely irritating and damaging suppositories resulted in a higher mortality, it was opted to select 50 mg as test amount to evaluate the local tolerance of suppositories. Furthermore, Novata® B served well as negative control, because the suppository base induced a low mucus production and protein release, and no enzyme release was detected. Novata® B/SLS 90/10 was selected as positive control, because it induced a high mucus production, a high protein release, and the release of enzymes.

The optimised test procedure was used to evaluate the local tolerance of a few suppositories. When the classification prediction model developed for the determination of the irritation and tissue damage category of bioadhesive powders (described in Chapter 5) was used to classify the suppositories, Novata® B and Suppocire® AM were classified as non-irritating formulations because of the low total mucus production. Furthermore, repeated treatment with Novata® B or Suppocire® AM caused minimal tissue damage as was demonstrated by low protein release and enzyme release below the detection limit. The total mucus production and mean protein release data of the slugs treated with Novata® B or Suppocire® AM were comparable to the ones of the negative control slugs described in Chapter 6. Fatty suppository bases are generally considered to be non-irritating to the rectal mucosa. Several authors reported that repeated rectal administration of suppositories containing semi-synthetic triglycerides did not provoke irritation of the rectal tissues of rabbits (Yahagi *et al.*, 2000; Peeters *et al.*, 2004), dogs (Neuwald and Meyer-Lohmann, 1959), and rats (Reid *et al.*, 1987; Thomas *et al.*, 1988). Although most of the literature on the

irritation and damage properties of fatty suppository bases describes that they are non-irritating, De Muynck *et al.* (1991) reported that a repeated treatment of rabbits with pure Novata® B or Suppocire® AM suppositories during 14 consecutive days unexpectedly caused severe mucosal damage.

A repeated treatment of the slugs with Novata® B/SLS 90/10 resulted in a high mucus production (± 31%), indicating severe irritation of the slug mucosa. Furthermore, the high protein release and increased enzyme release of these slugs indicated that Novata® B/SLS 90/10 caused also severe damage of the slug mucosa. The total mucus production, mean protein and enzyme release data of the slugs treated with Novata® B/SLS 90/10 were comparable to the ones of the positive control slugs described in Chapters 5 and 6.

Colitofalk® suppositories (containing mesalazine and a fatty suppository base) were classified by the Slug Mucosal Irritation test as non-irritating formulations. Furthermore, repeated treatment with Colitofalk® resulted in low protein release and enzyme release below the detection limit, indicating that minimal tissue damage occurred. The results obtained with the Slug Mucosal Irritation test are in good agreement with available preclinical data. Berryman *et al.* (2002) reported that twice daily administration of suppositories containing 500 mg mesalazine (similar to Colitofalk® suppositories) for 14 consecutive days to rabbits and dogs did not result in microscopically observable lesions of the rectum.

A repeated treatment with Pentasa® suppositories (containing mesalazine and a PEG suppository base) induced mild irritation and minimal damage of the slug mucosa as was indicated by the mucus production, protein and enzyme release data. After administration of Pentasa® suppositories once daily during 14-21 days, 11% of 132 patients with active idiopathic proctitis reported pain or anal irritation. Three of the six patients that stopped treatment because of intolerance, reported anal or rectal burning as reason for treatment discontinuation. Notwithstanding the adverse events, the overall acceptability was rated as good or fair (Lucidarme *et al.*, 1997). In another study, 77% of 125 patients with active cryptogenic proctitis reported that a once daily administration of Pentasa® suppositories for 14-21 days was well tolerated (Marteau *et al.*, 2000). It is interesting to note that the active substance in both Pentasa® and Colitofalk® suppositories is mesalazine. Pentasa® suppositories not only contain more mesalazine than Colitofalk® suppositories, but are also made of PEG, whereas the suppository base of Colitofalk® is a fatty base. The mild irritation potential of Pentasa® suppositories is probably due to the presence of PEG (see below).

A repeated treatment with PEG 1500/PEG 4000 3/7 induced severe irritation of the slug mucosa as was indicated by the high total mucus production. However, the suppository

formulation induced minimal tissue damage as was demonstrated by a low protein release and no detectable enzyme release. Throughout the experiments, it became clear that (grinded) suppositories containing PEG absorb water present in the slug mucosa. The PEG mixture was namely solid before the 30 minute contact period, whereas the PEG mixture became fluid after contact with the slug mucosa. In contrast with the dehydration observed after treatment with bioadhesive powders such as DDWM (Chapters 5 and 6), the dehydration induced by treatment with the PEG suppositories was much more pronounced. Ito (1980) suggested that the absorption of water present in the rectal mucosa by PEG is responsible for the irritation potential of PEG containing suppositories. Several authors reported that single or repeated administration of PEG suppositories resulted in irritation of the rectal mucosa. A repeated treatment of rabbits with PEG 1500/PEG 4000 3/7 suppositories during 14 consecutive days resulted in severe mucosal damage with severe mucosal hyperemia, diffuse infiltration of granulocytes, erosion, regeneration of the mucosal damage, and inflammatory polyps (De Muynck *et al.*, 1991). A single administration of PEG 1500/PEG 4000 1/3 suppositories to rabbits caused severe mucosal irritation with marked erosion and inflammatory cell infiltration of the lamina propria (Watanabe *et al.*, 1996). A single administration of 2.5 g PEG 1000/PEG 4000 suppositories to rabbits resulted in severe irritation with erosion of the mucosa, whereas 1.2 g PEG 1000/PEG 4000 suppositories generated moderate to severe erythema (Peeters *et al.*, 2004).

The results of this study indicate that the Slug Mucosal Irritation test seems to be a promising alternative to evaluate the local tolerance of suppositories intended for repeated administration. However, it has to be noted that the relevance and reliability of the optimised assay have to be further evaluated with additional suppository formulations that have been evaluated for their rectal irritating and damaging properties in animals and/or humans.

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CHAPTER 8: EVALUATION OF MUCOSAL TOLERANCE OF SEMI-SOLID VAGINAL FORMULATIONS

Based on Dhondt, M.M.M., Adriaens, E. and Remon, J.P. (2004) and on Dhondt, M.M.M., Adriaens, E., Van Roey, J. and Remon, J.P. (2005).

8.1 INTRODUCTION

To date, research on novel vaginal formulations is focused on local or systemic delivery of microbicides, spermicides, hormones, anti-infectious agents, proteins, and peptides. Vaginal formulations may be applied frequently over a period of months or years. Frequent use of some vaginal formulations can induce mucosal irritation and damage of the vaginal epithelium (Roddy *et al.*, 1993; Stafford *et al.*, 1998). Vaginal inflammation and ulceration might increase the susceptibility to sexually transmitted pathogens upon sexual intercourse (Stephenson, 2000; Fichorova *et al.*, 2001; Van Damme *et al.*, 2002). Therefore, the safety and vaginal tolerance of newly developed pharmaceutical products are important issues to be addressed by manufacturers and drug licensing companies.

The importance of an adequate safety assessment of vaginal formulations can be illustrated by the history of the non-ionic surfactant nonoxynol-9 (N-9). N-9 has been widely available as a contraceptive for many years and has been shown to be effective against a number of sexually transmitted pathogens in laboratory studies (Hicks *et al.*, 1985; Harrison and Chantler, 1998). Unfortunately, the repeated vaginal application of N-9 in humans has been associated with irritation or disruption of the vaginal and cervical epithelia (Roddy *et al.*, 1993; Stafford *et al.*, 1998), and with induction of an inflammatory response (Stafford *et al.*, 1998). Serious public health concerns have been raised by a recent phase II/III clinical trial that showed that a gel containing 3.5% N-9 (Advantage S®) may increase transmission of human immunodeficiency virus by causing lesions (Van Damme *et al.*, 2002).

8.2 VAGINAL MUCOSA

The human vagina is lined with a stratified squamous epithelium (Marieb, 1995) which is predominantly non-keratinised (Thompson *et al.*, 2001). Generally, no glands are present in the vaginal mucosa (Marieb, 1995; Beresford, 2005). Lubrication of the vaginal mucosa is provided by the cervical mucous glands (Marieb, 1995).

8.3 EVALUATION OF VAGINAL TOLERANCE OF FORMULATIONS

Regulatory authorities require that the mucosal tolerance of medicinal products intended for vaginal administration is evaluated by a repeated dose vaginal tolerance test. Local tolerance testing has to be conducted with the preparation being developed for human use (EC, 1990). It is generally recommended that both the active agent and the clinical formulation of the product are tested in a rabbit vaginal irritation model (standard 10-day application) early in the development process (IWGVM, 1996). During the treatment period, the animals are examined for clinical signs of irritation or pain and for vaginal discharge or bleeding. After administration, the animals are sacrificed and the vaginal mucosa is examined macroscopically and eventually microscopically (EC, 1990).

Several pre-clinical *in vivo* and *in vitro* models have been developed to study the irritating and damaging properties of formulations and their ingredients on the vaginal epithelium. Because rabbits have a simple cuboidal or columnar epithelium that is highly sensitive compared to the stratified squamous epithelium of the human vagina (Eckstein *et al.*, 1969), they are frequently used for vaginal irritation tests (Eckstein *et al.*, 1969; Chvapil *et al.*, 1980; Kaminsky and Willigan, 1982; Balzarini *et al.*, 1998; Gagné *et al.*, 1999; D'Cruz *et al.*, 2001; Zaneveld *et al.*, 2001; Dhondt *et al.*, 2005). Furthermore, histological changes of the vaginal mucosa induced by locally applied ingredients and formulations have been studied *in vivo* in mice (Neyts *et al.*, 2000; Achilles *et al.*, 2002; Milligan *et al.*, 2002; Catalone *et al.*, 2004), rats (Chvapil *et al.*, 1980; Kaminsky and Willigan, 1982), and non-human primates (Eckstein *et al.*, 1969; Patton *et al.*, 1998; Patton *et al.*, 1999; Zaneveld *et al.*, 2001) by light microscopy, scanning electron microscopy or by the release of markers.

The cytotoxicity of vaginally applied products and their ingredients has also been studied *in vitro* by evaluation of cell viability, histological changes or release of marker compounds. For this, primary human vaginal keratinocytes (Krebs *et al.*, 2000), immortalised human vaginal and cervical epithelial cells (Fichorova *et al.*, 1997; Maguire *et al.*, 2001;

Krebs *et al.*, 2002) or reconstructed three dimensional human vaginal tissue (Kubilus *et al.*, 2002; SkinEthic, 2005) are exposed to the products of interest. The cytotoxic effects of ingredients of vaginal formulations have also been investigated by *in vitro* erythrocyte hemolysis (Fowler *et al.*, 2003).

8.4 OBJECTIVES

In the present study, the five-day procedure of the Slug Mucosal Irritation test was optimised for the evaluation of the local tolerance of semi-solid vaginal formulations. It was determined if a repeated treatment on five successive days was necessary. The influence of the amount of semi-solid formulation on the end points of the test was evaluated. For this purpose, a gel containing 4.0% N-9 (Conceptrol®) was used as positive control like it is recommended by the International Working Group on Vaginal Microbicides (IWGVM, 1996). Nowadays, there is no generally accepted negative control vaginal formulation available; hence two gels that possibly could serve as negative control were selected. On the one hand hydroxyethyl cellulose gel was chosen, because Ballagh *et al.* (2002) used this gel as a negative control product. On the other hand Replens® was selected, because it was used as placebo in placebo-controlled trials (Van Damme *et al.*, 2000; Van Damme *et al.*, 2002).

Next, the repeatability of the optimised test procedure was assessed. For this purpose, the negative control and the positive control and some semi-solid vaginal formulations were tested on separate occasions. Finally, the optimised test procedure was used to evaluate the mucosal tolerance of semi-solid vaginal formulations that have been evaluated for the vaginal tolerance in animals and/or humans. Based on the results of this study on the one hand and on pre-clinical and clinical data on the other hand, a classification prediction model was developed for the determination of the irritation potential of semi-solid formulations.

8.5 MATERIALS AND METHODS

8.5.1 Semi-solid formulations

5% Hydroxyethyl cellulose (HEC) gel consisted of 5.0% (w/w) HEC, 10% (w/w) glycerol, 0.07% (w/w) methylparaben, 0.02% (w/w) propylparaben, 0.8% (w/w) propylene glycol, and water. The gels containing dapivirine, a human immunodeficiency virus-specific vaginal microbicide, were kindly provided by Tibotec BVBA (Mechelen, Belgium). The gels

were made up of 2.0% HEC and contained 22.5 µM, 225 µM or 10 mM dapivirine. The placebo dapivirine gel contained the same ingredients with the exception of dapivirine.

The over-the-counter (OTC) vaginal antifungal cream Monistat 7® (McNeil-PPC, Skillman, NJ, USA) contains 2% (w/w) miconazole nitrate, benzoic acid, cetyl alcohol, stearyl alcohol, isopropyl myristate, polysorbate 60, potassium hydroxide, propylene glycol, and purified water. The OTC vaginal lubricant Replens® (Columbia Laboratories, Hitchin, United Kingdom) contains carbomer, polycarbophil, glycerol, paraffin, hydrogenated palm oil glyceride, sorbic acid, sodium hydroxide, and 78.82% (w/w) purified water. K-Y® jelly (McNeil-PPC) is an OTC vaginal lubricant that is composed of chlorhexidine gluconate, HEC, glucono delta lactone, glycerol, methylparaben, sodium hydroxide, and purified water. The vaginal contraceptive Gynol II® (McNeil-PPC) contains 2.0% N-9, sodium carboxymethylcellulose, povidone, propylene glycol, sorbitol solution, sorbic acid, methylparaben, lactic acid, and purified water. Gynol II® Extra Strength (McNeil-PPC) is a vaginal contraceptive gel that is composed of 3.0% N-9, sodium carboxymethylcellulose, povidone, propylene glycol, sorbitol solution, sorbic acid, methylparaben, lactic acid, and purified water. The OTC vaginal contraceptive gel Advantage S® (Columbia Laboratories, NJ, USA) uses Replens® as a vehicle base and contains 3.5% N-9, carbomer 934P, polycarbophil, glycerol, paraffin, hydrogenated palm oil glyceride, methylparaben, sorbic acid, sodium hydroxide, and purified water. Protectaid® (Pirri Pharma Canada Inc., Montreal, Canada) is an OTC contraceptive sponge that is impregnated with a gel containing 0.125% N-9, 0.125% benzalkonium chloride, 0.5% sodium cholate, hydroxypropyl methylcellulose, glycerol, dimethicone, and purified water. The OTC vaginal contraceptive foam Delfen® (McNeil-PPC) contains 12.5% N-9, sodium carboxymethylcellulose, benzoic acid, cetyl alcohol, acetic acid, perfume, phosphoric acid, polyvinyl alcohol, propellant A-31, propylene glycol, stearamidoethyl diethylamine, stearic acid, sorbic acid, methylparaben, and purified water. Conceptrol® gel (McNeil-PPC) is an OTC vaginal contraceptive gel containing 4.0% N-9, sodium carboxymethylcellulose, propylene glycol, methylparaben, povidone, sorbitol solution, sorbic acid, lactic acid, and purified water.

