Scand. J. Lab. Anim. Sci. No. 3 . 1994 . Vol. 21

Local chemical irritation on mucosal surface, measured colorimetrically on the frog palate model

by *Sveinbjörn Gizurarson & Hrund Thorarinsdóttir,*
Department of Pharmacy, University of Iceland and Lyfjathroun hf,
The Icelandic Bio-Pharmaceutical Group, Taeknigardur, Dunhaga 5, IS-107 Reykjavik, Iceland.

Introduction

Investigations have demonstrated that the nasal mucosa might be a potential site for systemic absorption of drugs, as the surface of the mucosa is large and well provided with blood vessels. Products, for systemic drug therapy, utilizing drug absorption from the nasal cavity have already been developed, such as oxytoxin. Other products are still in clinical phases, such as insulin, glucagon and various vaccines. This route of administration requires various excipients, in order to slow down the self-cleaning capacity of the cilia, produce stable and sterile products and to enhance the absorption of molecules across the respiratorial membrane in the nasal cavity. Therefore it is important to evaluate the local toxicity and irritability of nasal drops and sprays early in the development phase. One of the tests, which have been used, is to investigate the effects on the mucociliary clearance apparatus or monitoring the toxic effects on the ciliary beat frequency (*Schipper et al.* 1992, *Batts & Marriott* 1988). Those studies give important information about the local effects or damage to the mucociliary system, the mucus or to the cilia. In previous experiment (*Gizurarson et al.* 1990), the effect of various excipients and absorption promoters were studied and their effect on the mucociliary clearance mechanism. During these experiments reddish colour appeared on the palate in all experiments, where the excipient was found irritating (unpublished results). Since that was not the aim of those studies, no notifications were made. In this present experiment the hypothesis is tested, if there may be direct relationship between the induction of colour on the mucosa and the grade of irritation (based on literature values), by the mean of colorimetric measurements using the frog palate model (*Gizurarson* 1992).

Materials and methods

Reagents and solutions

The control solution for all compounds was phosphate-buffered saline (PBS) prepared in distilled water. The test solutions were prepared by dissolving the required amount of compound in the appropriate control solution. Propylene glycol and sodium chloride were commercially available from Norsk Medisinaldepot (Oslo, Norway), tetraethyleneglycol was commercially available from Fluka Chemical Company (Buchs, Switzerland), glycofurolum was kindly provided by Hoffman-La Roche (Basle, Switzerland), sodium taurodihydrofusidate was kindly provided by Leo (Ballerup, Denmark), sodium chenodeoxycholate and cholera toxin B subunit were commercially available from Sigma Chemical Company (St. Louis, USA). Solutions, having pH between 0–14, were produced by adding either hydrochloric acid or sodium hydroxide, commercially available from Merck (Darmstadt, Germany), to isotonic saline and adjusted to appropriate pH by the mean of a pH-meter (Radiometer, Copenhagen, Denmark).

Method

The experimental arrangement for this experiment was similar as described in *Gizurarson* (1992). The frog (*Xenopus laevis*) was beheaded, the upper palate was exposed and introduced into a transparent chamber maintained at average room temperature with relative humidity of about 100 %. The palate surface was observed through a Nikon Type

102 stereo-microscope (Nikon Europe B. V. Badhoevedorp, The Netherlands), at 25 × magnification equipped with camera (Nikon FM, Nikon Europe B. V.).

Before each experiment, a control value (5 frogs were used in each experimental setup) was obtained for each frog palate, by applying 0.1 ml of PBS to the palate and leaving it in contact for about one minute before draining off. Hereafter the test substances was administered to the palate and left in contact for about one minute and drained off. Photographs were taken prior to each experiment after the administration of PBS, and after the administration of the test substances. During the experiments the palate was exposed with fixed light intensity (approx. 10 Mlx) using Schott KL1500 cold light source equipped with BG37 blue filter from Schott Glaswere (Wiesbaden, Germany).

Each film was developed identically and the intensity in colour on each picture was measured colorimetrically by the mean of a Gretag D186 Colorimeter (Gretag Ltd., Regensdorf, Switzerland), as the average intensity of four different colours: yellow, black, magneta and cyan. The colour was measured on three well defined areas (7.1 mm^2), on the anterior palate, anterior palate vein and on the right protrusion of eyeballs.

To evaluate the influence of each test substance, each frog was used as its own control. Calculations were performed according to standard statistical methods.

Results and discussion

Colours developed on mucosal surfaces, during the experiments, were either reddish due to irritation and increased blood circulation, dark red/brown due to cellular damage causing increased blood flow or pale or light due to a direct effect on the mucus, probably due to cross-binding of the mucoproteins and/or increased mucus secretion.

Fig. 1 shows the effect of pH on mucosal surface. The figure show that pH below 2 and above 12 caused significant colour changes in the mucosa, but between pH 2–12 no significant changes were measurable. The source for various pH were either hydrochloric acid or sodium hydroxide, adjusted to the specific pH. One factor, the type and character of the acid/base used, was not studied, but may have caused different pH-effect relationship such as damage below 4 or above 10. The effect of other substances, such as absorption promoters, may also depend on the surrounding pH. pH has been described as one of the influencing factors for successful absorption of drugs, as well as for the sense of irritation (*Hirai et al.* 1981).

