Title: New aspects of the Slug Mucosal Irritation assay: predicting nasal stinging, itching and burning sensations

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New aspects of the Slug Mucosal Irritation (SMI) assay:

Predicting nasal stinging, itching and burning sensations

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Short Abstract

The objective of this study was to evaluate the mucosal tolerance of several marketed nasal formulations using the slug *Arion lusitanicus*. None of the tested formulations resulted in tissue damage, however a clear effect on the mucus production of the slugs was observed, either due to the active ingredient, presence of benzalkonium chloride as a preservative or hyperosmolality of the formulation. The new 1-day protocol of the Slug Mucosal Irritation assay is a good tool to predict nasal clinical discomfort.

Abstract

Today, stinging, itching and/or burning (SIB) sensations cannot be detected by animal tests or *in vitro* models. In the past, the Slug Mucosal Irritation (SMI) assay demonstrated a relation between an increased mucus production in slugs and an elevated incidence of SIB sensations in humans. A new 1-day SMI-test procedure was developed focusing on the prediction of these short term sensations.

The objective of this study was to verify whether this new procedure is capable predicting mucosal tolerance of several marketed nasal formulations using the slug *Arion lusitanicus*. Irritation and tissue damage were quantified with a 5-day repeated exposure study by means of the mucus produced and proteins and enzymes released. On the other hand, the new protocol predicted SIB sensations by means of the mucus production. The effects of 6 liquid nasal formulations were tested with both protocols, while 5 physiologic saline solutions were only tested with the new protocol to optimize it. None of the tested liquid nasal formulations resulted in tissue damage, however the exposure to the different formulations had a clear effect on the mucus production of the slugs and moderate discomfort was observed in some cases. These effects were due to the active ingredient, the presence of benzalkonium chloride as a preservative or the hyperosmolality of the formulation. For the most part

results agreed with clinical data found in literature. It was concluded that the SMI assay and the new 1-day protocol in particular is a good tool to predict nasal clinical discomfort.

Key words: nasal formulations, discomfort, mucosal tolerance, Slug Mucosal Irritation assay, alternative testing method

Introduction

The nose serves as an efficient humidifier, heater and filter for inhaled air, thereby protecting the lower airways (Bousquet et al., 2001). Consequently, the nasal mucosa is constantly exposed to unconditioned and occasionally polluted inhaled air causing irritation, sneezing, reflex-mediated hyper-secretion and nasal blockage (Bousquet et al., 2001). The protection against invading microorganisms by the nasal mucosa includes an intact mucosal barrier, mucociliary transport and mucosal immunity (Mygind et al., 1990). Defects in any of these defense mechanisms may increase the susceptibility to infection (Bjerknes & Steinsvåg, 1993).

The nasal mucosa is also a potential site for drug absorption, as the surface of the mucosa is large and well provided with blood vessels (Hermens & Merkus, 1987). As a consequence, nasal drug formulations for local use are widely used as they are mostly 'over the counter' (OTC) drugs (Romeijn et al., 1996). A number of these OTC nasal formulations are used to relieve congestion in patients with allergic rhinitis (AR), non-allergic rhinitis, acute or chronic sinusitis, nasal polyposis, and rhinitis due to nasal septal deviation or obstruction (Graf, 2005; Åkerlund & Bende, 1991), even though some decongestive nose sprays contain components with potentially deleterious effects on the nasal mucosa (Talaat et al., 1981; van de Donk et al., 1981, 1982). Some of these formulations are even associated with the occurrence of rhinitis medicamentosa (RM), a condition first described by Kully (1945) as a therapy-resistant nasal blockage due to overmedication with nasal decongestants.

Suffering from RM, several histological changes may occur in the nose. Rhinitis medicamentosa may be induced either by the active ingredient of the formulation (e.g. oxymetazoline (Graf & Hallén, 1996; Min et al., 1996)) or preservatives (e.g. benzalkonium chloride (Graf, 1999; Graf et al., 1995; Graf & Hallén, 1996)). From most OTC-formulations only minimal effects are observed *in vivo*, but long term use may result in RM. This is the case, for example, when formulations with oxymetazoline are used longer than 10 days (Graf & Juto, 1994a, 1994b, 1995).