8.5.2 Local tolerance test procedure of the Slug Mucosal Irritation test

The local tolerance of the semi-solid vaginal formulations was assessed according to the procedure described in Chapter 6. During five successive days, the slugs were placed on the neat semi-solid formulation for 30 minutes and the mucus production of the slugs was

measured. One and two hours after each of the five contact periods, the samples were collected. The samples were analysed immediately for the presence of proteins, LDH, and ALP released from the body wall of the slugs.

8.5.3 Analytical procedures

The protein, LDH, and ALP determinations were performed according to the analytical procedures described in Chapter 3.

8.5.4 Data analysis

For each slug, the total mucus production, mean protein release, mean LDH release and mean ALP release were calculated as described in the data analysis section of Chapter 5. These values were used for the statistical analyses using the computer program SPSS (version 12.0; SPSS, Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

A two-way ANOVA was performed to investigate the test amount effect and formulation effect on the total mucus production and mean protein release. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If variances were unequal, the data were log-transformed. Because a significant interaction between both factors was present for the total mucus production, the test amount effect for each formulation and the formulation effect for each test amount were investigated with a Bonferroni post hoc test. No significant interaction was present for the mean protein release. Consequently, the main effects were interpreted separately from the interaction and a multiple comparison among pairs of means was performed using a Scheffé post hoc test.

Statistically significant differences between repeated experiments or different treatments were determined using a one-way ANOVA. The normal distribution of the data and the homogeneity of variances were tested with the tests mentioned above. If the variances were found to be not equal, the data were transformed to their logarithm. To further compare the effects of the different experiments or treatments, a multiple comparison among pairs of means was performed using a Scheffé test.

Using the cut-off values presented in Figure 5.2, the formulations were classified into one of the tissue damage categories. Based on in-house experience, cut-off values were established for the classification of semi-solid formulations into irritation categories.

8.6 RESULTS

8.6.1 Optimisation of the test procedure

The procedure was optimised for the evaluation of semi-solid vaginal formulations by testing several amounts (50, 100, 150, and 200 mg) of two gels that possibly could serve as negative control (HEC gel and Replens®) and a positive control gel containing 4.0% N-9 (Conceptrol®). All the slugs treated with the different amounts of HEC gel and Replens® survived a repeated treatment on five successive days. A daily treatment with 50 mg or 100 mg of Conceptrol® led to 20% mortality on day 5. 60% of the slugs treated with 150 mg or 200 mg Conceptrol® were dead on day 4 and all the slugs were finally dead on day 5.

Firstly, the data were analysed in order to find out if a repeated treatment on five successive days was necessary. The mucus production profiles of the slugs treated with 50 mg of each gel were comparable to those of the slugs treated with 100 mg of the same gel, hence only the latter are shown in Figure 8.1 (left graph). The mucus production profiles after treatment with 200 mg gel were similar to those of 150 mg gel and are presented in Figure 8.1 (right graph). Figure 8.1 illustrates that one treatment did not suffice to make a distinction between the mucus productions induced by the three gels. For the total mucus production, discrimination between the mucus productions of the slugs treated with the three gels became more pronounced after a repeated treatment and the best discrimination could be made after five treatments.

Only the protein release profiles after treatment with 100 mg gel are shown (Figure 8.2). After the first treatment, all gels induced a high protein release. However, from the second day on HEC gel and Replens® resulted in low protein release levels comparable to the ones of the negative control slugs in previous studies (Chapters 5, 6 and 7). The protein release by the Conceptrol® slugs increased with a repeated treatment. For the end point protein release, the best discrimination between the different semi-solid formulations could also be made after five successive treatments.

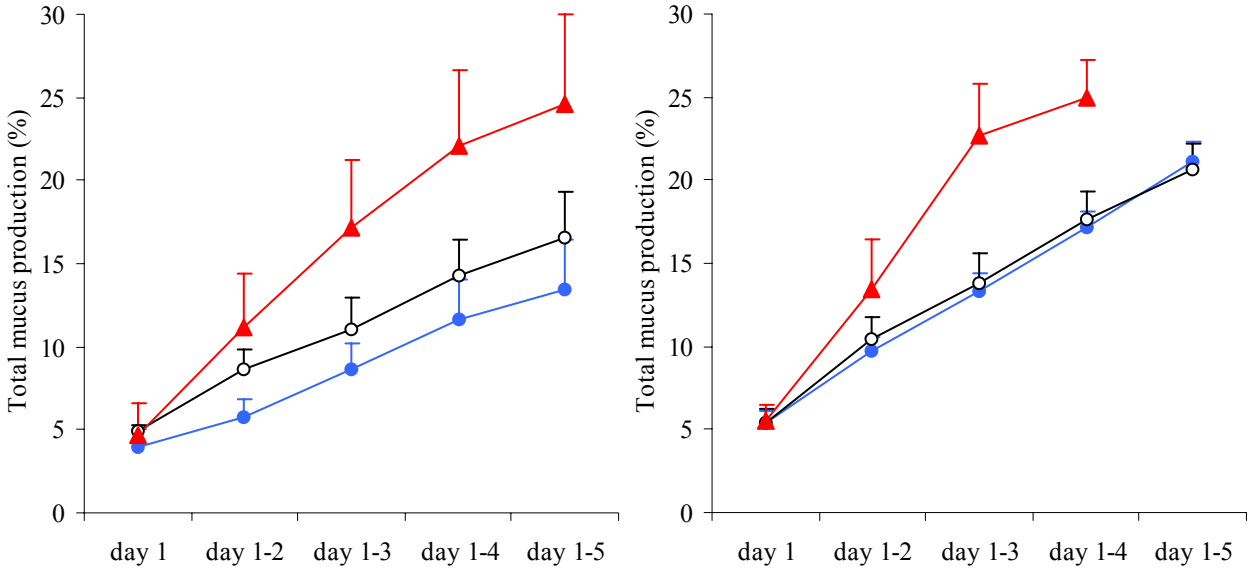

Figure 8.1 Total mucus production of the slugs treated with 100 mg (left graph) and 200 mg (right graph) HEC gel (●), Replens® (○) or Conceptrol® (▲) for 5 successive days. Data points are presented as the mean values (n = 5) and standard deviation bars are indicated.

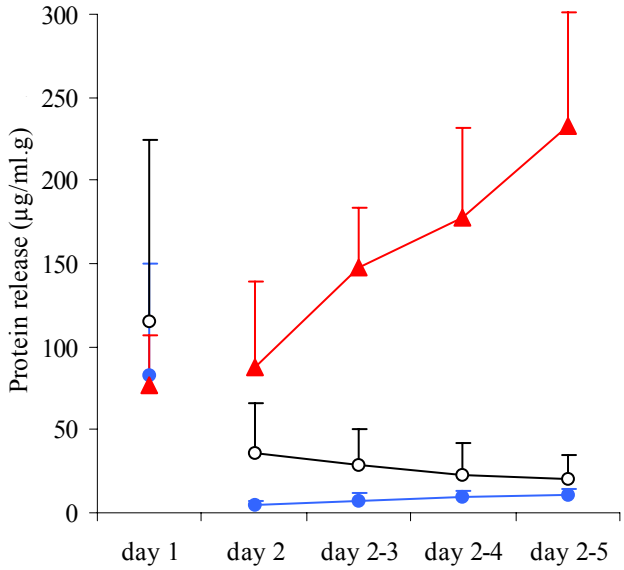

Figure 8.2 Mean protein release of the slugs treated with 100 mg HEC gel (●), Replens® (○) or Conceptrol® (▲) for 5 successive days. Data points are presented as the mean values (n = 5) and standard deviation bars are indicated.

Enzyme release was only detected for the slugs treated with Conceptrol®. 50 mg Conceptrol® induced LDH release after the third contact period. Amounts of 100 mg Conceptrol® and more resulted in LDH release after the second contact period. ALP release

appeared one day after LDH release except for 150 mg Conceptrol® that induced ALP release after the second contact period. Figure 8.3 illustrates that the LDH and ALP release induced by Conceptrol® increased with a repeated treatment.

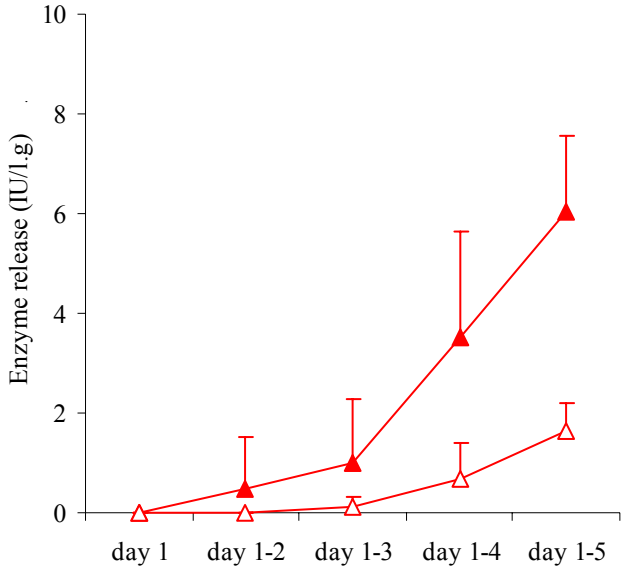

Figure 8.3 Mean LDH release (▲) and ALP release (Δ) of the slugs treated with 100 mg Conceptrol® for 5 successive days. Data points are presented as the mean values (n = 5) and standard deviation bars are indicated.

The results indicated that with respect to the irritation and tissue damage potential, the best discrimination between the different semi-solid formulations can be made after a repeated treatment on five successive days. As described in Chapter 6, prolongation of the experiment was not useful, because the mortality of the slugs treated with irritating formulations would increase.

Next, the effect of the amount of semi-solid formulation on the end points of the test was evaluated (Table 8.1). For the total mucus production, two-way ANOVA testing revealed a significant interaction between the test amount and the semi-solid formulation ($p = 0.029$). Firstly, a Bonferroni post hoc analysis was performed to investigate the test amount effect for each formulation. Bonferroni testing revealed (1) that the total mucus production induced by 50 mg and 100 mg of 5% HEC gel was significantly lower than the total mucus production induced by 150 mg and 200 mg of this gel, (2) that the total mucus production induced by 50 mg of Replens® was significantly lower than the mucus production induced by 150 mg and 200 mg of this gel and (3) that the total mucus productions induced by the four test amounts of Conceptrol® were not significantly different. Furthermore, a Bonferroni post hoc analysis

was performed to investigate the formulation effect for each test amount. Bonferroni testing revealed that the total mucus production of slugs treated with 50 mg or 100 mg of 5% HEC gel or Replens® was significantly lower than the total mucus productions of the slugs treated with the corresponding amount of Conceptrol®. However, neither the total mucus productions of the slugs treated with 150 mg of the three gels, nor the total mucus productions of the slugs treated with 200 mg of the three gels were significantly different. The largest difference in mucus production between the slugs exposed to Conceptrol® and the other two gels was obtained when the slugs were placed on 100 mg gel.

Table 8.1 Influence of the test amount and the semi-solid formulation on the total amount of mucus produced by slugs during a repeated treatment on 5 successive days

Formulation	Total mucus production (%)				
	50 mg	100 mg	150 mg	200 mg	**Average formulation**
5% HEC gel	11.4 ± 2.3 [a, c]	13.4 ± 3.1 [a, c]	19.0 ± 1.9 [b, c]	21.1 ± 1.3 [b, c]	**16.2 ± 4.5**
Replens®	14.0 ± 1.6 [a, c]	16.6 ± 2.7 [a, b, c]	19.9 ± 3.6 [b, c]	20.6 ± 1.6 [b, c]	**17.7 ± 3.6**
Conceptrol®	20.5 ± 5.3 [a, d]	24.6 ± 5.3 [a, d]	22.5 ± 4.2 [a, c]	25.0 ± 2.2 [a, c]	**23.1 ± 4.5**
Average test amount	**15.3 ± 5.1**	**18.2 ± 6.1**	**20.5 ± 3.5**	**22.2 ± 2.6**	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 20 and 15 slugs, respectively.

Two-way ANOVA testing revealed a significant interaction and a significant test amount and formulation effect ($p < 0.05$).

[a, b] For this formulation, total mucus productions marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

[c, d] For this test amount, total mucus productions marked with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni test).

Table 8.2 shows that treatment with each of the amounts of HEC gel and Replens® resulted in low protein release, whereas treatment with an increasing amount of Conceptrol® induced increased protein release. For the mean protein release, two-way ANOVA testing revealed no significant interaction between the test amount and the semi-solid formulation ($p = 0.707$). The test amount had no significant effect on the mean protein release ($p = 0.140$). However, there was a significant main effect of the formulation ($p < 0.001$). Scheffé post hoc testing suggested that treatment with Conceptrol® induced a significantly increased protein release compared to HEC gel and Replens®. Treatment of the slugs with different amounts of Conceptrol® resulted in comparable mean LDH release data and in comparable ALP release data (One-way ANOVA, $p > 0.05$).

Table 8.2 Influence of the test amount and the semi-solid formulation on the mean protein release induced by a repeated treatment of the slugs on 5 successive days

Formulation	Mean protein release (µg/ml.g)				
	50 mg	100 mg	150 mg	200 mg	**Average formulation**
5% HEC gel	11 ± 5	10 ± 4	12 ± 8	17 ± 12	**13 ± 8** [a]
Replens®	17 ± 9	20 ± 15	21 ± 13	23 ± 18	**20 ± 13** [a]
Conceptrol®	144 ± 102	233 ± 69	228 ± 151	306 ± 92	**228 ± 115** [b]
Average test amount *	**57 ± 84**	**88 ± 113**	**92 ± 135**	**115 ± 148**	

Values are the mean ± standard deviation of 5 slugs; bold values in column and row represent the mean ± standard deviation of 20 and 15 slugs, respectively.

Two-way ANOVA testing revealed no significant interaction and no significant test amount effect ($p > 0.05$), but a significant formulation effect ($p < 0.05$).

[a, b] Same letter indicates no significant difference between the semi-solid formulations ($p > 0.05$, Scheffé test).

* There are no significant differences between the mean protein release data induced by the four test amounts ($p > 0.05$, Two-way ANOVA).

Based on the fact that the largest difference between the mucus productions induced by the three semi-solid formulations was obtained when the slugs were placed on 100 mg gel and on the fact that the statistical analyses revealed no significant differences between the protein release data induced by the four test amounts, 100 mg was selected as test amount to evaluate the local tolerance of semi-solid formulations. Furthermore, Conceptrol® was a good positive control, because the gel induced a high mucus production, a high protein release, and enzyme release. 5% HEC gel and Replens® induced a low mucus production and protein release, and no enzyme release was detected. Because 5% HEC gel induced a (non-significantly) lower mucus production than Replens®, 5% HEC gel was chosen as negative control.

8.6.2 Repeatability of the test procedure

In order to investigate the repeatability of the test results obtained with the optimised test procedure for local tolerance testing of semi-solid vaginal formulations, the negative control and the positive control were tested independently on three separate occasions. Table 8.3 shows the total mucus production and mean protein and enzyme release of the negative control slugs measured in each of the repeated experiments and the mean of the three experiments. All the slugs treated with 5% HEC gel survived a repeated treatment on five

successive days. For the negative control, ANOVA testing revealed no significant difference in total mucus production and in mean protein release between the repeated experiments ($p > 0.05$).