In Fig. 2 the osmolality versus colour increment is drawn up, using only sodium chloride as a source for osmotic pressure. Low

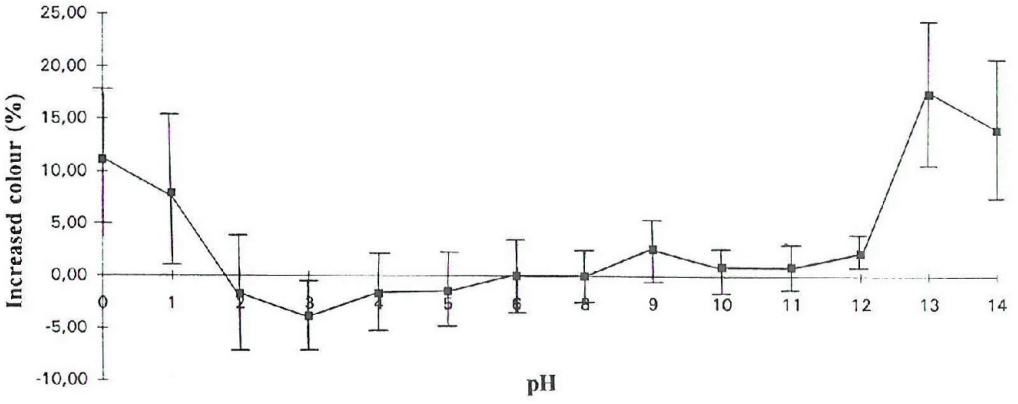

Figure 1. Average colour increasment and S.D. for different pH values. Measured on the frog palate model.

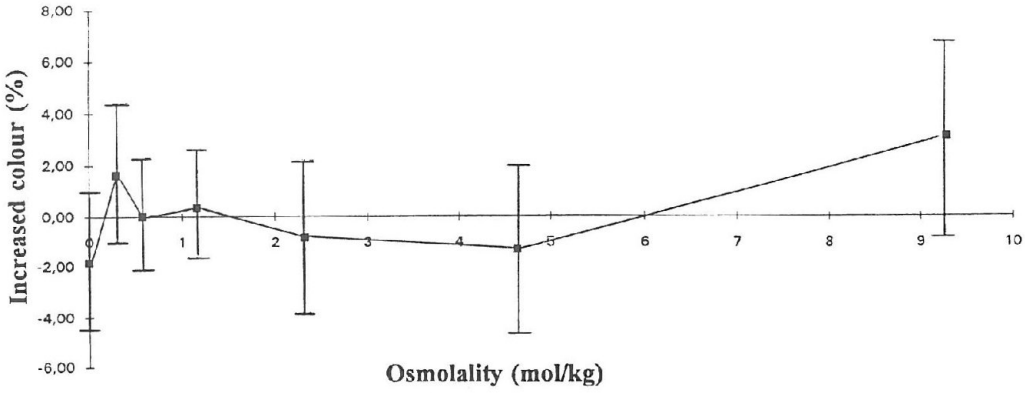

Figure 2. Average colour increasment and S.D. for different osmolality (mol/kg) values. Measured on the frog palate model.

values (below ÷) may indicate that the vehicle was irritating, causing increased mucus secretion, which is seen as decrease in the colour intensity. On the other hand, vehicles with high osmotic pressure may be damaging, causing increased blood flow which result in increased colour intensity. That would also be expected from hypoosmotic vehicles. Great variation was seen within all data, resulting in no significance at all. As for pH, other substances than sodium chloride, may affect the osmotic pressure in a different manner. Each substance or formulation should be studied individually for irritation and toxicity e.g. by the mean of combined colorimetric and mucociliary clearance study. *Ohwaki et al.* (1989) describes that the maximum absorption through the nasal epithelial membrane (*in vitro*) was observed at a sodium chloride concentration of 0.87 mol/kg, where e.g. the local irritation and mucus secretion is minimal. In another study, *Tengamnuay & Mitra* (1990) showed that by changing the ionic strenght of solutions, they were able to increase the absorption enhancing effects of bile salt-fatty acid mixed micelles.

Table 1 show that substances, which are known to be irritating, such as pure propylene glycol, pure glycofurolum, sodium taurodihydrofusidate and sodium chenodeoxycholate were able to increase the colour density, significantly, on the mucosal surface, changing from pale yellow to light or even dark red colour. Other substances, such as cholera toxin B subunit, which also is known to be irritating and causes increase in nasal secretion when administered intranasally (unpublished results), coused decrease in the colour intensity, probably by the mean of increasing the mucus secretion.

Photographs and measurements of the colours intesity may be a helpful tool to evaluate the irritating effect of various substances on biological tissues. The investigator

Table 1. Analysis of the effect of different excipients on the increased colour formation, when mucosal membrane, in the frog palate system, is exposed to these components.