In general, the development of some pharmaceutical formulations and cosmetics can be ceased in a later phase in the development when they cause stinging, itching and/or burning sensations during clinical studies. These discomforts are only seen in this stage since neither animal tests nor *in vitro* models were able to detect these

effects. This was the case for the Nasalide® nasal spray, containing the topical intranasal corticosteroid flunisolide. Trangsrud et al. (2002) noted that Nasalide® was associated with more reports of nasal stinging and burning. According to early reports of tolerability, up to 45% of patients noted nasal burning with Nasalide® nasal spray (Dura Pharmaceuticals Inc., 2000; Mabry, 1995). Some of these problems were due to the formulation. In the 1980s the drug was reformulated to contain less propylene glycol (Nasarel®) which resulted in a significantly lower frequency of nasal burning and stinging as well as throat irritation (Greenbaum et al., 1988; Meltzer et al., 1990). Hence, a screening method for clinical discomfort would be very helpful in the development and refinement process of formulations which are usually tolerated well. In the past, the Slug Mucosal Irritation (SMI) test, which is an alternative mucosal irritation method, demonstrated a relation between an increase in mucus production in slugs and an elevated incidence of stinging, itching and burning (SIB) in humans (Adriaens & Remon, 2008). The principle of the SMI-test is the fact that a higher mucus production is observed when slugs are exposed to more irritant substances, and that when tissue damage occurs, there will be protein and enzyme release. By means of the SMI-test we are able to predict the stinging, itching and burning (SIB) sensations since slugs react on these stimuli by an increase in mucus production.

In this present study, the mucosal tolerance of several nasal formulations was evaluated with a 5-day repeated exposure study. Additionally, the effect of tonicity and the concentration of benzalkonium chloride (BAC) were investigated into detail. In the second part of this study, a newly developed 1-day protocol of the SMI assay is suggested as a new method to predict short term sensations (stinging, itching and burning) in man. To optimize this new protocol, several marketed physiological saline solutions with different tonicity were tested in addition to the nasal formulations tested with the 5-day protocol.

Materials and methods

Materials

Nasal formulations and controls

Four nasal solutions were selected: Nesivine® and Nesivine® Baby (oxymetazoline HCl - Merck, Overijse, Belgium), Allergodil® (azelastine HCl - Meda Pharma, Brussels, Belgium) and Syntaris® (flunisolide - Norton Healthcare Ltd., London, United Kingdom). Additionally, effects of 2 nasal suspensions Flixonase Aqua™ (fluticasone propionate - GlaxoSmithKline, Genval, Belgium) and Nasonex® (mometasone furoate - Schering-Plough NV/SA, Brussels, Belgium) were also investigated. All nasal formulations were studied in undiluted form. The details of the tested formulations are listed in Table 1.

Additionally, isotonic nasal physiological salt solutions Naaprep® (GlaxoSmithKline, Mary-le-Roi, France), Rhina-Care (Sanofi-Aventis, Diegem, Belgium) and Physiomer® Normal Jet (Goëmar, Saint-Malo, France) were also tested with the newly developed protocol. Moreover, possible effects of the hypertonic solutions Sinomarin® (Belobal, Paris, France – 2.3% NaCl) and Physiomer® Sinus (Goëmar, Saint-Malo, France – 2.2% NaCl) were investigated as well.

In the experiments phosphate buffered saline (PBS, pH 7.4) was used as a negative control, whereas a 1% (w/v) dilution of benzalkonium chloride (BAC) (both delivered by Sigma, St Louis, MO, USA) was used as a positive control.

Methods

Local tolerance test procedure of the SMI-test

The slugs (*Arion lusitanicus*) were born in the lab in October-December 2008 and bred in an acclimatized room (18-20°C). 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 18-20°C. During the isolation period, the body wall of the slugs was daily wetted with 1 ml PBS using a micropipette.

The effect of the 6 selected nasal formulations, negative and positive controls on the mucosal tissue of the slugs was investigated. All nasal formulations were tested pure.

The slugs and Petri dishes, where they were put on during the contact period (CP), were weighed at the beginning of the experiment. Subsequently, the slugs were placed individually during 30 minutes in a Petri dish on 100 µl of test medium. For each series, five slugs were used. Each experimental run also contained a negative (PBS) and a positive control (BAC, 1% w/v). After the CP, the amount of mucus produced was measured by reweighing the Petri dishes containing the test medium (without the slugs). The mucus production (MP) is expressed as a percentage (w/w) of the initial 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 and were analyzed immediately for the presence of proteins, lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) released from the slug body wall. Finally, the slugs were transferred to a fresh Petri dish and again 1 ml PBS was added. The second PBS samples were collected after 60 minutes. Again, the samples were analyzed immediately for the presence of proteins, LDH, and ALP. This procedure was repeated for 5 successive days.