Table 8.3 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the negative control slugs treated with 100 mg HEC gel on 5 successive days

Experiment	Total mucus production (%) *	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
Experiment 1	13.4 ± 3.1	10 ± 4	–	0	5
Experiment 2	12.9 ± 2.6	6 ± 6	–	0	5
Experiment 3	12.2 ± 1.3	8 ± 7	–	0	5
Inter-experiment mean	**12.9 ± 0.6**	**8 ± 2**	–		**3**

Total mucus production and mean protein release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 3 experiments; -, below the detection limit.

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

A daily treatment with Conceptrol® led to 20%, 0% and 60% mortality by day 5 in the first, second and third experiment, respectively. The total mucus production of the slugs treated with Conceptrol® in the second experiment was significantly higher than that of the slugs in the first and third experiment (Scheffé test, $p < 0.05$) (Table 8.4). For the positive control, ANOVA testing revealed no significant difference in mean protein release and in mean LDH release between the repeated experiments ($p > 0.05$).

Table 8.4 Intra- and inter-experiment variability for the total mucus production, mean protein and enzyme release of the positive control slugs treated with 100 mg Conceptrol® on 5 successive days

Experiment	Total mucus production (%)	Mean protein release (µg/ml.g) *	Mean LDH release (IU/l.g) *	Number of slugs with ALP release	n
Experiment 1	24.6 ± 5.3 [a]	233 ± 69	6.0 ± 1.5	5	5
Experiment 2	37.9 ± 5.7 [b]	147 ± 63	4.2 ± 4.2	5	5
Experiment 3	27.6 ± 3.3 [a]	228 ± 148	2.7 ± 2.2	3	5
Inter-experiment mean	**30.0 ± 7.0**	**202 ± 48**	**4.3 ± 1.7**		**3**

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 5 slugs; bold values represent the mean ± standard deviation of 3 experiments.

[a, b] For this end point, the values marked with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

* For this end point, there are no significant differences between the test results of the repeated experiments ($p > 0.05$, One-way ANOVA).

8.6.3 Evaluation of local tolerance of semi-solid vaginal formulations

The local tolerance of semi-solid vaginal formulations was evaluated by placing the slugs on 100 mg semi-solid formulation for 30 minutes during five successive days. All the slugs survived a repeated treatment with 5% HEC gel, Monistat 7®, placebo dapivirine gel, 22.5 µM dapivirine gel, 225 µM dapivirine gel, 10 mM dapivirine gel, Replens®, K-Y® jelly, Gynol II®, Advantage S®, Protectaid® or Gynol II® Extra Strength. A repeated treatment with Delfen® caused 20% mortality by day 5. A daily treatment with Conceptrol® led to 20%, 0% and 60% mortality by day 5 in the first, second and third experiment, respectively.

Placebo dapivirine gel, Replens®, Gynol II®, Protectaid®, and Gynol II® Extra Strength were tested independently on two or more separate occasions. ANOVA testing resulted in no significant differences in the total mucus production, in mean protein release, and in mean LDH release between the repeated experiments ($p > 0.05$).

The effects of a repeated treatment on the total mucus production, mean protein and enzyme release are presented in Table 8.5. Slugs treated with Monistat 7®, placebo dapivirine gel, 22.5 µM dapivirine gel, 225 µM dapivirine gel, 10 mM dapivirine gel or Replens® produced an amount of mucus comparable to that of the negative control slugs treated with 5% HEC gel ($p > 0.05$, Scheffé test). K-Y® jelly, Gynol II®, Advantage S®, Protectaid®, Gynol II® Extra Strength, and Delfen® induced a mucus production that was comparable to the

mucus production of the positive control slugs treated with Conceptrol® ($p > 0.05$, Scheffé test).

The protein release of the slugs treated with Monistat 7®, placebo dapivirine gel, 22.5 µM dapivirine gel, 225 µM dapivirine gel, 10 mM dapivirine gel, Replens®, K-Y® jelly or Advantage S® was comparable to that of the negative control slugs ($p > 0.05$, Scheffé test). Gynol II® and Protectaid® induced a protein release that was significantly higher than the protein release of the negative control slugs, but significantly lower than the protein release of the positive control slugs ($p < 0.05$, Scheffé test). The protein release of the slugs treated with Gynol II® Extra Strength or Delfen® was comparable to that of the positive control slugs ($p > 0.05$, Scheffé test). Slugs treated with Gynol II®, Protectaid®, Gynol II® Extra Strength, Delfen® or Conceptrol® also released LDH and ALP. The mean LDH release of the slugs treated with Delfen® was comparable to the mean LDH release of the positive control slugs ($p > 0.05$, Scheffé test).

Table 8.5 Effect of a repeated treatment for 5 successive days with 100 mg of different semi-solid vaginal formulations on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
5% HEC gel	12.9 ± 2.3 [a]	8 ± 6 [a]	–	0	15
Monistat 7®	9.8 ± 1.2 [a]	5 ± 3 [a]	–	0	5
Placebo dapivirine gel	13.3 ± 2.1 [a]	15 ± 6 [a]	–	0	10
22.5 µM Dapivirine gel	10.6 ± 1.4 [a]	16 ± 8 [a]	–	0	4
225 µM Dapivirine gel	10.0 ± 2.2 [a]	12 ± 3 [a]	–	0	5
10 mM Dapivirine gel	12.0 ± 1.7 [a]	11 ± 4 [a]	–	0	5
Replens®	17.9 ± 2.4 [a]	16 ± 15 [a]	–	0	15
K-Y® jelly	20.9 ± 2.3 [b]	5 ± 2 [a]	–	0	5
Gynol II®	23.4 ± 4.3 [b]	37 ± 26	0.5 ± 0.4	3	10
Advantage S®	32.3 ± 1.4 [b]	31 ± 29 [a]	–	0	5
Protectaid®	25.8 ± 6.6 [b]	36 ± 31	0.3 ± 0.3	4	10
Gynol II® Extra Strength	27.7 ± 5.5 [b]	67 ± 45 [b]	1.0 ± 1.1	5	10
Delfen®	46.6 ± 9.7 [b]	241 ± 146 [b]	4.4 ± 3.0 [b]	4	5
Conceptrol®	30.0 ± 7.4 [b]	202 ± 102 [b]	4.3 ± 3.0 [b]	13	15

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a] For this end point, data are not statistically different from 5% HEC gel ($p > 0.05$, Scheffé test).

[b] For this end point, data are not statistically different from Conceptrol® ($p > 0.05$, Scheffé test).

8.6.4 Development of prediction model

Based on the results of this study on the one hand and on pre-clinical and clinical safety data on the other hand, a classification prediction model was developed for the determination of the irritation category of semi-solid formulations. Semi-solids that induce a low total mucus production (< 15%) are classified as non-irritating. Semi-solids that cause a mucus production between 15% and 20% are predicted as mildly irritating formulations. Semi-solid formulations that result in 20% to 25% mucus production are classified as moderately irritating semi-solids, whereas semi-solids that cause ≥ 25% mucus production are classified as severely irritating (Figure 8.4) (Adriaens *et al.*, 2004).

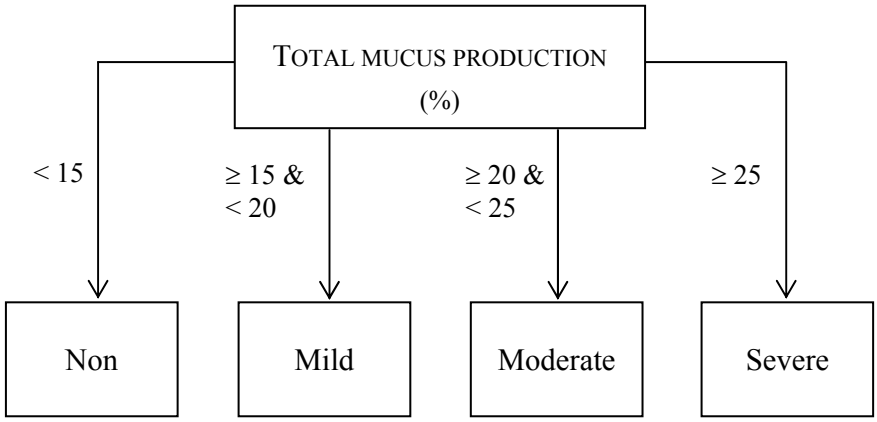

Figure 8.4 Classification prediction model for the determination of the irritation category of semi-solid formulations. The total mucus production is calculated after a repeated 30-minute treatment of *A. lusitanicus* on five successive days with 100 mg semi-solid formulation. (Based on Adriaens *et al.*, 2004)

8.7 DISCUSSION

The vaginal route offers a promising approach for the administration of for instance microbicides and spermicides. Frequent use of some vaginal formulations can damage the vaginal epithelium (Roddy *et al.*, 1993; Stafford *et al.*, 1998). Mucosal irritation and damage might favour the entry of sexually transmitted pathogens (Stephenson, 2000; Fichorova *et al.*, 2001; Van Damme *et al.*, 2002). Therefore, pre-clinical safety studies that document the safety and local tolerance of newly developed vaginal formulations are required by regulatory authorities. The objective of this study was to optimise and evaluate the five-day procedure of the Slug Mucosal Irritation test for local tolerance testing of semi-solid vaginal formulations.

It was investigated if a repeated treatment on five successive days was necessary by testing two semi-solid formulations that possibly could serve as negative control (5% HEC gel and Replens®) and a positive control semi-solid formulation (Conceptrol®). The results showed that a repeated treatment on five successive days was necessary to obtain a good discrimination between the mucus production, protein release and enzyme release data of the slugs exposed to HEC gel or Replens® on the one hand and the data of the slugs treated with Conceptrol® on the other hand.

Next, the influence of the amount of semi-solid formulation on the end points of the Slug Mucosal Irritation test was investigated. Based on the fact that the largest difference between the total mucus productions induced by the three semi-solid formulations was obtained when the slugs were placed on 100 mg gel and on the fact that the statistical analyses revealed no significant differences between the mean protein release data induced by the four test amounts, 100 mg was selected as test amount to evaluate the local tolerance of semi-solid formulations.

The results obtained with the Slug Mucosal Irritation test were compared with available *in vivo* (pre-) clinical local tolerance data. Based on the results of this study on the one hand and on pre-clinical and clinical data on the other hand, a classification prediction model was developed for the determination of the irritation potential of semi-solid formulations. As mentioned previously, the major obstacle to interpreting the available clinical data was the difference in study design. Furthermore, genital lesions were often assessed by means of colposcopy which is a visual diagnostic tool with several limitations (Van Damme *et al.*, 2000).

Slugs treated with 5% HEC gel produced a low amount of mucus in each of the three experiments, indicating that no irritation of the slug mucosa occurred. Furthermore, repeated treatment with 5% HEC gel caused minimal tissue damage as was demonstrated by low protein release and enzyme release below the detection limit. 5% HEC gel was selected as negative control. Indeed, the non-irritating properties of HEC gel were reported by several authors. Ballagh *et al.* (2002) reported that vaginal application of HEC gel once daily for seven consecutive days by 20 sexually inactive women resulted in genital heat and burning in 5% of the cases. 10% of the women reported itching. Colposcopic examination revealed no serious lesions that were not related to applicator use. HEC gel was found to be acceptable in that study (Ballagh *et al.*, 2002). Furthermore, microscopic examination of the vaginal and cervical tissues of five rabbits treated intravaginally for 10 consecutive days with a placebo gel containing 2% HEC revealed an intact epithelium and the lack of leukocyte influx. It was

concluded that intravaginal administration of HEC gel to rabbits did not cause vaginal irritation (Dhondt *et al.*, 2005).

A repeated treatment of the slugs with Conceptrol® resulted in high mucus production in each of the three repeated experiments, indicating severe irritation of the slug mucosa. Furthermore, the high protein release and increased LDH and ALP release of these slugs indicated that Conceptrol® caused severe damage of the slug mucosa. Because it is recommended that a gel containing 4.0% N-9 is used as a positive control in the rabbit vaginal irritation model (IWGVM, 1996), Conceptrol® was selected as positive control for mucosal tolerance testing of semi-solid vaginal formulations with the Slug Mucosal Irritation test. Several *in vivo* studies showed vaginal irritation and damage after single or repeated use of Conceptrol®. A single vaginal application of Conceptrol® resulted in the entry of inflammatory leukocytes into the vagina of mice within four hours (Milligan *et al.*, 2002). Intravaginal administration of Conceptrol® to five rabbits once daily for 10 consecutive days caused epithelial loss and atrophy and leukocyte infiltration in the vaginal and cervical tissues (Dhondt *et al.*, 2005). When monkeys were treated daily for three days, not treated for two days and then treated for three additional days with Conceptrol®, epithelial disruption and inflammatory response were noted (Patton *et al.*, 1999). Twice daily use of Conceptrol® for 14 days by 90 sexually active women resulted in genital burning and itching in 8% and 19% of the cases, respectively; epithelial disruptions were observed in 13% of the women (Hoffman *et al.*, 2004). Poindexter *et al.* (1996) reported that the use of Conceptrol® by 31 sexually inactive women once daily for seven consecutive nights caused cervical redness in 19% of the subjects and a combination of redness and ulceration in 7% of the women.

Monistat® 7 Vaginal Cream (containing 2% (w/w) miconazole nitrate) did not irritate the slug mucosa, as was indicated by an amount of mucus comparable to that of the negative control slugs. Furthermore, the formulation was classified as a minimally tissue damaging formulation by the Slug Mucosal Irritation test. The results obtained with the Slug Mucosal Irritation test are in good agreement with clinical data. Only 2% of 114 women suffering from vulvovaginal candidiasis that applied a vaginal cream containing 2% miconazole nitrate once daily for seven days complained of increased vulvovaginal irritation (burning and itching) (Brown *et al.*, 1999). Upmalis *et al.* (2000) described two studies that evaluated the safety of vaginal application of Monistat® 7 Vaginal Cream once daily for seven successive days by 265 patients suffering from vulvovaginal candidiasis. Less than 10% of all adverse events (such as vaginal irritation or burning) were considered to be treatment-related.

The placebo dapivirine gel and the 22.5 µM, 225 µM and 10 mM dapivirine gels (each containing 2% HEC) were classified as non-irritating formulations based on the mucus production of the slugs. Furthermore, repeated treatment with these gels resulted in minimal damage of the slug mucosa. These data are in good agreement with results obtained with the rabbit vaginal irritation test. Microscopic examination of the vaginal and cervical tissues of rabbits treated intravaginally once daily for 10 successive days with these gels revealed an intact vaginal and cervical epithelium and no leukocyte infiltration. Each of the gels was considered to be non-irritating to the rabbit vaginal tissue (Dhondt *et al.*, 2005).