Substances	% Increment
PBS, isotonic (as reference)	0.0.
Excipient	
Propylene glycol (pure)	7.1 ± 5.2
Glycofurolum (pure)	3.9 ± 8.8
Enhancers	
Sodium taurodihydrofusidate (10 mg/ml)	8.9 ± 4.1
Sodium chenodeoxycholate (10 mg/ml)	10.2 ± 2.1
Toxins	
Cholera toxin B subunit (20 µg/ml)	– 5.1 ± 6.6

will be able to digitalize the change in colour intensity instead of objective evaluation with scores. This study shows that colorimetric measurement may be used together with other experiments, to evaluate the effect of chemical substances, excipients or vehicle systems on biological surface. In diagnostic cytology the investigator need to evaluate the mucosa objectively, ranging from pink or pale to red or blue purplish (*Jalowayski* 1991). In such studies, each investigator may see different colours differently, where pictures may be used to evaluate the density of colours with more accuracy.

Acknowledgements
Dept. of Bacterial Vaccines, State Seruminstitute (Copenhagen, Denmark) is kindly thanked for providing the frogs, Dept. of microbiology, Institute for biology, University of Iceland for providing the microscope, Mr. Sigfús Bl. Cassata in Focus hf, Reykjavik, Iceland for providing the camera, Morgunbladid for providing the colorimeter and Students Innovation Fund is thanked for their support.

Summary
The effect of chemicals, toxins, allergens etc. on biological tissues are usually evaluated in microscopes as well as objective by giving different scores for different signs. When visual evaluation is used, the investigations may see each colour or each sign in a different manner. Experiments were conducted in order to evaluate the possibility of measuring the irritation and mucus secretion caused by irritation by the mean of digitalizing the colour density in the palatal mucosal membrane of the frog, using a colorimeter. Increased redness on the mucosal surface was seen as increased colour density, giving high values, whereas increased mucus secretion resulted in decrease in colour density, giving lower values than seen for normal mucosa.

Resumé
Påvirkning af kemiske substanser, toxiner, allergener o.s.v. på biologisk væv er normalt evalueret mikroskopisk såvel som objektivt, hvor der gives forskellige scores for forskellige tegn. Når visuel evaluering bruges, kan hver farve eller tegn beskrives på forskellig vis, afhængig af forskeren. Et forsøg er blevet udført for at undersøge muligheden for at måle farveintensiteten på frøganer ved hjælp af en farvemåler. På den måde kan irritation eller øget slimproduktion forårsaget af irritation digitaliseres. Irritation i form af øget rødme på slimhinden kunne ses som stigning i farveintensiteten, som i forsøget gav høje værdier. Derimod gav øget slimproduktion et fald i farveintensiteten, hvor de målte værdier lå under det, som kunne ses for normale slimhinder.

Ágrip
Áhrif efna, eiturefna, ofnæmisvaldandi efna o.fl. á lifandi vefi eru yfirleitt skoðuð míkróskópiskt með aðstoðar smásja eða i formi hlutdrægs mats, þar sem gefnar eru einkunnir fyrir viss einkenni. Þegar vísinðamaður á að leggja mat á einhver áhrif, fer þaðeftir því hver á í hlut, hvernig þau eru skráð Nokker tilraunir hafa verið framkvæmdar til þess að kanna hvort hægt sé að mæla ertingu eða aukna slímmyndun af völdum ertingu, á gómum froska, með aðstoðar litgreinis. Erting i formi aukins roða mátti sjá í auknum litastyrk, sem jafnframt gaf há tölugildi, en erting í formi aukinnar slímmyndunar, lýsti sér í litastyrksfalli.

References
Batts, AH & C Marriott: The preservation of nasal preparations. Int. Biodeterioration 1988, *24*, 7–15.

Gizurarson S, C Marriott, GP Martin & E Bechgaard: The influence of insulin and some excipients used in nasal insulin preparations on mucociliary clearance. Int. J. Pharm. 1990, *65*, 243–247.

Gizurarson S: In situ method to estimate local effect of nasal preparations. Scand. J. Lab. Anim. 1992, *19*, 129–135.

Hirai S, T Ikenaga & T Matsuzawa: Nasal absorption of insulin in dogs. Diabetes 1981, *27*, 296–299.

Jalowayski AA: A procedureal guide for diagnostic nasal cytology. Booklet from Synbiotics Corporation, San Diego, California, USA, 1991, p. 7.

Ohwaki T, M Ishii, S Aoki, K Tatsuishi & M Kayano: Effect of dose, pH and osmolarity on nasal absorption of secretin in rats. III. *In Vitro* membrane permeation test and determination of apparent partition coefficient of secretin. Chem. Pharm. Bull. 1989, *37*, 3359–3362.

Schipper NGM, J Verhoef, SG Romeijn & FWH M Merkus: Absorption enhancers in nasal insulin delivery and their influence on nasal ciliary functioning. J. Contr. Rel. 1992, *21*, 173–186.

Tengamnuay P & AK Mitra: Bile salt-fatty acid mixed micelles as nasal absorption promoters of peptides. I. Effects of ionic strenght, adjuvant composition and lipid structure on the nasal absorption of [D-Arg^2]kyotorphin. Pharm. Res. 1990, *7*, 127–1133.