Additionally, the influence of osmolality and BAC-concentration was investigated to deduce the possible effect of these factors. A concentration-response experiment was conducted testing the irritation potency of BAC in a concentration of (w/v) 0.02%, 0.1%, 0.5% and 1%, while the effect of tonicity was investigated testing 6 different concentrations (w/v) of NaCl (0.05%, 0.5%, 0.9%, 2.5%, 5% and 10%). These experiments followed the procedure described above.

Analytical procedures

- Protein determination

The total protein concentration present in the PBS-samples was determined with a NanoOrange® protein quantification kit (Invitrogen™, Merelbeke, Belgium) and expressed as µg/ml per gram body weight. The NanoOrange® reagent allows accurate detection of proteins in solution at concentrations between 10 ng/ml and 10 µg/ml (Harvey et al., 2001). The 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. Bovine serum albumin was used as a standard.

- Enzyme activity

The lactate dehydrogenase activity (LDH, EC 1.1.1.27) and alkaline phosphatase activity (ALP, EC 3.1.3.1) were measured using commercial kits (DG 1340-UV and DG 1245-UV, respectively, Sigma Diagnostics, Bornem, Belgium), and expressed as IU/l per gram body weight. The reagents measure the enzyme activity using an optimized method based on the standard method recommended by the German Society for Clinical Chemistry (DGKC, 1972). The activity measurements were conducted on a Cobas Plus analyser (ABX, Brussels, Belgium) at 37 °C.

Stinging, itching and burning (SIB) test procedure of the SMI-test

This new test procedure is a modified version of the previously described method, only focusing on the occurrence of SIB-sensations. In this 1-day experiment, there are 3 CPs of 15 min, with 1 h rest/ recovery period in between. For each series, 3 slugs are used. During each CP, the slugs are placed on a Petri dish on 100 µl of the undiluted test substance. Body weight and MP are determined as described for the local tolerance experiment. No samples are taken to analyze protein or enzyme release. In the experiments PBS was used as a negative control, whereas a 1% (w/v) dilution of BAC was used as a positive control, as were in the 5-day protocol.

Osmolality measurements

The osmolality of the test compounds was measured using an Advanced Micro Osmometer (Model 3300 Advanced Instruments Inc, Norwood, MA, US) by the freezing-point method. Clinitrol™ 290 was used as reference solution (Advanced Instruments). The measurements were performed in triplicate (on 20-µL aliquots) and mean values used for analysis. Results are represented as mOsm/kg. Samples with an osmolality > 1200 mOsm/kg were diluted for measurement.

Data analysis

For the 5-day procedure the irritation potency was predicted based on the total amount of mucus produced (total MP) during the repeated 30 min CPs. Total MP is expressed as a percent of the body weight of the slugs. For each slug, total MP is calculated by adding up the mucus produced during each 30 min CP, and a mean value for the slugs in each treatment was calculated. The cut-off values for classification are shown in Table 2. Tissue damage is predicted by: the number of slugs in each treatment group (out of the 5 per treatment) that show ALP release; the mean LDH release of all the samples; and the mean protein release excluding the samples taken on day 1 (Fig. 1).

Classification in the newly developed test method for SIB is based on the MP only. The cut-off values are based on the mean total MP of the 3 CPs (Table 2).

Results

Local tolerance of nasal formulations

The 5-day test procedure was used to evaluate the mucosal tolerance of liquid nasal formulations. The effects of a daily 30 min exposure to the test substances on the total MP, mean protein and enzyme release are presented in Table 3.

None of the tested liquid nasal formulations caused tissue damage (mean protein release < 25 µg/ml.g; no LDH or ALP release) as did the negative control PBS. On the other hand, severe tissue damage was observed in the positive control BAC 1% (mean protein release > 100 µg/ml.g; LDH > 4 IU/g; ALP observed in 1 slug). From day 1 on, LDH release was already observed. Release of this marker increased in function of time. On the other hand, ALP release was detected on day 4 in only 1 of the 5 slugs treated with this solution. The next day this slug was found dead, indicating some severe tissue damage had occurred.

The exposure to the different formulations had a clear effect on the MP of the slugs. Mometazone furoate (Nasonex®) and fluticasone propionate (Flixonase Aqua™) showed a comparable total MP and were classified as mild irritants. Oxymetazoline HCl 0.01% (Nesivine® Baby) and oxymetazoline HCl 0.05% (Nesivine®) were both classified as moderate irritants (total MP between 5 and 10%). A slight concentration-response effect was observed, with MP in oxymetazoline HCl 0.05% (Nesivine®) slightly higher than for oxymetazoline HCl 0.01% (Nesivine® Baby). Both azelastine HCl (Allergodil®) and flunisolide (Syntaris®) caused severe irritation in the slugs (total MP > 10%).