Based on the results of the Slug Mucosal Irritation test, Replens® was classified as a mildly irritating formulation that causes minimal tissue damage. On the one hand, Replens® is considered to be safe and without adverse effects on the vaginal or cervical mucosa. After a daily administration of Replens® for 10 consecutive days to one rabbit, light microscopic examination revealed a normal non-keratinised squamous epithelium (Balzarini *et al.*, 1998). On the other hand, application of Replens® once daily for 14 consecutive days by 178 healthy sexually active women resulted in genital itching and vaginal discharge in 9% and 19% of the women, respectively. Treatment with Replens® induced a higher incidence of oedema than the no-treatment control group. Notwithstanding these results, it was concluded that Replens® was fairly innocuous (Van Damme *et al.*, 1998).

Based on the slug mucus production, protein and enzyme release data, K-Y® jelly was classified as a moderately irritating formulation that induces minimal tissue damage. The moderate irritation of the mucosal tissue might be due to the presence of chlorhexidine gluconate, because a single 15-minute exposure to a 0.5% chlorhexidine gluconate gel damaged a major portion of the vaginal epithelium of mice (Achilles *et al.*, 2002). Vaginal administration of K-Y® jelly to rabbits for 10 consecutive days has also been shown to cause mild irritation to the vagina (personal communication, Emilia Lonardo, Personal Products Company, 2000) (Mauck *et al.*, 2001). Vaginal application of K-Y® jelly by 12 healthy sexually inactive women once daily for six successive nights resulted in product-related colposcopic findings (disruption of epithelium or blood vessels) in 25% of the women. The colposcopic findings and signs of irritation reported during follow-up were lower than in the Conceptrol® group (Mauck *et al.*, 2001).

A repeated treatment of the slugs with Gynol II® resulted in moderate irritation of the slug mucosa. Furthermore, the mean protein release of the slugs treated with Gynol II® was significantly higher than that of the negative control slugs, but significantly lower than that of the positive control slugs. Gynol II® was classified as a mildly tissue damaging formulation.

The irritating and tissue damaging properties of Gynol II® are probably due to the presence of 2% N-9 and are also reported by Ballagh *et al.* (2002). Vaginal application of Gynol II® once daily for seven consecutive days by 20 sexually inactive women resulted in genital heat or burning in 25% of the cases. 40% of the women reported itching. Colposcopic examination revealed serious vaginal lesions that were not related to applicator use (multiple areas of epithelial loss) in 10% of the women (Ballagh *et al.*, 2002).

Treatment of the slugs with Advantage S® (containing 3.5% N-9) induced severe irritation of the mucosa as was demonstrated by a high total mucus production which was comparable to that of the positive control slugs. Furthermore, the formulation was classified as mildly tissue damaging based on the (non-significantly) increased protein release of the slugs. It is interesting to note that Advantage S® is formulated by the addition of 3.5% N-9 to the vaginal lubricant Replens® and that it contains less carbomer than Replens® (Van Damme *et al.*, 2002). So the irritation and tissue damage caused by Advantage S® is probably due to the presence of N-9. Various phases of clinical trials were completed before the adverse effects of Advantage S® became clear. The application of Advantage S® once daily for 14 consecutive days by 179 healthy sexually active women induced genital itching and vaginal discharge in 14% and 32% of the women, respectively. Ulceration and abrasion – lesions associated with epithelial disruption – were observed in 2% of the women, but did not occur significantly more than in the Replens® and the no-treatment control group (Van Damme *et al.*, 1998). A phase II trial involving the application of Advantage S® multiple times a day by female sex workers revealed no difference between Advantage S® and Replens® with respect to incidence of lesions (Van Damme *et al.*, 2000). A recent phase II/III trial in the same target population finally concluded that frequent use of Advantage S® increased women's susceptibility to human immunodeficiency virus infection by causing lesions (Van Damme *et al.*, 2002). Another recent publication reported that vaginal application of Advantage S® by 296 healthy sexually active women upon intercourse during seven months resulted in vulvar or vaginal irritation in 22% of the subjects (Raymond *et al.*, 2004).

A repeated treatment with Protectaid® induced severe irritation of the slug mucosa as was demonstrated by a high total mucus production which was comparable to that of the positive control slugs. Furthermore, the mean protein release induced by Protectaid® was significantly higher than that induced by the negative control, but significantly lower than the protein release induced by Conceptrol®. Protectaid® was classified as a mildly tissue damaging formulation. The observed irritation and tissue damage may be due to the presence of 0.125% N-9, 0.125% benzalkonium chloride and 0.5% sodium cholate. Patton *et al.* (1999) reported

that a repeated application of N-9 plus benzalkonium chloride induced a more robust inflammatory response in monkeys than repeated applications of either N-9 or benzalkonium chloride alone. Our results contrast with studies involving the vaginal use of the Protectaid® sponge by women. Creatsas *et al.* (2002) concluded that use of the sponge by 15 women upon intercourse during one year caused no colposcopic findings of vaginal lesions or irritation. The apparent discrepancy in results may be explained by the fact that we tested only the gel that is impregnated in the Protectaid® sponge. A sponge releases smaller amounts of gel over a long period of time, possibly leading to less exposure to gel (Poindexter *et al.*, 1996).

Treatment of the slugs with Gynol II® Extra Strength (containing 3% N-9) induced severe irritation of the mucosa as was demonstrated by a high total mucus production which was comparable to that of the positive control slugs. Furthermore, the formulation caused a protein release comparable to the positive control slugs. Gynol II® Extra Strength was classified as a moderately tissue damaging formulation by the Slug Mucosal Irritation test. Use of Gynol II® Extra Strength once daily for seven consecutive days resulted in vaginal irritation in 87% of the 15 women. Product-related colposcopic findings (especially erythema) were observed in 47% of the women. Furthermore, the vaginal and cervical epithelia or blood vessels of 20% of the women were disrupted (Mauck *et al.*, 2004). The irritating and tissue damaging properties of Gynol II® Extra Strength are possibly due to the presence of 3% N-9.

A repeated treatment with Delfen® (containing 12.5% N-9) induced severe irritation of the slug mucosa as was demonstrated by a high total mucus production which was comparable to that of the positive control slugs. Furthermore, the formulation caused a protein and LDH release comparable to the positive control slugs and ALP release in four out of the five slugs which indicated severe tissue damage. Taking into account the reported irritation and tissue damage induced by a semi-solid formulation containing 4% N-9 (Conceptrol®) (Poindexter *et al.*, 1996; Patton *et al.*, 1999; Mauck *et al.*, 2001; Milligan *et al.*, 2002; Hoffman *et al.*, 2004; Dhondt *et al.*, 2005), the severely irritating and tissue damaging properties of Delfen® can be ascribed to the presence of 12.5% N-9.

The Slug Mucosal Irritation test seems to be able to classify the formulations into more irritation categories than commonly practiced *in vitro* cytotoxicity tests. Maguire *et al.* classified several vaginal formulations as non-irritating or irritating by using an immortalized cell line derived from human vaginal cells. This *in vitro* study concluded that Replens® and K-Y® jelly were not cytotoxic to human vaginal cells, whereas Gynol II® and Advantage S® had a cytotoxic effect on the cells. Gynol II® and Advantage S® namely exhibited a 50% cytotoxic effect at concentrations of 300 µg/ml and 250 µg/ml, respectively (Maguire *et al.*, 2001).

In order to compare the Slug Mucosal Irritation test data with the clinical data in a more structured way, those clinical studies were selected that lasted one to two weeks and that mentioned the number of patients experiencing vaginal burning or itching during treatment with the semi-solid vaginal formulations. Table 8.6 shows that for clinical studies that included at least 90 women, an increasing irritation category predicted by the Slug Mucosal Irritation test corresponds with an increasing percentage of women that reported vaginal burning and/or itching. The results indicate that the mucus production of the slugs is related to the burning and itching sensation that is reported by women in clinical studies. Hitherto, no other pre-clinical tests are available that can predict the burning/itching sensation induced by formulations. The results of this study indicate that the Slug Mucosal Irritation test seems to be a promising alternative to evaluate the mucosal tolerance of semi-solid vaginal formulations intended for repeated administration.

Table 8.6 Comparison of irritation categories obtained with the Slug Mucosal Irritation test on the one hand with clinical data concerning vaginal burning and itching induced by a repeated treatment (1-2 weeks) with some semi-solid vaginal formulations on the other hand

Formulation	Irritation category obtained with Slug Mucosal Irritation test	Clinical data		
		Women with vaginal burning or itching (%)	Women (n)	Source
HEC gel	No irritation	5% burning, 10% itching	20	Ballagh *et al.*, 2002
Monistat 7®	No irritation	2% burning and itching	114	Brown *et al.*, 1999
Replens®	Mild irritation	9% itching	178	Van Damme *et al.*, 1998
Gynol II®	Moderate irritation	25% burning, 40% itching	20	Ballagh *et al.*, 2002
Advantage S®	Severe irritation	14% itching	179	Van Damme *et al.*, 1998
Conceptrol®	Severe irritation	8% burning, 19% itching	90	Hoffman *et al.*, 2004

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CHAPTER 9: EVALUATION OF MUCOSAL TOLERANCE OF LIQUID NASAL FORMULATIONS

9.1 INTRODUCTION

Nasal decongestants, antihistamines, and steroids are widely used topical drugs that often are administered as liquid formulations. Such formulations may be applied frequently over a period of months or years. Some substances can damage the nasal mucosa or affect the nasal mucociliary defensive system (Quadir *et al.*, 1999). Because irritation and damage of the nasal mucosa can break the protective barrier against micro-organisms and noxious substances, it is important that nasal formulations do not irritate or damage the nasal mucosa and that their local tolerance is evaluated before clinical use.

9.2 NASAL MUCOSA

The anterior part of the human nasal cavity is lined with a stratified squamous epithelium without cilia and transitional epithelium. The olfactory epithelium covers the upper part of the nasal cavity (Mygind and Dahl, 1998; Quadir *et al.*, 1999). This epithelium is pseudostratified and contains olfactory receptor cells, columnar supporting cells, and basal cells (Marieb, 1995). The remaining part of the nasal cavity is lined with pseudostratified respiratory epithelium that comprises ciliated and non-ciliated columnar cells with microvilli, goblet cells, and basal cells (Mygind and Dahl, 1998; Quadir *et al.*, 1999).

An important nasal mechanism contributing to the body's primary non-specific defence mechanism is nasal mucociliary clearance. By this, potentially hazardous materials (as dust and micro-organisms) and cellular debris are captured within the mucus layer, are then moved by the coordinated beating of the cilia within the periciliary liquid toward the pharynx and are subsequently swallowed or expectorated (Proctor, 1977).

9.3 EVALUATION OF NASAL TOLERANCE OF FORMULATIONS

Nasal tolerance testing is required by regulatory authorities for medicinal products intended for nasal administration on the one hand and for products which are not envisaged for nasal delivery but can come accidentally into contact with the nasal mucosa on the other hand. Local tolerance testing is usually conducted with the definitive formulation consisting of the drug in its vehicle at the concentration intended for human use (EC, 1990).

Several pre-clinical *in vivo* and *in vitro* models have been developed to study the impact of drugs and excipients on the nasal epithelium. Histological changes of the nasal mucosa induced by locally applied ingredients and formulations have been investigated *in vivo* in rabbits (Suh *et al.*, 1995; Ugwoke *et al.*, 2000; Callens *et al.*, 2001; Cüreoglu *et al.*, 2002; Çankaya *et al.*, 2003), rats (Marttin *et al.*, 1996; Zhou and Donovan, 1996; Berg *et al.*, 1997; Lebe *et al.*, 2004), pigs (Tas *et al.*, 2005), and monkeys (Ainge *et al.*, 1994) by light microscopy or scanning electron microscopy. The effect of compounds and formulations on the nasal mucosa has also been evaluated by the release of markers from the nasal cavity of rats and rabbits *in vivo* or *in situ* (Shao and Mitra, 1992; Shao *et al.*, 1992; Krishnamoorthy *et al.*, 1995; Marttin *et al.*, 1995; Tengamnuay *et al.*, 2000; Callens *et al.*, 2001). Furthermore, the effects of ingredients of nasal formulations on the nasal mucociliary clearance have been studied in the frog palate model (Batts *et al.*, 1989; Gizurarson *et al.*, 1990).

The irritating and tissue damaging properties of nasal compounds and formulations have also been studied *in vitro* by evaluation of histological changes and release of marker compounds. For this, cultured human adenoid tissue (Berg *et al.*, 1995; Steinsvag *et al.*, 1996) or reconstructed three dimensional human nasal tissue (El-Shafy *et al.*, 2001) is exposed to the products of interest. The ciliary beat frequency has been evaluated *in vitro* using human nasal epithelial cells cultured as monolayer (Hermens *et al.*, 1990) or suspension (Agu *et al.*, 2000; Ugwoke *et al.*, 2000), freshly harvested chicken embryo trachea (Van de Donk *et al.*, 1980; Merkus *et al.*, 1991; Boek *et al.*, 1999), and cryopreserved human sphenoidal sinus mucosa (Boek *et al.*, 1999). The cytotoxic effects of absorption enhancers have been investigated by *in vitro* erythrocyte hemolysis (Martin *et al.*, 1992; Vermehren and Hansen, 1998) and using intestinal Caco-2 cell monolayers (Anderberg and Artursson, 1993). However, it has to be noted that *in vitro* cells and tissues are generally less resistant to the effects of nasal compounds and formulations than the corresponding *in vivo* tissues because of several reasons. Firstly, the normal overlaying protective mucous layer is usually absent in *in vitro* models, so that under *in vitro* conditions compounds and formulations are not diluted by

mucus and come directly into contact with the cells or tissues (Merkus *et al.*, 1991; Boek *et al.*, 1999; Merkus *et al.*, 1999; Lebe *et al.*, 2004). Furthermore, the exposure time of *in vitro* cells or tissues to the nasal products is generally longer than that of *in vivo* tissues because of the absence of the nasal mucociliary clearance mechanism under *in vitro* conditions (Merkus *et al.*, 1991; Steinsvag *et al.*, 1996; Boek *et al.*, 1999; Merkus *et al.*, 1999; Lebe *et al.*, 2004). Thirdly, reversion of irritation or damage due to constant renewal of epithelial cells is also not possible *in vitro* (Ohashi *et al.*, 1991; Schipper *et al.*, 1991; Lebe *et al.*, 2004).

9.4 OBJECTIVES

Previously, the irritation potential of dilutions of various absorption enhancers used in nasal pharmaceutical formulations was evaluated with the Slug Mucosal Irritation test. The results were in agreement with other *in vivo* and *in vitro* nasal irritation data (Adriaens and Remon, 1999). In the present study, a slightly modified version of the five-day procedure described by Adriaens and Remon (1999) was used to evaluate the mucosal tolerance of liquid nasal formulations. The 15-minute contact period was namely prolonged to 30 minutes in analogy with previous studies (Chapters 5, 6, 7 and 8). Furthermore, only 500 µl of undiluted test formulation was used instead of 2 ml because of the low availability of certain liquid test formulations.

In analogy with previous studies (Adriaens and Remon, 1999), PBS was used as negative control, whereas benzalkonium chloride was selected as positive control.