The effects on the MP are in most cases due to the active ingredient, while for Syntaris® the high tonicity (1592 ± 28 mOsm/kg) is the probable cause. It is quite striking that the elevated MP for Syntaris® was not accompanied with protein and enzyme release, indicating stinging, itching, and/or burning might have occurred without any tissue damage.

Possible influence of BAC-concentration and osmolality

The influence of osmolality and the BAC-concentration was also investigated with the 5-day test procedure. Results are presented in Table 4.

A concentration-response experiment was conducted testing the irritation potency of BAC in concentrations of 0.02%, 0.1%, 0.5% and 1%. There was a clear effect of BAC-concentration on the reaction of the slugs. Irritation was observed in all tested concentrations. Even BAC 0.02% resulted in moderate irritation (total MP between 5% and 10%), while the other 3 concentrations were classified as severe irritant (total MP > 10%). There was no tissue damage in BAC 0.02% and BAC 0.1% (mean protein release < 25 µg/ml.g; no LDH and ALP release). On the other hand, BAC 0.5% and BAC 1% resulted both in severe tissue damage (mean protein release > 100 µg/ml.g; ALP and LDH release).

Another concentration-response experiment with 6 different NaCl-concentrations was conducted as well. This was done to deduce the effect of tonicity on the reaction of the slugs. First, none of the NaCl-solutions resulted in tissue damage (mean protein release < 25 µg/ml.g; no LDH or ALP release). A second observation is the fact that the 3 lowest concentrations (0.05%, 0.5% and 0.9%) did not cause any irritation either (total MP < 0%). For all 3 concentrations, total MP was even negative, probably due to the osmotic effect, since hypotonicity results in some fluid take-up by the slugs, giving a very low or even negative MP, as is the case here. The 2.5%-NaCl solution resulted in moderate irritation, while both 5%- and 10%-NaCl solutions caused severe irritation (total MP > 10%).

Stinging, itching and burning of liquid nasal formulations

In this first part, the same liquid nasal formulations as tested with the local tolerance test procedure were investigated. Results are presented in Table 5. All formulations showed a higher MP than the negative control (PBS). With this protocol mometasone furoate (Nasonex®), fluticasone propionate (Flixonase Aqua™), oxymetazoline 0.01% (Nesivine® Baby), and oxymetazoline 0.05% (Nesivine®) were all classified as causing mild discomfort (total MP between 3% and 8%). Azelastine HCl (Allergodil®) and flunisolide (Syntaris®) showed a more elevated MP (total MP between 8% and 15%) and were therefore classified as causing moderate discomfort. It is clear that in most of the formulations the elevated MP is caused as a response to the active ingredient, while in the case of flunisolide (Syntaris®) the

reaction is associated with the high osmolality of the formulation. All the other formulations are practically isotonic.

Stinging, itching and burning of physiological saline solutions

In a second part, nasal physiological saline solutions were also tested with the newly developed protocol. Results are also presented in Table 5. All practically isotonic solutions had a MP comparable with the negative control (total MP < 3%). Therefore, Naaprep®, Rhina-Care and Physiomer® Normal Jet were classified as causing no discomfort. Hypertonic saline solutions (630 mOsm/kg < osmolality < 750 mOsm/kg) (Physiomer® Sinus and Sinomarin®) resulted in an elevated MP (total MP between 3% and 8%), and consequently being classified as causing mild discomfort (total MP between 3% and 8%).

Discussion

In this study, the mucosal tolerance of several nasal formulations was evaluated with a 5-day repeated exposure study using the SMI assay. In the second part, the newly developed 1-day protocol of the SMI assay for the detection of short term sensations (stinging, itching and burning) was evaluated by testing several marketed physiological saline solutions with a different tonicity, as well as the nasal formulations tested with the 5-day protocol. Additionally, the effect of tonicity and the concentration of BAC on the slugs were investigated into detail with both testing procedures.

In many multidose topical aqueous nose drop and spray formulations, BAC is used as a preservative to prevent bacterial contamination and maintain the safety of the preparations (Graf, 2005; Graf et al., 1995, 1999; Marple et al., 2004; Steinsvåg et al., 1996). Benzalkonium chloride acts by damaging the cell wall of micro-organisms, by altering its permeability (Richards & Cavill, 1976). In multidose marketed nasal products, BAC is usually added in concentrations of 0.005 - 0.02% (w/v) (Riechelmann et al., 2004; Verse et al., 2003), while the American College of Toxicology concluded that BAC can be used safely in a concentration up to 0.1% (w/v). However, some reports suggest that the presence of BAC in a formulation may induce severe morphological and histological changes, both *in vitro* and *in vivo* (e.g. Berg et al., 1995; Cüreoğlu et al., 2002; Kuboyama et al., 1997; Lebe et al., 2004; Riechelmann et al., 2004). In addition, according to some authors, decongestant nasal sprays with BAC aggravate rhinitis medicamentosa by causing increased swelling of nasal epithelium (Graf, 1999; Graf et al., 1995; Hallén & Graf, 1995). Results from the SMI-tests reveal a concentration response effect, which has also been observed by Berg et al. (1995), Lebe et al. (2004) and Marple et al. (2004). In slugs, high concentrations of BAC result in a very high MP and cause significant tissue damage, while for lower concentrations only an elevated MP is seen, without tissue damage being observed. This increased MP in lower concentrations is presumably induced by a stinging or burning sensation. Riechelmann et al. (2004) evaluated the adverse effects of BAC on human nasal mucosa, both *in vitro* and *in vivo*. *In vitro*, BAC in a concentration of 0.01% and 0.025% appeared to be ciliotoxic for human nasal respiratory epithelia from healthy, non-allergic volunteers. *In vivo* however, BAC did not interfere with nasal mucus transport and did not induce nasal

inflammatory alterations compared to the placebo. However, directly after application, 0.05% BAC caused nasal irritation, hypersecretion and a burning sensation. Moreover, scores for persistent nasal irritation were higher for BAC than the placebo.

In this current study, all tested liquid nasal formulations only demonstrated an effect on the MP, without inducing tissue damage. This increase in MP may be caused by the irritability and toxicity of a certain ingredient on the one hand, as described above for BAC, while on the other hand tonicity of the preparations may play an important role as well. A mild irritating effect in slugs was observed for mometasone furoate (Nasonex®) and fluticasone propionate (Flixonase Aqua®) nasal sprays. Probably these reactions were mainly caused by the presence of BAC in the formulation, since an isotonic 0.02% BAC solution alone induced a comparable reaction in slugs, as was seen for the mometasone furoate and fluticasone propionate nasal sprays. Generally, mometasone furoate and fluticasone propionate nasal sprays are clinically well tolerated (e.g. Bronsky et al., 1997; Grossman et al., 1993; Hebert et al., 1996; Holm et al., 1998; Kerwin et al., 2008; Mandl et al., 1997) with a very low frequency of reported local adverse events, which in most cases was comparable to the control group (Table 6).

Exposure of the slugs to an oxymetazoline HCl spray induced a higher MP than mometasone furoate and fluticasone propionate sprays. Two concentrations of oxymetazoline HCl were tested (0.01% (Nesivine® Baby) and 0.05% (Nesivine®)), with the highest concentration containing BAC as a preservative in an unknown concentration. The slugs' reaction was concentration dependent, and might even be enhanced by the presence of BAC, resulting in a greater MP observed for 0.05% oxymetazoline HCl. These results show that oxymetazoline as such also induces a mildly irritating effect. In literature, the (over)use of oxymetazoline HCl is often described to be related with RM (e.g. Graf & Juto, 1994a, 1994b, 1995). Opinions vary as to how long topical decongestants can safely be used without risking the development of RM (Graf et al., 1999), as the recommended usage of topical decongestants varies globally (Morris et al., 1997). In the current study, a slight time dependent effect of oxymetazoline HCl was observed in the 5-day test. After the fifth CP protein release exceeded 25 µg/ml.g, a phenomenon which has never been observed in slugs treated with PBS or other non-irritating substances. In this context,

a 2- or 3-week lasting SMI-experiment might provide insight in the effect of oxymetazoline HCl on the longer term.

The tested azelastine HCl nasal spray (Allergodil®) induced an elevated MP in slugs, probably caused by the active ingredient itself, since the spray did not contain BAC as a preservative. Generally, azelastine HCl is well tolerated, however some commonly reported adverse events associated with the use of intranasal azelastine include irritation of the nasal mucosa and application site (e.g. Grossman et al., 1994; Lassig et al., 1996; Mösges et al., 1995; Wober et al., 1997), and nasal burning (e.g. Lumry et al., 2007; Newson-Smith et al., 1997) (Table 6). Nevertheless, all clinical trials cited above were done with patients suffering from (seasonal) AR, whose nasal mucosa is already affected, which may also influence the reaction of the patients.