9.5 MATERIALS AND METHODS

9.5.1 Liquid formulations

The OTC nasal solution Naaprep® (GlaxoSmithKline, Genval, Belgium) contains 0.9% (w/v) sodium chloride and purified water. The homeopathic nasal solution Luffeel® (Homeoden-Heel, Ghent, Belgium) is composed of *Luffa operculata* D4, *Luffa operculata* D12, *Luffa operculata* D30, *Galphimia glauca* D4, *Galphimia glauca* D12, *Galphimia glauca* D30, Histaminum D12, Histaminum D30, Histaminum D200, Sulphur D12, Sulphur D30, Sulphur D200, 0.01% benzalkonium chloride, and isotonic solution. The nasal suspension Flixonase Aqua™ (GlaxoSmithKline, Genval, Belgium) contains 0.05% (w/w) fluticasone propionate, glucose, microcrystalline cellulose, sodium carboxymethylcellulose, polysorbate,

hydrochloric acid, phenylethyl alcohol, benzalkonium chloride, and purified water. Nasonex® (Schering-Plough NV/SA, Brussels, Belgium) is a nasal suspension containing 0.05% mometasone furoate, colloidal cellulose BP 65 cps, glycerol, sodium citrate dihydrate, citric acid monohydrate, polysorbate 80, phenylethyl alcohol, benzalkonium chloride, and purified water. The OTC nasal solution Nesivine® (Merck KGaA, Darmstadt, Germany) contains 0.05% (w/v) oxymetazoline hydrochloride, monobasic sodium phosphate, dibasic sodium phosphate, sodium hydroxide, sodium edetate, 0.015% (w/v) benzalkonium chloride, and purified water. Allergodil® (Viatris, Brussels, Belgium) is an OTC nasal solution containing 0.1% (w/v) azelastine hydrochloride, methyl hydroxypropylcellulose, sodium edetate, citric acid, dibasic sodium phosphate, sodium chloride, and purified water. The nasal solution Syntaris® (Northon Healthcare Ltd, London, United Kingdom) contains 0.025% (w/v) flunisolide, citric acid, butyl hydroxytoluene, PEG 400, sodium citrate, polysorbate 20, propylene glycol, sorbitol, sodium edetate, benzalkonium chloride, and purified water.

PBS was used as negative control, whereas a 1% (w/v) dilution of benzalkonium chloride (Sigma, St. Louis, MO, USA) was used as a positive control.

9.5.2 Local tolerance test procedure of the Slug Mucosal Irritation test

The local tolerance of the liquid nasal solutions was assessed using the procedure described in Chapter 6. During five successive days, the slugs were placed in a Petri dish on a quarter of a membrane filter (cellulose acetate, 0.45 µm, 90 mm, Sartorius AG, Goettingen, Germany) moistened with 500 µl of the undiluted test formulation for 30 minutes and the mucus production of the slugs was measured. One and two hours after each of the five contact periods, the samples were collected. The samples were analysed immediately for the presence of proteins, LDH, and ALP released from the body wall of the slugs.

9.5.3 Analytical procedures

The protein, LDH, and ALP determinations were performed according to the analytical procedures described in Chapter 3.

9.5.4 Data analysis

For each slug, the total mucus production, mean protein release, mean LDH release and mean ALP release were calculated as described in the data analysis section of Chapter 5. These values were used for the statistical analyses using the computer program SPSS (version 12.0; SPSS, Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Statistically significant differences between different treatments were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov-Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal, the data were transformed to their logarithm. To compare further the effects of the different treatments, a multiple comparison among pairs of means was performed with a Scheffé test.

Using the cut-off values presented in Figure 5.2, the formulations were classified into one of the tissue damage categories. Based on in-house experience, cut-off values were established for the classification of liquid formulations into irritation categories.

9.6 RESULTS

9.6.1 Evaluation of local tolerance of nasal liquid formulations

The five-day test procedure was used to evaluate the mucosal tolerance of liquid nasal formulations. All the slugs survived a repeated treatment with PBS, Naaprep®, Luffeel®, Flixonase Aqua™, Nasonex®, Nesivine®, Allergodil®, and Syntaris®. Only a daily treatment with 1% benzalkonium chloride caused 20% mortality by day 3 and finally all the slugs were dead by day 5.

The effects of a daily treatment on the total mucus production, mean protein and enzyme release are presented in Table 9.1. Scheffé post hoc testing suggested the following four homogeneous subsets for the total amount of mucus secreted: (1) Luffeel®, PBS and Naaprep®, (2) Flixonase Aqua™, Nasonex®, Nesivine®, and Allergodil®, (3) Syntaris® and (4) 1% benzalkonium chloride.

Furthermore, a repeated treatment with Naaprep®, Luffeel®, Flixonase Aqua™, Nasonex®, Nesivine®, Allergodil® or Syntaris® resulted in a protein release comparable to that of the negative control slugs treated with PBS ($p > 0.05$, Scheffé test). Moreover, the release of LDH and ALP by slugs treated with these liquid nasal formulations was under the detection

limit. Only treatment with 1% benzalkonium chloride caused an increased protein, LDH and ALP release from the slug mucosa. ALP release was detected for four out of the five slugs treated with 1% benzalkonium chloride. ALP release occurred one day after LDH release and increased with a repeated treatment.

Table 9.1 Effect of a repeated treatment for 5 successive days with 500 µl of different liquid nasal formulations on the end points of the Slug Mucosal Irritation test

Formulation	Total mucus production (%)	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	n
PBS	-8.2 ± 3.4 [a]	13 ± 6 [a]	–	0	5
Naaprep®	-8.9 ± 2.2 [a]	15 ± 27 [a]	–	0	5
Luffeel®	-17.5 ± 5.0 [a]	8 ± 6 [a]	–	0	5
Flixonase Aqua™	4.7 ± 2.6	5 ± 2 [a]	–	0	5
Nasonex®	5.2 ± 2.5	3 ± 0 [a]	–	0	5
Nesivine®	8.0 ± 2.5	6 ± 2 [a]	–	0	5
Allergodil®	8.9 ± 2.5	5 ± 2 [a]	–	0	5
Syntaris®	18.4 ± 2.7	30 ± 9 [a]	–	0	5
1% benzalkonium chloride	28.3 ± 3.3 [b]	349 ± 137 [b]	8.5 ± 3.9	4	5

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation; -, below the detection limit.

[a] For this end point, data are not significantly different from PBS ($p > 0.05$, Scheffé test).

[b] For this end point, data are not significantly different from 1% benzalkonium chloride ($p > 0.05$, Scheffé test).

9.6.2 Development of prediction model

Based on the results of this study and the studies of Adriaens and Remon (1999), Adriaens (2000), and Adriaens *et al.* (2001) on the one hand and on pre-clinical and clinical safety data on the other hand, a classification prediction model was developed for the determination of the irritation category of liquid formulations. Liquids that induce a low total mucus production (< 0%) are classified as non-irritating. Liquids that cause a mucus production between 0% and 5% are predicted as mildly irritating formulations. Liquid formulations that result in 5% to 10% mucus production are classified as moderately irritating, whereas liquids that cause ≥ 10% mucus production are classified as severely irritating (Figure 9.1) (Adriaens *et al.*, 2004).

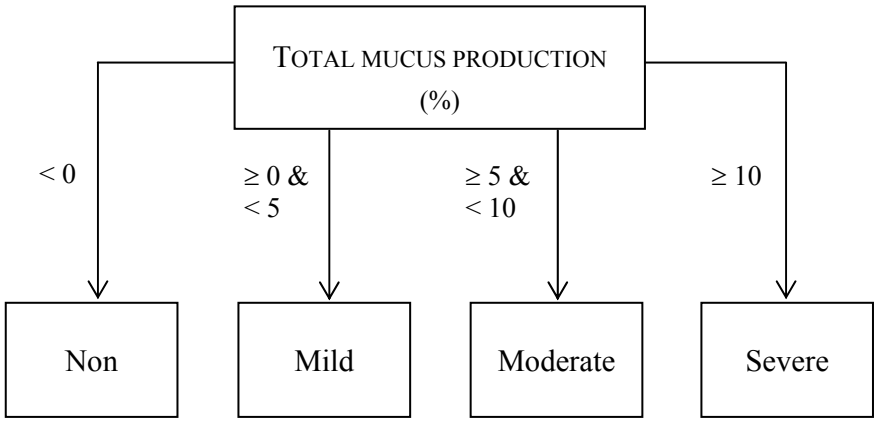

Figure 9.1 Classification prediction model for the determination of the irritation category of liquid formulations. The total mucus production is calculated after a repeated 30-minute treatment of *A. lusitanicus* with liquid formulation on five successive days. (Based on Adriaens *et al.*, 2004)

9.7 DISCUSSION

Irritation and damage of the nasal mucosa can break the protective barrier against micro-organisms and noxious substances. Therefore, repeated dose local tolerance testing of nasally applied formulations is important. The objective of this study was to evaluate the five-day procedure of the Slug Mucosal Irritation test for local tolerance testing of liquid formulations intended for repeated nasal administration. The results of the Slug Mucosal Irritation test were compared with available clinical and pre-clinical *in vivo* and *in vitro* data on the safety of the tested liquids. Based on the results of this study and the studies of Adriaens and Remon (1999), Adriaens (2000), and Adriaens *et al.* (2001) on the one hand and on pre-clinical and clinical data on the other hand, a classification prediction model was developed for the determination of the irritation potential of liquid formulations. It has to be stressed that comparing the available clinical data was difficult because of the difference of study design on the one hand and because the nasal mucosa in patients suffering from allergic rhinitis generally differs morphologically from the corresponding tissue in healthy subjects (Lim *et al.*, 1995; Mygind *et al.*, 1974) on the other hand. The protective function of the mucociliary transport mechanism may not be effective in patients with rhinitis (Toremalm, 1980; Wilson *et al.*, 1985). This can consequently result in an increased exposure time of the nasal mucosa to topically administered formulations (Berg *et al.*, 1995). On the other hand, the amount of nasal mucus produced in patients with allergic rhinitis can be increased, so that

nasally applied substances may be more diluted and better tolerated in noses with rhinitis than in healthy noses (Lebe *et al.*, 2004).

Slugs treated with PBS produced a low amount of mucus, indicating that no irritation of the slug mucosa occurred. Furthermore, repeated treatment with PBS caused minimal tissue damage as was demonstrated by low protein release and enzyme release below the detection limit. These results are in agreement with *in vitro* data. Exposure of human adenoid tissue *in vitro* to PBS once a day during 10 days did not change the epithelium. A normal pseudostratified epithelium consisting of ciliated and non-ciliated columnar cells with microvilli and goblet cells was detected (Steinsvag *et al.*, 1996).

A repeated treatment of the slugs with a 1% (w/v) dilution of benzalkonium chloride – which was used as positive control – resulted in a high mucus production (± 28%), indicating severe irritation of the slug mucosa. Furthermore, the high protein release and increased LDH and ALP release of these slugs indicated that 1% benzalkonium chloride caused severe damage of the slug mucosa. The safety of benzalkonium chloride as preservative in liquid nasal formulations has been extensively studied. However, most of the studies established the irritation and tissue damage potential of benzalkonium chloride at concentrations lower than 1%, because this compound is generally present in liquid nasal formulations at concentrations ranging from 0.005 to 0.01%. With regard to the safety of benzalkonium chloride at these low concentrations, conflicting reports have been published. It was opted to summarise especially the results of those studies which evaluated the effect of solutions containing benzalkonium chloride without additional active compounds, because the presence of other compounds can influence the effect on the nasal mucosa. Nasal application of 0.001% benzalkonium chloride to rabbits twice daily for 14 and 28 days resulted in squamous cell metaplasia of the nasal epithelium. Furthermore, a reduction of the height of the epithelium and a decrease of the number of ciliated cells and goblet cells were observed. However, no morphological changes occurred in the subepithelial tissue (Cüreoglu *et al.*, 2002). In the frog palate model, one or two treatments with 0.01% benzalkonium chloride caused an irreversible ciliostatic effect (Batts *et al.*, 1989). A 0.01% benzalkonium chloride solution intranasally administered to anaesthetised rats for 15 minutes resulted in minor to major morphological changes (Marttin *et al.*, 1996). Nasal administration of 0.05% or 0.1% benzalkonium chloride eight times on one day to rats induced nasal lesions such as epithelial desquamation, degeneration, oedema or neutrophilic cellular infiltration (Kuboyama *et al.*, 1997). On the contrary, nasal administration of 0.01% benzalkonium chloride to six healthy volunteers was well tolerated and did not change nasal clearance after a single administration (Batts *et al.*, 1991).

Furthermore, a cosmetic ingredient review panel concluded that benzalkonium chloride can be safely used as preservative at concentrations up to 0.1% (Liebert, 1989).

Naaprep® (containing 0.9% sodium chloride) did not irritate the slug mucosa as was indicated by a mucus production comparable to that of the negative control slugs treated with PBS. Furthermore, Naaprep® was classified as a minimally damaging formulation based on the protein and enzyme release data. The results obtained with the Slug Mucosal Irritation test are in good agreement with available *in vitro* and *in vivo* data. Exposure of human adenoid tissue *in vitro* to 0.9% sodium chloride once a day during 10 days did not change the epithelium. A normal pseudostratified epithelium consisting of ciliated and non-ciliated columnar cells with microvilli and goblet cells was detected (Steinsvag *et al.*, 1996). Treatment of the nasal mucosa of healthy rats and rabbits with 0.9% sodium chloride solution twice daily for 21 to 28 days revealed no histological alterations (Berg *et al.*, 1997; Cüreoglu *et al.*, 2002; Lebe *et al.*, 2004). The thick pseudostratified epithelium consisted of tall columnar cells with cilia and goblet cells and was covered with a mucus layer. Furthermore, normal submucosal structures were observed (Berg *et al.*, 1997; Cüreoglu *et al.*, 2002). Only two adverse events (pain) were reported in a multicentre study of 209 rhinosinusitis patients who irrigated two to six times daily for 20 days with isotonic saline (Seppey *et al.*, 1995).

Based on the results obtained with the Slug Mucosal Irritation test, Luffeel® was classified as a non-irritating formulation that causes minimal tissue damage. These results are in agreement with *in vivo* data obtained after daily treatment for six weeks of 72 patients suffering from seasonal allergic rhinitis with Luffa comp.-Heel™ nasal spray (containing the same ingredients as Luffeel®). Only 1% of the patients reported mild to moderate nasal burning and 1% of the cases reported minor, intermittent epistaxis. These adverse events were possibly, probably or very probably related to treatment. However, all adverse events disappeared spontaneously (Weiser *et al.*, 1999).