As mentioned before, an increased MP might also be explained as an effect of tonicity. Adriaens & Remon (2008) indicated that an increased osmolality resulted in more irritation in slugs. This is clearly the case for the flunisolide nasal spray (Syntaris® - 1592 ± 28 mOsm/kg). It induced a total MP of 19.7 ± 1.3% in the 5-day protocol and 12.4 ± 1.1% in the 1-day test. There is a strong similarity between these results and those of the tested 5%-NaCl solution (1544 ± 11 mOsm/kg), for which the MP totaled 19.1 ± 3.0% and 12.6 ± 0.7%, for the 5- and 1-day test respectively. These results indicate that we may conclude that the irritation of the flunisolide nasal spray is due to its hypertonicity. Supporting evidence of the effect of hypertonicity is given by the studies of, Adam et al. (1998), Baraniuk et al. (1999), Hauptman & Ryan (2007), Rabago et al. (2002) and Shoseyov et al. (1998) (Table 7). Thus, although generally safe, daily hypertonic nasal irrigation may be associated with some clinically minor side effects.

Conclusion

We can conclude that the SMI-assay is a good tool to predict nasal clinical discomfort. The 5-day testing procedure is able to investigate the effect of a repeated exposure, focusing on both irritation and tissue damage, while the newly developed 1-day procedure is capable to predict the likely occurrence of stinging, itching and/or burning sensations in man, quantified by total mucus production of the slugs. None of the tested marketed nasal formulations appeared to result in tissue damage, although moderate discomfort was observed in some cases. Generally there is a good agreement between the data obtained with the SMI-tests and published clinical data. Both SMI-test procedures gave the same ranking of the tested formulations; however the ranges were smaller, due to a difference in contact period, and consequently the total amount of mucus produced. Active ingredients, preservatives and osmolality appeared to play an important role in the reaction of the slugs.

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Tables

Table 1 Overview of the tested nasal formulations and their ingredients

Product name	Active ingredient	Other ingredients
Nasonex®*[a]	Mometasone furoate (MOMF) 0.05%	colloidal cellulose, glycerol, sodium citrate dehydrate, citric acid monohydrate, polysorbate 80, BAC, purified water
Flixonase Aqua™*[a]	Fluticasone propionate (FP) 0.5%	glucose, microcrystalline cellulose, sodium carboxymethyl-cellulose, polysorbate 80, hydrochloric acid, phenylethyl alcohol, BAC, purified water
Nesivine Baby®[b]	Oxymetazoline hydrochloride (OXY) 0.01%	monobasic sodium phosphate, dibasic sodium phosphate, sodium hydroxide, purified water
Nesivine®*[b]	Oxymetazoline hydrochloride (OXY) 0.05%	sodium edate, BAC, monobasic sodium phosphate, dibasic sodium phosphate, sodium hydroxide, purified water
Allergodil®[b]	Azelastine hydrochloride (AZE) 0.1%	methyl hydroxyl propylcellulose, sodium edate, citric acid, dibasic sodium phosphate, sodium chloride and purified water
Syntaris®*[b]	Flunisolide (FS) 0.025%	citric acid, BAC, butyl hydroxytoluene, polyethylene glycol 400, sodium citrate, sodium edate, polysorbate 20, propylene glycol, sorbitol, purified water

*containing benzalkonium chloride (BAC); [a] suspension; [b] solution

Table 2 Classification criteria for irritation of the total mucus production (expressed as a % of initial body weight) for the 1-day stinging, itching and burning (SIB) protocol and 5-day local tolerance (LT) protocol

1-day SIB	5-day LT	Irritation
< 3%	< 0%	Not
3 – 8%	0 – 5%	Mild
8 – 15%	5 – 10%	Moderate
> 15%	> 10%	Severe

Table 3 Effect of a repeated treatment for 5 successive days with 100 µl of different liquid nasal formulations and control solutions on the endpoints of the Slug Mucosal Irritation (SMI) test

Formulation	Total mucus production (%)	Irritation	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	Tissue damage	Osmolality (mOsm/kg)
PBS[a]	-2.7 ± 1.2	No	9 ± 3	-	0	No	280 ± 1
MOMF 0.05%*[b]	4.3 ± 1.8	Mild	24 ± 10	-	0	No	307 ± 4
FP 0.05%*[b]	4.4 ± 1.1	Mild	12 ± 8	-	0	No	338 ± 1
OXY 0,01%*[a]	6.8 ± 3.4	Moderate	13 ± 5	-	0	No	296 ± 2
OXY 0,05%*[a]	8.4 ± 1.8	Moderate	12 ± 2	-	0	No	296 ± 2
AZE 0.1%[a]	10.8 ± 1.6	Severe	21 ± 15	-	0	No	279 ± 2
FS 0.025%*[a]	19.7 ± 1.3	Severe	16 ± 10	-	0	No	1592 ± 28
1% BAC[a]	28.8 ± 9.8	Severe	113 ± 24	7.9 ± 2.5	1	Severe	291 ± 2

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 5 slugs; - , below the detection limit; PBS: phosphate buffered saline; BAC: benzalkonium chloride; MOMF: mometasone furoate; FP: fluticasone propionate; OXY: oxymetazoline HCl; AZE: azelastine HCl; FS: flunisolide; *: contains BAC; [a]: solution; [b]: suspension

Table 4 Effect of a repeated treatment for 5 successive days with 100 µl of BAC- and NaCl-solutions on the endpoints of the Slug Mucosal Irritation (SMI) test

Formulation	Total mucus production (%)	Irritation	Mean protein release (µg/ml.g)	Mean LDH release (IU/l.g)	Number of slugs with ALP release	Tissue damage	Osmolality (mOsm/kg)
BAC 0,02%	5.1 ± 2.7	Moderate	14 ± 4	-	0	No	279 ± 0
BAC 0,1%	15.2 ± 2.7	Severe	19 ± 17	-	0	No	278 ± 2
BAC 0,5%	32.2 ± 4.2	Severe	114 ± 53	3 ± 2	2	Severe	284 ± 3
BAC 1%	25.1 ± 1.7	Severe	151 ± 41	8 ± 4	2	Severe	291 ± 2
NaCl 0.05%	-10.5 ± 1.7	No	11 ± 5	-	0	No	15 ± 1
NaCl 0.5%	-5.3 ± 1.2	No	14 ± 4	-	0	No	162 ± 1
NaCl 0.9%	-1.8 ± 2.1	No	19 ± 12	-	0	No	288 ± 6
NaCl 2.5%	6.5 ± 1.2	Moderate	18 ± 6	-	0	No	763 ± 7
NaCl 5%	19.1 ± 3.0	Severe	23 ± 11	-	0	No	1544 ± 11
NaCl 10%	37.3 ± 8.5	Severe	24 ± 12	-	0	No	2912 ± 45

Total mucus production, mean protein release and mean LDH release data are presented as the mean ± standard deviation of 5 slugs; -: below detection limit; BAC: benzalkonium chloride

Table 5 Effect of a repeated treatment (3 contact periods of 15 min on the same day) of 6 liquid nasal formulations, 5 physiological saline solutions and several concentrations of BAC and NaCl on the endpoints of the Slug Mucosal Irritation (SMI) test to detect SIB sensations

Product	Total mucus production (%)	Discomfort	Osmolality (mOsm/kg)
PBS[a]	0.0 ± 1.0	No	280 ± 1
BAC 1%*[a]	25.3 ± 3.4	Severe	291 ± 2
MOMF 0.05%*[b]	3.6 ± 1.3	Mild	307 ± 4
FP 0.05%*[b]	4.8 ± 0.1	Mild	338 ± 1
OXY 0,01%*[a]	5.6 ± 0.5	Mild	296 ± 2
OXY 0,05%*[a]	6.0 ± 0.1	Mild	296 ± 2
AZE 0.1%[a]	7.6 ± 0.9	Mild	279 ± 2
FS 0.025%*[a]	12.4 ± 1.1	Moderate	1592 ± 28
Naaprep® (0.9% NaCl[a])	0.4 ± 0.7	No	287 ± 2
Rhina-Care (0.9% NaCl[a])	0.4 ± 0.5	No	297 ± 3
Physiomer® Normal Jet (0.9% NaCl[a])	0.8 ± 1.2	No	326 ± 2
Physiomer® Sinus (2.2% NaCl[a])	3.9 ± 0.2	Mild	638 ± 5
Sinomarin® (2.3% NaCl[a])	4.0 ± 1.0	Mild	726 ± 3
BAC 0,02%*[a]	4.1 ± 0.8	Mild	279 ± 0
BAC 0,1%*[a]	10.8 ± 0.9	Moderate	278 ± 2
BAC 0,5%*[a]	22.9 ± 3.4	Severe	284 ± 3
NaCl 0.05%[a]	-3.7 ± 0.5	No	15 ± 1
NaCl 0.5%[a]	-1.9 ± 0.3	No	162 ± 1
NaCl 0.9%[a]	-0.2 ± 0.5	No	288 ± 6
NaCl 2.5%[a]	3.5 ± 0.5	Mild	763 ± 7
NaCl 5%[a]	12.6 ± 0.7	Moderate	1544 ± 11
NaCl 10%[a]	19.2 ± 2.4	Severe	2912 ± 45