A repeated treatment with Flixonase Aqua™ induced mild irritation and minimal damage of the slug mucosa. Local adverse effects, such as irritation of the nose and throat or minor epistaxis, have been associated with the use of Flixonase Aqua™ (GlaxoSmithKline, 2000). Nasal burning and epistaxis were respectively reported by 4% and 4% of 25 patients with seasonal allergic rhinitis that were treated with fluticasone propionate nasal spray (100 µg/day) during four weeks (van As *et al.*, 1991). A daily treatment with fluticasone propionate nasal spray (200 µg/day) during 6 months resulted in nasal irritation, nasal burning, and epistaxis in respectively 0%, 5% and 8% of 128 patients suffering from perennial allergic rhinitis (Banov *et al.*, 1994). With regard to the tissue damage potential of

Flixonase Aqua™, *in vitro* and *in vivo* data are not in line with each other. Exposure of human respiratory mucosa *in vitro* to undiluted fluticasone propionate spray for 10 minutes once a day during 10 days resulted in loss of the morphological characteristics of the tissue fragments. However, the authors suggest that formulations cause generally more pronounced effects under *in vitro* conditions than under *in vivo* conditions (Steinsvag *et al.*, 1996). Indeed, several *in vivo* studies demonstrated that a repeated treatment with fluticasone propionate nasal spray did not damage the nasal mucosa. Neither reduction of the number of ciliated cells, nor damage of the ciliated cells was microscopically observed after treatment of monkeys for 28 successive days with fluticasone propionate nasal spray (400 µg/day) (Ainge *et al.*, 1994). Treatment of 22 patients suffering from perennial allergic rhinitis with fluticasone propionate spray (200 µg/day) for six weeks caused neither decrease of the number of ciliated cells nor damage of the nasal mucosa (Braat *et al.*, 1995). In addition, nasal biopsies taken before and after 12 months treatment of 16 patients suffering from perennial allergic rhinitis with intranasal fluticasone propionate (200 µg/day) revealed no damage of the nasal mucosa (Holm *et al.*, 1998).

The amount of mucus produced by the slugs treated with Nasonex® was significantly larger than that of the negative control slugs, but significantly smaller than that of the positive control slugs. The formulation was classified as moderately irritating. Furthermore, Nasonex® induced minimal damage of the slug mucosa as was demonstrated by the protein and enzyme release data. In 2%, 2%, 8% and 1% of the cases, the use of Nasonex® has been respectively associated with nasal irritation, nasal burning, epistaxis, and nasal ulceration (Schering-Plough, 2000). After administration of Nasonex® once daily during 28 days, 6% of 126 patients with seasonal allergic rhinitis reported mild to moderate nasal burning rated as at least possibly related to treatment; 3% of the patients reported epistaxis (Hebert *et al.*, 1996). A daily treatment with Nasonex® during 12 months of adult patients with perennial rhinitis was not associated with changes in the nasal mucosa. Evaluation of pre-treatment and post-treatment nasal biopsy samples revealed no significant differences in epithelial atrophy. Furthermore, comparison of the nasal mucosa before and after treatment with Nasonex® revealed a higher percentage of pseudostratified ciliated columnar epithelium after treatment (Minshall *et al.*, 1998).

A repeated treatment of the slugs with Nesivine® (a 0.05% (w/v) oxymetazoline hydrochloride nasal solution) resulted in a total mucus production that was significantly larger than that of the negative control slugs, but significantly smaller than that of the positive control slugs. Nesivine® was classified as a moderately irritating formulation that induced

minimal damage of the slug mucosa. Local adverse effects, such as nasal stinging, have been associated with the use of Nesivine® (Merck, 1996). Treatment of 20 healthy volunteers three times daily for 30 days with 0.05% (w/v) oxymetazoline nasal spray (containing 0.01% benzalkonium chloride) led to epistaxis in 10% of the cases and a statistically significant increase in mucosal swelling and stuffiness (Graf *et al.*, 1995). With regard to the tissue damage potential of Nesivine®, *in vitro* and rabbit data are not in line with each other. Exposure of human respiratory mucosa *in vitro* to undiluted 0.05% (w/v) oxymetazoline hydrochloride spray for one to 30 minutes once daily during 14 days resulted in viability loss of all the tissue fragments and in reduction of the number of ciliated microvillous cells. However, tissue fragments exposed for 10 minutes during 14 days to a 3% (v/v) dilution of the spray did not change morphologically compared to those exposed to 0.9% sodium chloride solution (Berg *et al.*, 1995). Nasal administration of 0.05% (w/v) oxymetazoline hydrochloride spray twice daily for 1 week to healthy rabbits resulted in mild to moderate ciliary loss and mild epithelial ulceration. The ciliary loss and epithelial ulceration were more pronounced with increasing administration duration (Suh *et al.*, 1995).

The amount of mucus produced by the slugs treated with Allergodil® (a 0.1% (w/v) azelastine hydrochloride nasal solution) was significantly larger than that of the negative control slugs, but significantly smaller than that of the positive control slugs. Allergodil® was classified as a moderately irritating formulation by the Slug Mucosal Irritation test. Allergodil® induced minimal tissue damage as was indicated by the protein and enzyme release data. Symptoms of rhinitis or irritation of the nasal mucosa occurred in 8% of 489 children with perennial or seasonal allergic rhinitis after twice daily treatment with Allergodil® for four weeks. Epistaxis was reported by 1.2% of the 489 children (Lassig *et al.*, 1996). During a twice daily treatment with Allergodil® for six months, 6.5% of 185 patients with perennial allergic rhinitis reported itching or burning of the nasal mucosa. Epistaxis occurred in 0.5% of the patients. Rhinoscopic examination of the nasal mucosa and nasal biopsies revealed no evidence of drug related mucosal damage (Gastpar *et al.*, 1993).

A repeated treatment with Syntaris® (a nasal solution containing 0.025% (w/v) flunisolide) induced severe irritation of the slug mucosa as was demonstrated by the high total mucus production. Furthermore, the formulation caused a (non-significantly) increased protein release compared to the negative control slugs which is an indication of mild tissue damage. Adverse effects seen in patients treated with Syntaris® were nasal burning, nasal irritation, epistaxis, runny and stuffy nose, throat irritation, change or loss in the sense of smell or taste, and nasal septal perforation. During clinical studies, the dose needed to be

reduced or the treatment had to be discontinued in 3% of the subjects because of the occurrence of nasal burning. Rarely, a permanent loss in the sense of smell and/or taste was reported (Roche, 1995). Exposure of human respiratory mucosa *in vitro* to undiluted 0.025% flunisolide spray for 10 minutes once a day during 10 days resulted in loss of cilia of the tissue fragments and even loss of the continuous epithelial lining (Steinsvag *et al.*, 1996). Nasal administration of 0.025% (w/v) flunisolide nasal spray twice daily for 21 days to rats resulted in squamous cell metaplasia of the nasal epithelium. Furthermore, a reduction of the height of the epithelium, a decrease of the number of ciliated cells, a reduction of the number of goblet cells, and the absence of the mucus layer were observed. However, no morphological changes occurred in the subepithelial tissue (Berg *et al.*, 1997).

Table 9.2 Comparison of irritation categories obtained with the Slug Mucosal Irritation test on the one hand with clinical data concerning nasal irritation or burning induced by a repeated treatment (4-6 weeks) with some liquid nasal formulations on the other hand

Formulation	Irritation category obtained with Slug Mucosal Irritation test	Clinical data		
		Patients with nasal irritation or burning (%)	Patients (n)	Source
Luffeel®	No irritation	1	72	Weiser *et al.*, 1999
Flixonase Aqua™	Mild irritation	4	25	van As *et al.*, 1991
Nasonex®	Moderate irritation	6	126	Hebert *et al.*, 1996
Allergodil®	Moderate irritation	8	489	Lassig *et al.*, 1997

In order to compare the Slug Mucosal Irritation test data with the clinical data in a more structured way, those clinical studies were selected that lasted four to six weeks and that mentioned the number of patients experiencing nasal irritation or burning during treatment with the liquid nasal formulations. Table 9.2 shows that an increasing irritation potential predicted by the Slug Mucosal Irritation test corresponds with an increasing percentage of patients that reported nasal irritation or burning. The results indicate that the mucus production of the slugs is also related to the burning sensation that is reported by patients in clinical studies. As mentioned before, no other pre-clinical tests that can predict the burning sensation induced by formulations are hitherto available. So it can be concluded that the results of this study indicate that the Slug Mucosal Irritation test seems to be a promising alternative to evaluate the mucosal tolerance of liquid nasal formulations intended for repeated administration.

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CHAPTER 10: GENERAL CONCLUSIONS

The first objective of this research was the optimisation and validation of the Slug Mucosal Irritation test procedure and prediction model for the evaluation of the eye irritation and damage potential of chemicals. For this purpose, 28 chemicals with known rabbit eye irritation data were used. The test procedure was reduced to one day by increasing the test concentration of the second contact period to 3.5%. A classification prediction model was developed that classifies the chemicals based on the amount of mucus produced during a 60-minute contact period with a 1% dilution of the chemical on the one hand and on the protein and LDH release of the slugs induced by the two contact periods (score for tissue damage) on the other hand. The mean κ-value for the agreement between the irritation categories of five repeated trials was 0.74, indicating substantial reproducibility. 71% of the 28 chemicals were correctly predicted into the three EU eye irritation categories with a sensitivity and specificity of 94% and 75%, respectively. Evaluation of the data of the 28 chemicals and 12 additional chemicals revealed that chemicals with anaesthetizing properties were generally classified differently by the Slug Mucosal Irritation test compared to the rabbit eye irritation test. Lauric acid (a chemical that causes mechanical injury), sodium lauryl sulphate, and Triton X-100® were also classified differently. For most of these differently classified chemicals, however, the results obtained with the Slug Mucosal Irritation test are in good agreement with available *in vivo* data or data of other alternative eye irritation tests. The eye irritation and damage test procedure of the Slug Mucosal Irritation test seems to be a promising screening method in a stepwise scheme for eye irritation testing. In this way, the number of rabbits used for eye irritation tests can be reduced and the rabbits' suffering can be limited. It is interesting to note that the transferability of the optimised test still has to be evaluated.

In order to evaluate the robustness of the Slug Mucosal Irritation test, the effect of the slug population and species on the test end points and the eye irritation classification was investigated using the optimised one-day test procedure. The results indicate that the tested *A.*

lusitanicus populations influence neither the test end points nor the classification. However, because the results demonstrate that the use of other slug species instead of *A. lusitanicus* can influence the test end points and the eye irritation classification, it is important to optimise and validate the test procedure and prediction model if another slug species is used.

Another research objective was the optimisation of the five-day procedure of the Slug Mucosal Irritation test for local tolerance testing of solid, semi-solid and liquid formulations intended for repeated administration via the ocular, buccal, nasal, rectal or vaginal route. Because no list of reference formulations intended for screening the mucosal tolerance is available, formulations with known *in vivo* or *in vitro* irritation data were selected for this purpose. The test procedure was optimised for each of the different formulation types (i.e. bioadhesive powders, suppositories, semi-solids, and liquids) by selecting an appropriate test amount, negative and positive control. A classification prediction model was developed that classifies the formulations into four irritation and tissue damage categories (non/minimal, mild, moderate and severe). Investigation of the repeatability of the test procedures by means of the negative and positive control and some formulations shows that the test results and classifications are reproducible. A good agreement between the Slug Mucosal Irritation test results and available *in vivo* and *in vitro* data indicates that the test seems to be a promising screening tool to evaluate the mucosal tolerance of formulations intended for repeated administration. The Slug Mucosal Irritation test can be used to perform concentration response experiments of excipients or drugs. Because substances can be tested on several days, the test can identify slow-acting irritants (i.e. irritants with a delayed response). Taking into account that the Slug Mucosal Irritation test can even predict mucosal burning and stinging and levels of mild irritation, the test can be a very helpful tool in prioritising formulation compounds or concentrations of compounds early in the drug development phase before pre-clinical studies in vertebrates and clinical studies. The test procedures can be further modified on demand of the customer that appeals to the Slug Mucosal Irritation test. However, it has to be noted that the relevance and reliability of the optimised test procedures have to be further evaluated with additional formulations with known *in vivo* mucosal tolerance data. Establishment of a data bank containing information about formulations and their mucosal irritating and damaging properties would benefit the validation of alternative mucosal tolerance tests.

It can be concluded that the Slug Mucosal Irritation test seems to be a promising method for the evaluation of the eye irritating properties of chemicals and for mucosal tolerance testing of solid, semi-solid and liquid formulations.

SUMMARY

Because irritation and damage of the mucosa can break the protective barrier against micro-organisms and noxious substances, it is important to evaluate the mucosal irritation potential of chemicals and pharmaceutical formulations which can come into contact with the mucosa. Regulatory authorities generally require that the eye irritation potential of chemicals and the mucosal tolerance of pharmaceutical formulations are assessed in vertebrates. However, the use of vertebrates for safety studies is criticised. Consequently, there is a great interest in developing alternative methods such as *in vitro* methods and the use of 'lower' organisms as test organisms. Before an alternative method is accepted by researchers and regulators, the relevance and the reliability of the test have to be investigated using reference standards (Chapter 1 and Chapter 2).

Within this scope, an alternative mucosal irritation test using slugs (i.e. *Arion lusitanicus*) was developed. The slug was selected as test organism, because the mucosal tissue of interest is located at the outside of the slug and because the slug mucosa histologically resembles the human mucosa. The mucus production of the slugs was chosen as end point to evaluate the irritation potential of substances; the release of proteins and enzymes from the slug body wall was used as measure of tissue damage. This research aimed at the optimisation and validation of the procedure and prediction model of the Slug Mucosal Irritation test for the evaluation of the eye irritation and damage potential of chemicals after single exposure. Another objective was the optimisation of the five-day procedure for local tolerance testing of solid, semi-solid and liquid formulations intended for repeated administration (Chapter 1 and Chapter 2).

In Chapter 3, reference chemicals (with known rabbit eye irritation data) were used to optimise and validate the Slug Mucosal Irritation test procedure and prediction model for the evaluation of eye irritation and damage. The results of this study demonstrate that by increasing the test concentration of the second contact period to 3.5%, the Slug Mucosal

Irritation test procedure can be reduced to one day. A classification prediction model was developed that classifies the chemicals first based on the amount of mucus produced during a 60 minute contact period with a 1% dilution of the chemical. Chemicals that do not affect this end point are classified based on the tissue damage induced by the first treatment and by a second treatment with a 3.5% dilution of the chemical. Repeated testing of 28 chemicals on five separate occasions revealed good intra-laboratory reproducibility. Only for four test substances, there was a switch between non-irritant and R36, whereas there was no switch between non-irritant and R41. When the 28 chemicals were divided into non-irritants or irritants, a sensitivity and specificity of 94% and 75% were respectively obtained. Furthermore, 71% of the 28 chemicals were correctly predicted into the three EU eye irritation categories. Evaluation of the data of the 28 chemicals and 12 additional chemicals revealed that chemicals with anaesthetizing properties were generally classified differently by the Slug Mucosal Irritation test compared to the rabbit eye irritation test. Lauric acid (a chemical that causes mechanical injury), sodium lauryl sulphate, and Triton X-100® were also classified differently. For most of these differently classified chemicals, however, the results obtained with the Slug Mucosal Irritation test correspond with available *in vivo* data or data of other alternative eye irritation tests.

In Chapter 4, the effects of the slug population and species on the test end points and on the eye irritation classification were investigated. Comparison of the results of one Belgian and two Swiss *A. lusitanicus* populations indicates that the geographic and ecological origins of the tested slug populations influence neither the mucus production, nor the score for tissue damage. Slug species-specific effects on the test end points were investigated by comparing the data of Belgian *A. lusitanicus* with similar data of Belgian *L. flavus* and *L. maximus*. *L. flavus* and *L. maximus* produced more mucus than *A. lusitanicus*, so that the mucus production cut-off values had to be increased. Therefore, the results indicate that the test procedure and the prediction model have to be optimised and validated, if other slug species are used instead of *A. lusitanicus*.