Total mucus production presented as the mean ± standard deviation of 3 slugs; BAC: benzalkonium chloride; MOMF: mometasone furoate; FLP: fluticasone propionate; OXY: oxymetazoline; AZE: azelastine HCl; FLS: flunisolide; *: contains BAC; [a]: solution; [b]: suspension

Table 6 Comparison if irritation categories obtained with the Slug Mucosal Irritation assay (SIB protocol) with clinical data concerning nasal irritation or burning sensation induced by a repeated treatment (1 week up to 1 year) with some liquid nasal formulations

Active ingredient	Discomfort category SMI	Dose	Clinical data		
			Patients with nasal irritation or burning (%)	Patients (n)	Source
Mometasone furoate (MOMF)	0.05% = mild	placebo, 50 µg, 100 µg, 200 µg, 800 µg	2, 5, 4, 3, 4	95, 96, 95, 98, 95	Bronsky et al. (1997)
		placebo, 100 µg, 200 µg	5, 6, 3	123, 126, 126	Hebert et al. (1996)
		placebo, 200 µg	7, 3	184, 181	Mandl et al. (1997)
Fluticasone propionate (FP)	0.05% = mild	placebo, 100 µg, 200 µg	0, 4, 1	85, 84, 81	Grossman et al. (1993)
		placebo, 100 µg bid	57*, 62*	12, 17	Holm et al. (1998)
		placebo, 250 µg	1, 0	212, 212	Kerwin et al. (2008)
		placebo, 200 µg	7, 3	184, 183	Mandl et al. (1997)
Azelastine HCl (AZE)	0.1% = mild	140 µg per nostril bid	8	489	Lassig et al. (1996)
		280 µg per nostril bid (14 days, 31 days)	1.4, 1.2	3680, 4002	Wober et al. (1997)
		placebo, 2 mg bid	84*, 136*	65, 68	Grossman et al. (1994)
		placebo, 137 µg per nostril bid	0, 0.4	278, 276	Lumry et al. (2007)
		placebo, 280 µg per nostril bid	0, 1	77, 83	Newson-Smith et al. (1997)
		1 mg/ml per nostril bid	5.1	119	Mösges et al. (1995)

*: represent total % of all reported adverse experiences

Table 7 Comparison of irritation categories obtained with the Slug Mucosal Irritation assay (SIB protocol) with clinical data concerning nasal irritation or burning sensations or general adverse events induced by a (repeated) treatment (1 day up to 6 months) with liquid nasal saline solutions

Concentration NaCl (%)	Discomfort category SMI	Clinical data		
		Patients with nasal irritation or burning (%) or *general adverse events or observations if not specified otherwise*	Patients (n)	Source
0.9 & 3.5	0.9% = no, 5% = moderate	*0.9% < 3.5%*	15, 15	Shoseyov et al. (1998)
0.9 & 2	0.9% = no, 2.5% = mild	13, 32	31, 33	Adam et al. (1998)
2	2.5% = mild	18	44	Rabago et al.(2002)
0.9 & 3	0.9% = no, 2.5% = mild	*0.9% < 3%*	40, 40	Hauptman & Ryan (2007)
0.9, 2.7, 5.4, 10.8	0.9% = no, 2.5% = mild, 5% = moderate, 10% = severe	*0.9% < 2.7% < 5.4% < 10.8%*	29	Baraniuk et al. (1999)

Figures

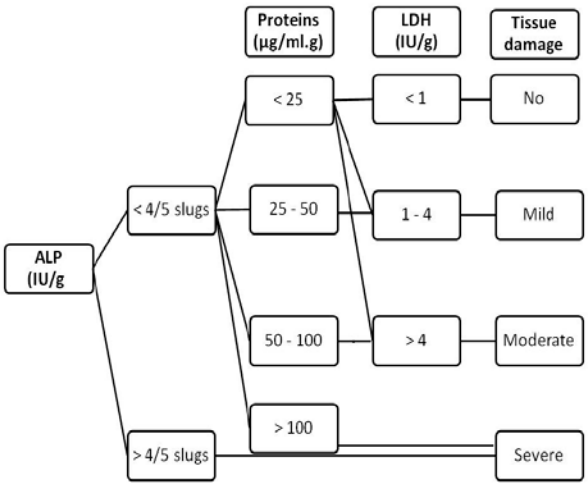

Figure 1 Classification prediction model for 5-day Local Tolerance testing procedure for tissue damage