In Chapter 5, the local tolerance of bioadhesive powder formulations intended for repeated buccal administration (containing Amioca® starch and linear polyacrylic acid or Carbopol® 974P) was evaluated. For this purpose, the five-day procedure of the Slug Mucosal Irritation test – currently utilised for local tolerance testing of nasal bioadhesive powders at the start of this research work – was used. A classification prediction model was developed which classifies the formulations into four irritation categories based on the total mucus production and into four tissue damage categories based on the protein and enzyme release.

The test enabled to investigate the concentration effect of ingredients of buccal powder formulations on the mucosal tissue. The mucosal irritation and damage potential of the cross-linked polyacrylic acid (Carbopol® 974P) was more pronounced that of the linear polyacrylic acid. Furthermore, the irritation and tissue damage potential increased with increasing polyacrylic acid content. The results are in good agreement with available (pre-) clinical data on the buccal tolerance. These concentration response experiments can be a very helpful tool in prioritising concentrations of formulation compounds in the drug development phase.

Chapter 6 describes the optimisation of the five-day procedure of the Slug Mucosal Irritation test for the evaluation of the local tolerance of bioadhesive powders intended for repeated ocular administration and their ingredients. The results indicate that a repeated treatment with 20 mg powder on five successive days – similar to the procedure used in Chapter 5 – enables the best discrimination between the mucus production, protein and enzyme release data of the negative and positive control slugs. DDWM and DDWM/SLS 80/20 were selected as negative and positive control, respectively. A repeated treatment with the ingredients DDWM, sodium stearyl fumarate or Amioca® resulted in no irritation of the slug mucosa. Ciprofloxacin HCl and gentamycin sulphate were classified as mildly irritating compounds. Vancomycin HCl induced severe irritation of the slug mucosa. Treatment with each of the previous compounds resulted in minimal damage of the mucosa. Carbopol® 974P induced severe irritation and moderate damage of the slug mucosa. The powders containing DDWM, 5% Carbopol® 974P, 1% sodium stearyl fumarate, and up to 10% ciprofloxacin HCl were classified as non-irritating formulations, whereas the powders containing Amioca®, 1% sodium stearyl fumarate, 5% gentamycin sulphate, 5% vancomycin HCl, and 4.45% or 13.35% Carbopol® 974P were classified as mildly irritating powders. For the tested ocular powders and their ingredients, the local tolerance data obtained with the Slug Mucosal Irritation test are in good agreement with existing *in vivo* data on the ocular tolerance.

In Chapter 7, the five-day procedure of the Slug Mucosal Irritation test was optimised for the evaluation of the local tolerance of suppositories. The results indicate that a repeated treatment with 50 mg grinded suppository enables good discrimination between the mucus production, protein and enzyme release data of the negative and positive control slugs. Novata® B and Novata® B/SLS 90/10 were selected as negative and positive control, respectively. The prediction model developed for bioadhesive powders was used to classify the suppositories. A repeated treatment of the slugs with Novata® B, Suppocire® AM or Colitofalk® suppositories – each containing a fatty suppository base – resulted in no irritation and minimal damage of the slug mucosa. Pentasa® suppositories – containing PEG as base –

and PEG 1500/PEG 4000 3/7 were respectively classified as mildly and severely irritating formulations. Neither Pentasa® nor PEG 1500/PEG 4000 3/7 resulted in damage of the slug mucosa. For the tested suppositories, the local tolerance data obtained with the Slug Mucosal Irritation test are in line with (pre-) clinical rectal tolerance data.

Chapter 8 deals with the optimisation of the five-day procedure of the Slug Mucosal Irritation test for the evaluation of the local tolerance of semi-solid vaginal formulations. Based on the results, 100 mg was selected as test amount to evaluate the mucosal tolerance of semi-solids. 5% HEC gel and Conceptrol® were selected as negative and positive control, respectively. A classification prediction model was developed for the determination of the irritation category of semi-solid formulations. A repeated treatment of the slugs with 5% HEC gel, Monistat® 7 or gels containing up to 10 mM dapivirine induced no mucosal irritation. Replens® and K-Y® jelly were respectively classified as mildly and moderately irritating semi-solids. None of the previous formulations induced damage of the mucosa. The irritation potential of the semi-solid formulations containing N-9 as only active compound (Gynol II®, Gynol II® Extra Strength, Advantage S®, Conceptrol®, and Delfen®) increased with increasing concentration of N-9. The latter formulations induced mild, moderate or severe damage of the slug mucosa. Protectaid® was classified as a severely irritating formulation that causes mild mucosal damage. For all the tested semi-solids with the exception of Protectaid®, the local tolerance data obtained with the Slug Mucosal Irritation test are in good agreement with existing *in vivo* data concerning the vaginal tolerance. The overestimation of the irritating and damaging properties of Protectaid® by the Slug Mucosal Irritation test may be explained by the fact that we tested only the gel that is impregnated in the Protectaid® sponge whereas application of the Protectaid® sponge by women leads to less exposure to gel.

In Chapter 9, the five-day procedure of the Slug Mucosal Irritation test was evaluated for the evaluation of the local tolerance of liquid nasal formulations. A classification prediction model was developed for the determination of the irritation category of liquid formulations. A repeated treatment of the slugs with Naaprep® or Luffeel® induced no mucosal irritation. Flixonase Aqua™ was classified as a mildly irritating formulation, whereas Nasonex®, Nesivine®, and Allergodil® were classified as moderately irritating formulations. None of the previous formulations induced damage of the slug mucosa. Syntaris® induced severe irritation and mild damage of the slug mucosa. For the tested liquid nasal formulations, local tolerance data obtained with the Slug Mucosal Irritation test are in good agreement with (pre-) clinical nasal tolerance data.

SAMENVATTING

De mucosa vormt een beschermende barrière tegen micro-organismen en schadelijke stoffen. Bijgevolg is het belangrijk om voor chemische producten en farmaceutische formulaties die met de mucosa in contact komen na te gaan of ze mucosale irritatie en beschadiging veroorzaken. De regelgevende instanties vereisen over het algemeen dat de lokale tolerantie van producten in vertebraten wordt beoordeeld. Het gebruik van vertebraten voor veiligheidstests wordt echter sterk in vraag gesteld. Bijgevolg is er een grote belangstelling voor de ontwikkeling van alternatieve methodes zoals *in vitro*methodes en voor het gebruik van lagere organismen als testorganismen. Alvorens een alternatieve methode door onderzoekers en regelgevers wordt aanvaard, moeten de relevantie en de betrouwbaarheid van de test onderzocht worden met behulp van referentiestoffen (Hoofdstuk 1 en Hoofdstuk 2).

In dit raam werd een alternatieve mucosale irritatietest ontwikkeld die gebruik maakt van naaktslakken (namelijk *Arion lusitanicus*). De naaktslak werd gekozen als testorganisme, omdat de mucosa zich aan de buitenzijde van de slak bevindt en omdat de mucosa van de slak histologisch lijkt op de humane mucosa. De mucusproductie van de slakken werd geselecteerd als eindpunt om de potentieel irriterende effecten van een substantie te evalueren; de vrijstelling van proteïnen en enzymen uit de lichaamswand van de slakken werd gekozen om de weefselbeschadiging te beoordelen. Dit onderzoek beoogde de optimalisatie en validatie van de testprocedure en het predictiemodel van de mucosale irritatietest voor de evaluatie van de oogirritatie en -beschadiging die kunnen veroorzaakt worden door een éénmalige blootstelling aan chemische producten. Een ander objectief was de optimalisatie van de vijf dagen procedure voor de evaluatie van de lokale tolerantie van vaste, halfvaste en vloeibare formulaties bestemd voor herhaalde toediening (Hoofdstuk 1 en Hoofdstuk 2).

In Hoofdstuk 3 werden referentiechemicaliën (waarvan gegevens over oogirritatie bij konijnen beschikbaar waren) gebruikt om de mucosale irritatie testprocedure te optimaliseren

en valideren voor de evaluatie van oogirritatie en -beschadiging. De resultaten van deze studie tonen dat de testprocedure kan worden verkort tot één dag door de testconcentratie van de tweede contactperiode te verhogen tot 3.5%. Er werd een predictiemodel ontwikkeld dat de chemicaliën eerst klasseert op basis van de hoeveelheid mucus die geproduceerd wordt tijdens een 60 minuten durende behandeling met een 1% verdunning van het chemische product. De chemicaliën die dit eindpunt niet beïnvloeden worden geklasseerd op basis van de weefselbeschadiging die wordt veroorzaakt door de eerste behandeling en door een tweede behandeling met een 3.5% verdunning van het product. Herhaald testen van 28 chemicaliën in vijf afzonderlijke experimenten toonde een goede intralaboratorium reproduceerbaarheid. Slechts vier chemicaliën werden zowel niet-irriterend als R36 geklasseerd in de herhaalde experimenten, terwijl geen enkele chemische stof zowel niet-irriterend als R41 geklasseerd werd. Wanneer de 28 chemicaliën werden ingedeeld in niet-irriterende of irriterende stoffen, werden een gevoeligheid en specificiteit van respectievelijk 94% en 75% bekomen. Bovendien werden 71% van de 28 chemische producten correct voorspeld in de drie EU oogirritatiecategorieën. Evaluatie van de data van de 28 chemicaliën en 12 bijkomende chemicaliën leerde dat chemische producten met verdovende eigenschappen over het algemeen verschillend geklasseerd worden met de mucosale irritatietest dan met de oogirritatietest bij konijnen. Laurylzuur (een chemische stof die mechanische beschadiging veroorzaakt), natriumlaurylsulfaat en Triton X-100® werden ook verschillend geklasseerd. Voor de meeste van de verschillend geklasseerde chemicaliën zijn de resultaten bekomen met de mucosale irritatietest echter in overeenstemming met beschikbare *in-vivo*gegevens of resultaten bekomen met andere alternatieve oogirritatietests.

In Hoofdstuk 4 werden de effecten van slakkenpopulatie en -species op de eindpunten van de test en op de oogirritatieclassificatie nagegaan. Vergelijking van de resultaten van één Belgische en twee Zwitserse *A. lusitanicus* populaties leert dat de geografische en ecologische oorsprong van de geteste populaties noch de mucusproductie, noch de score voor weefselbeschadiging beïnvloedt. De species specifieke effecten op de testeindpunten werden onderzocht door de gegevens van Belgische *A. lusitanicus* slakken te vergelijken met gelijkaardige gegevens van Belgische *L. flavus* en *L. maximus* slakken. *L. flavus* en *L. maximus* produceerden meer mucus dan *A. lusitanicus*, zodat de grenswaarden van de mucusproductie moesten worden verhoogd. De resultaten tonen bijgevolg aan dat de testprocedure en het predictiemodel dienen geoptimaliseerd en gevalideerd te worden als andere slakkenspecies gebruikt worden in plaats van *A. lusitanicus*.

In Hoofdstuk 5 werd de lokale tolerantie geëvalueerd van bioadhesieve poeders bestemd voor herhaalde buccale toediening (die Amioca® zetmeel en lineair polyacrylzuur of Carbopol® 974P bevatten). Hiervoor werd de vijf dagen procedure van de mucosale irritatietest – die bij het begin van dit onderzoek aangewend werd voor de evaluatie van de lokale tolerantie van nasale bioadhesieve poeders – gebruikt. Er werd een predictiemodel ontwikkeld dat de formulaties klasseert in vier irritatiecategorieën op basis van de totale mucusproductie en in vier weefselbeschadigingcategorieën op basis van de vrijstelling van proteïnen en enzymen. De test liet toe om het concentratie-effect van ingrediënten van buccale poederformulaties te evalueren. Het vernet polyacrylzuur (Carbopol® 974P) veroorzaakte meer mucosale irritatie en beschadiging dan het lineaire polyacrylzuur. Bovendien nam het irriterend en beschadigend vermogen toe met een stijgende concentratie aan polyacrylzuur. De resultaten zijn in overeenstemming met beschikbare (pre)klinische data over de buccale tolerantie. Deze concentratie-respons experimenten kunnen een zeer nuttig hulpmiddel zijn bij de selectie van concentraties van componenten die bestemd zijn voor de ontwikkeling van geneesmiddelen.

Hoofdstuk 6 beschrijft de optimalisatie van de vijf dagen procedure van de mucosale irritatietest voor de evaluatie van de lokale tolerantie van bioadhesieve poeders bestemd voor herhaalde oculaire toediening en hun ingrediënten. De resultaten toonden aan dat een herhaalde behandeling met 20 mg poeder op vijf opeenvolgende dagen – gelijkaardig aan de procedure gebruikt in Hoofdstuk 5 – best discrimineert tussen de gegevens over de mucusproductie en de proteïnen- en enzymen-vrijstelling van de negatieve en positieve controleslakken. DDWM en DDWM/SLS 80/20 werden respectievelijk als negatieve en positieve controle gekozen. Een herhaalde behandeling met de ingrediënten DDWM, natriumstearylfumaraat of Amioca® resulteerde niet in irritatie van de mucosa van de slakken. Ciprofloxacine HCl en gentamycinesulfaat werden als mild irriterende componenten geklasseerd. Vancomycine HCl induceerde sterke irritatie van de mucosa. Behandeling met elk van de voorgaande componenten resulteerde in minimale beschadiging van de mucosa. De poeders die DDWM, 5% Carbopol® 974P, 1% natriumstearylfumaraat en tot 10% ciprofloxacine HCl bevatten werden geklasseerd als niet-irriterende formulaties. De poeders die Amioca®, 1% natriumstearylfumaraat, 5% gentamycinesulfaat, 5% vancomycine HCl en 4.45% of 13.35% Carbopol® 974P bevatten werden geklasseerd als mild irriterende poeders. De lokale tolerantiedata van de geteste poeders en hun ingrediënten bekomen met de mucosale irritatietest zijn in goede overeenstemming met bestaande *in-vivo*data over de oculaire tolerantie.

In Hoofdstuk 7 werd de vijf dagen procedure van de mucosale irritatietest geoptimaliseerd voor de evaluatie van de lokale tolerantie van suppositoria. De resultaten tonen aan dat een herhaalde behandeling met 50 mg fijngemalen suppositorium goed discrimineert tussen de gegevens over de mucusproductie en de proteïnen- en enzymen-vrijgave van de negatieve en positieve controleslakken. Novata® B en Novata® B/SLS 90/10 werden respectievelijk als negatieve en positieve controle gekozen. Het predictiemodel ontwikkeld voor bioadhesieve poeders werd gebruikt om de suppositoria te klasseren. Een herhaalde behandeling van de slakken met Novata® B, Suppocire® AM of Colitofalk® suppositoria – die elk een vette suppobasis bevatten – resulteerde niet in irritatie of beschadiging van de mucosa van de slakken. Pentasa® suppositoria – met PEG als basis – en PEG 1500/PEG 4000 3/7 werden respectievelijk als mild en sterk irriterende formulaties geklasseerd. Noch Pentasa®, noch PEG 1500/PEG 4000 3/7 resulteerde in beschadiging van de mucosa. De lokale tolerantiedata van de geteste suppositoria bekomen met de mucosale irritatietest zijn in goede overeenstemming met beschikbare (pre)klinische data over de rectale tolerantie.

Hoofdstuk 8 behandelt de optimalisatie van de vijf dagen procedure van de mucosale irritatietest voor de evaluatie van de lokale tolerantie van halfvaste vaginale formulaties. Op basis van de resultaten werd 100 mg geselecteerd als testhoeveelheid om de mucosale tolerantie van halfvaste formulaties te beoordelen. 5% HEC gel en Conceptrol® werden respectievelijk als negatieve en positieve controle gekozen. Er werd een predictiemodel opgesteld dat de irritatiecategorie van halfvaste formulaties voorspelt. Een herhaalde behandeling van de slakken met 5% HEC gel, Monistat® 7 of gelen die tot 10 mM dapivirine bevatten induceerde geen mucosale irritatie. Replens® en K-Y® jelly werden respectievelijk als mild en matig irriterende formulaties geklasseerd. Geen van voorgaande formulaties induceerde mucosale beschadiging. Het irriterend vermogen van de halfvaste formulaties die N-9 als enige actief bestanddeel bevatten (Gynol II®, Gynol II® Extra Strength, Advantage S®, Conceptrol® en Delfen®) nam toe met toenemende concentratie aan N-9. Deze formulaties induceerden milde, matige of sterke beschadiging van de mucosa van de slakken. Protectaid® werd geklasseerd als een sterk irriterende formulatie die milde mucosale beschadiging veroorzaakt. Voor alle geteste halfvaste formulaties met uitzondering van Protectaid® zijn de data bekomen met de mucosale irritatietest in goede overeenstemming met bestaande *in-vivo*data over de vaginale tolerantie. De overschatting van de irriterende en beschadigende eigenschappen van Protectaid® door de mucosale irritatietest kan verklaard worden door het

feit dat we enkel de gel testten die aanwezig is in de Protectaid® spons. Gebruik van de Protectaid® spons door vrouwen resulteert echter in een beperktere blootstelling aan de gel.

In Hoofdstuk 9 werd nagegaan of de vijf dagen procedure van de mucosale irritatietest kan gebruikt worden voor de evaluatie van de lokale tolerantie van vloeibare nasale formulaties. Er werd een predictiemodel ontwikkeld dat de irritatiecategorie van vloeibare formulaties voorspelt. Een herhaalde behandeling van de slakken met Naaprep® of Luffeel® induceerde geen mucosale irritatie. Flixonase Aqua™ werd geklasseerd als een mild irriterende formulatie, terwijl Nasonex®, Nesivine® en Allergodil® geklasseerd werden als matig irriterende formulaties. Geen van voorgaande formulaties induceerde beschadiging van de mucosa van de slakken. Syntaris® induceerde sterke irritatie en milde beschadiging van de mucosa. De lokale tolerantiedata van de geteste vloeibare nasale formulaties bekomen met de mucosale irritatietest zijn in goede overeenstemming met (pre)klinische data over de nasale tolerantie.

RÉSUMÉ

Il est important d'évaluer le potentiel irritatif des produits chimiques et des formulations pharmaceutiques qui peuvent entrer en contact avec la muqueuse, parce que l'irritation et les dégâts à la muqueuse peuvent détruire la barrière protectrice contre les substances nocives et les micro-organismes. Les autorités réglementaires exigent généralement que l'irritation oculaire des produits chimiques et la tolérance muqueuse des formes pharmaceutiques soient évaluées en utilisant des vertébrés. Cependant, l'utilisation des vertébrés pour des études de toxicologie est fortement critiquée. En conséquence, le développement de méthodes alternatives telles que les méthodes *in vitro* et l'utilisation des invertébrés suscitent un grand intérêt. Avant qu'une méthode alternative ne soit acceptée par des chercheurs et des législateurs, la pertinence et la fiabilité de l'essai doivent être étudiées en utilisant des substances de référence (Chapitre 1 et Chapitre 2).

Dans ce cadre, un test alternatif pour l'évaluation du potentiel irritatif a été développé en utilisant des limaces (notamment *Arion lusitanicus*). La limace a été choisie, parce que le tissu muqueux est situé à l'extérieur des limaces et que la muqueuse des limaces ressemble histologiquement à la muqueuse humaine. La production de mucus des limaces a été choisie comme paramètre pour évaluer le potentiel irritatif des substances; la libération de protéines et d'enzymes de la muqueuse des limaces a été sélectionnée pour évaluer les dégâts au tissu. Cette thèse de doctorat a visé l'optimisation et la validation de la procédure et du modèle de prédiction du test d'irritation muqueuse pour l'évaluation de l'irritation oculaire et des dégâts oculaires qui peuvent être causés par une exposition unique aux produits chimiques. Un autre objectif de cette thèse était l'optimisation de la procédure de cinq jours pour l'évaluation de la tolérance locale des formulations solides, semi-solides et liquides destinées à l'administration répétée (Chapitre 1 et Chapitre 2).

Dans le Chapitre 3, des produits chimiques de référence (dont les données sur l'irritation oculaire chez le lapin étaient disponibles) ont été employés pour optimiser et

valider la procédure et le modèle de prédiction du test d'irritation muqueuse pour l'évaluation de l'irritation oculaire et des dégâts oculaires. Les résultats de cette étude démontrent que la procédure du test d'irritation muqueuse peut être réduite à un jour en augmentant la concentration d'essai de la deuxième période d'exposition à 3.5%. On a développé un modèle de prédiction qui classe d'abord les agents chimiques selon la quantité de mucus produite pendant une période d'exposition de 60 minutes à une dilution de 1% du produit. Les produits chimiques qui n'affectent pas la production de mucus sont classés selon les dégâts au tissu induits par le premier traitement et par un deuxième traitement avec une dilution de 3.5% du produit. L'évaluation répétée de 28 agents chimiques à cinq périodes séparées a permis de démontrer la bonne reproductibilité intra-laboratoire. Seulement quatre substances ont été classées comme non irritantes et R36 aux expériences répétées, tandis qu'aucune substance n'a été classée à la fois comme non irritant et R41. Quand les 28 produits chimiques ont été classés en deux classes (non irritant ou irritant), une sensibilité et une spécificité de 94% et de 75% ont été respectivement obtenues. En outre, 71% des 28 agents chimiques ont été correctement classés dans les trois catégories d'irritation oculaire d'UE. L'évaluation des données des 28 produits chimiques et de 12 produits chimiques additionnels a indiqué que les produits ayant des propriétés anesthésiques ont généralement été classés différemment par le test d'irritation muqueuse par comparaison au test d'irritation oculaire de Draize. L'acide laurique (causant des dégâts mécaniques), le lauryl sulfate de sodium et le Triton X-100® ont également été classés différemment. Cependant, les résultats obtenus à l'aide du test d'irritation muqueuse correspondent à ceux obtenus par d'autres essais *in vivo* or alternatifs pour la plupart des produits classés différemment.

Dans le Chapitre 4, les effets des populations et des espèces de limaces sur les points limites du test et sur la classification de l'irritation oculaire ont été étudiés. La comparaison des résultats d'une population belge et de deux populations suisses d'*A. lusitanicus* indique que les origines géographiques et écologiques des populations de limaces n'influencent ni la production de mucus, ni le score des dégâts au tissu. Les effets des espèces de limaces sur les points limites du test ont été étudiés en comparant les données d'*A. lusitanicus* belge aux données semblables du *L. flavus* et du *L. maximus* belges. *L. flavus* et *L. maximus* ont produit plus de mucus qu'*A. lusitanicus*, de sorte que les valeurs limites de la production de mucus ont dû être augmentées. Par conséquent, les résultats indiquent que la procédure et le modèle de prédiction doivent être optimisés et validés, si d'autres espèces de limaces sont employées au lieu d'*A. lusitanicus*.

Dans le Chapitre 5, la tolérance locale des poudres bioadhésives destinées à l'administration buccale répétée (contenant l'amidon Amioca® et l'acide polyacrylique linéaire ou le Carbopol® 974P) a été évaluée. A cet effet, la procédure de cinq jours du test d'irritation muqueuse – qui a été employée au début de cette étude pour l'évaluation de la tolérance locale des poudres bioadhésives nasales – a été utilisée. On a développé un modèle de prédiction qui classe les formulations en quatre catégories d'irritation sur la base de la production de mucus et en quatre catégories de dégâts au tissu sur la base de la libération de protéines et d'enzymes. L'essai a permis d'étudier l'effet de la concentration des ingrédients des poudres buccales sur le tissu muqueux. L'irritation muqueuse et les dégâts à la muqueuse causés par l'acide polyacrylique réticulé (Carbopol® 974P) étaient plus prononcés que ceux causés par l'acide polyacrylique linéaire. En outre, le potentiel irritatif des poudres augmentait quand le contenu en acide polyacrylique s'élevait. Les résultats concordent bien avec les données (pré-) cliniques disponibles sur la tolérance buccale. Ces expériences de concentration-réponse peuvent être un outil très utile pour sélectionner des concentrations de composants de formulation dans le développement des médicaments.

Le Chapitre 6 décrit l'optimisation de la procédure de cinq jours du test d'irritation muqueuse pour l'évaluation de la tolérance locale des poudres bioadhésives destinées à l'administration oculaire répétée et leurs ingrédients. Les résultats indiquent qu'un traitement répété avec 20 mg de la poudre pendant cinq jours successifs – semblable à la procédure utilisée dans le Chapitre 5 – permet la meilleure discrimination entre les productions de mucus et entre les données sur la libération de protéines et d'enzymes des contrôles négatifs et positifs. Le DDWM et le DDWM/SLS 80/20 ont été respectivement choisis comme contrôle négatif et positif. Un traitement répété avec le DDWM, le stéarylfumarate de sodium et l'Amioca® n'a pas causé de l'irritation de la muqueuse des limaces. Le chlorhydrate de ciprofloxacine et le sulfate de gentamycine ont été classés comme des composants légèrement irritants. Le chlorhydrate de vancomycine a induit une irritation sévère de la muqueuse des limaces. Le traitement avec chacun des composants précédents a causé des dégâts minimes. Le Carbopol® 974P a induit une irritation sévère et des dégâts modérés à la muqueuse des limaces. Les poudres contenant du DDWM, 5% de Carbopol® 974P, 1% de stéarylfumarate de sodium et jusqu'à 10% de chlorhydrate de ciprofloxacine ont été classées comme des poudres non irritantes, tandis que les poudres contenant de l'Amioca®, 1% de stéarylfumarate de sodium, 5% de sulfate de gentamycine, 5% de chlorhydrate de vancomycine et 4.45% ou 13.35% de Carbopol® 974P ont été classées comme des poudres légèrement irritantes. En ce qui concerne les poudres ophtalmiques et leurs ingrédients examinés, les données de tolérance

locale obtenues à l'aide du test d'irritation muqueuse correspondent bien aux données *in vivo* existantes concernant la tolérance oculaire.

Le Chapitre 7 examine l'optimisation de la procédure de cinq jours du test d'irritation muqueuse pour l'évaluation de la tolérance locale des suppositoires. Les résultats indiquent qu'un traitement répété avec 50 mg de poudre de suppositoire permet la bonne discrimination entre les productions de mucus et entre les données sur la libération de protéines et d'enzymes des contrôles négatifs et positifs. Novata® B et Novata® B/SLS 90/10 ont été respectivement choisis comme contrôle négatif et positif. Le modèle de prédiction développé pour les poudres bioadhésives a été employé pour classer les suppositoires. Un traitement répété des limaces avec des suppositoires de Novata® B, de Suppocire® AM ou de Colitofalk® – chacun contenant une base lipophile de suppositoire – n'a pas causé de l'irritation ou des dégâts à la muqueuse des limaces. Les suppositoires de Pentasa® – contenant PEG comme base – et PEG 1500/PEG 4000 3/7 ont été respectivement classés comme des formulations légèrement et sévèrement irritantes. Ni Pentasa® ni PEG 1500/PEG 4000 3/7 n'ont causé des dégâts à la muqueuse des limaces. En ce qui concerne les suppositoires examinés, les données de tolérance locale obtenues à l'aide du test d'irritation muqueuse concordent bien avec les données (pré-) cliniques disponibles sur la tolérance rectale.

Le Chapitre 8 décrit l'optimisation de la procédure de cinq jours du test d'irritation muqueuse pour l'évaluation de la tolérance locale des formulations semi-solides vaginales. Sur la base des résultats, 100 mg a été choisi comme quantité d'essai pour évaluer la tolérance muqueuse des formulations semi-solides. 5% de gel HEC et Conceptrol® ont été respectivement choisis comme contrôle négatif et positif. Un modèle de prédiction a été développé pour déterminer la catégorie d'irritation des formulations semi-solides. Un traitement répété des limaces avec 5% de gel HEC, Monistat® 7 ou des gels contenant jusqu'à 10 mM de dapivirine n'a induit aucune irritation muqueuse. Replens® et K-Y® jelly ont été respectivement classés comme des formulations légèrement et modérément irritantes. Aucune des formulations précédentes n'a induit des dégâts à la muqueuse. Le potentiel irritatif des formulations semi-solides contenant seulement N-9 comme composant actif (Gynol II®, Gynol II® Extra Strength, Advantage S®, Conceptrol® et Delfen®) augmentait quand la concentration de N-9 s'élevait. Les dernières formulations ont induit des dégâts légers, modérés ou sévères à la muqueuse des limaces. Protectaid® a été classé comme formulation sévèrement irritante qui endommage légèrement la muqueuse. En ce qui concerne toutes les formulations semi-solides examinées excepté Protectaid®, les données de tolérance locale obtenues à l'aide du test d'irritation muqueuse correspondent bien aux données *in vivo*

existantes concernant la tolérance vaginale. La surestimation des caractéristiques d'irritation et de dégâts de Protectaid® par le test d'irritation muqueuse peut être expliquée par le fait que nous avons examiné seulement le gel qui est imprégné dans l'éponge de Protectaid®, tandis que l'application de l'éponge de Protectaid® par des femmes entraîne une moindre exposition au gel.

Dans le Chapitre 9, la procédure de cinq jours du test d'irritation muqueuse a été évaluée pour l'évaluation de la tolérance locale des formulations nasales liquides. Un modèle de prédiction a été développé pour déterminer la catégorie d'irritation des formulations liquides. Un traitement répété des limaces avec Naaprep® ou Luffeel® n'a pas induit de l'irritation muqueuse. Flixonase Aqua™ a été classé comme une formulation légèrement irritante, tandis que Nasonex®, Nesivine® et Allergodil® ont été classés comme des formulations modérément irritantes. Aucune des formulations précédentes n'a induit des dégâts à la muqueuse des limaces. Syntaris® a induit de l'irritation sévère et de légers dégâts à la muqueuse. En ce qui concerne les formulations liquides nasales examinées, les données de tolérance locale obtenues à l'aide du test d'irritation muqueuse concordent bien avec les données pré-cliniques et cliniques disponibles sur la tolérance nasale.