

Using the Slug Mucosal Irritation assay to investigate the tolerability of tablet excipients on human skin in the context of the use of a nipple shield delivery system

Tolerability of tablet excipients on the nipple

Richard Kendall^{1,2*}, Joke Lenoir³, Stephen Gerrard⁴, Rebekah L. Scheuerle⁴, Nigel K.H. Slater⁴, Catherine Tuleu¹

¹ School of Pharmacy, University College London, London, UK

² School of Pharmacy, Keele University, Keele, UK

³ Faculty of pharmaceutical sciences University of Ghent, Gent, Belgium

⁴ Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK

* corresponding author: Dr Richard Kendall, Lecturer in Pharmaceutics, School of Pharmacy [HNB 2.26] Keele University, Staffordshire ST5 5BG, UK. Tel +441782 733877; Fax +441782 733326; r.a.kendall@keele.ac.uk

Purpose

Neonates are particularly challenging to treat. Recently, a novel and patented drug delivery device containing a rapidly disintegrating tablet held within a modified nipple shield (NSDS) was designed to deliver medication to infants during breastfeeding. In the present study an *in vivo* Slug Mucosal Irritation (SMI) assay, previously validated for prediction of ocular and nasal irritation, was used as an alternative to vertebrate models to anticipate the potential maternal dermatological tolerability issues to NSDS tablet components.

Methods

This first SMI study evaluated excipients used in solid oral dosage forms. Zinc sulphate was selected as the model drug, for its ability to reduce infant mortality from diarrhoeal disease. It was blended with GRAS excipients commonly used to manufacture rapidly disintegrating solid oral dosage forms. Slugs were exposed to blends slurried in human breast milk to assess their stinging, itching or burning potential, using objective values such as mucus production to categorize irritation potency

Results

There was no irritancy detected for any of the excipients tested, however, mild irritancy was observed when excipients were combined with zinc sulphate.

Conclusion

These promising results provide evidence to support the continued investigation of the tested excipients within NSDS rapidly disintegrating tablet formulations.

198 words

KEY WORDS:

Nipple Shield Delivery System, Slug Mucosal Irritation Assay, Skin tolerability, tablet excipients, pediatric

ABBREVIATIONS

API: Active Pharmaceutical Ingredient

BAC: Benzalkonium Chloride

CP: Contact Period

GRAS: Generally Recognised as Safe

HIV: Human Immunodeficiency Virus

HLTV: human T-lymphotropic virus

HBM: human breast milk

MP: Mucus Production

NC: Negative control

NSDS: Nipple Shield Delivery System

PC: positive control

REC: Research ethic committee

PBS: phosphate buffered saline

SIB: Stinging, itching or burning

SMI: Slug Mucosal Irritation

INTRODUCTION

Identifying the paediatric drug product technology gap

The development of age appropriate medicines which deliver an active pharmaceutical ingredient (API) to children at the required rate and extent is a complex process. Neonates are a particularly challenging sub-population to address

for formulation scientists due to issues including dysphagia, taste aversion, and the need for frequent dose modifications. (1)

Liquid formulations have been typically the dosage forms of choice for paediatric drug administration, but are often not practical in developing countries because of high cost, lack of access to refrigeration, contamination issues and limited shelf life. (2, 3, 4) They may also be unpalatable and contain undesirable or unsafe preservatives and solvents. (5) Solid oral dosage forms for infants are often scaled down from adult doses, and there is currently a debate on the limitations of clinical work performed to demonstrate suitability of the dose to the infant. (6, 7) Dispersible tablets can also be used, but require clean sources of water for reconstitution. They also require administration devices for which the volume of reconstituted suspension is calculated based upon body weight, body surface area or age, depending on the therapeutic index of the drug. However there is currently a WHO recommendation if not push for solid oral dosage forms to maximise drug product stability, particularly in developing countries. (8)

A major contributor to this unacceptable statistic is diarrhoeal disease, the second leading cause of mortality in this age group, being responsible for 760,000 deaths yearly. (9) A significant proportion of the 1.7 billion annual cases of diarrhoeal disease reported globally could be prevented through ready access to clean drinking water alongside better sanitation and hygiene, both of which also limit the utility of medicines designed to be administered post reconstitution in drinking water.

A potential solution to the paediatric drug product technology gap in neonates

A novel Nipple Shield Delivery System (NSDS) has been proposed as a means to address some of these challenges. (10) This thin disposable device (Figure 1) adapted from an existing nipple shield breastfeeding aid (11) to contain a fast dispersible tablet, is placed over the mother's breast just before infant feeding. When the human breast milk (HBM) passes through the device it releases an API to the infant via the milk. Based on patient need, a wide-range of APIs could be delivered to infants using the NSDS, such as antibiotics, antivirals, antimalarials, vitamins, nutrients, and probiotics. The APIs, which are stored in a dry form prior to reconstitution in HBM during administration, could therefore have longer shelf lives than other dosage forms.

The NSDS has the potential to compliment dispersible tablet use in environments where infants breastfeed exclusively and remove many of the issues which can cause contamination of reconstituted drugs delivered to infants in resource-limited countries. More than 5.9 million children under 5 years of age died in 2015. Many of these could have been prevented with access to appropriate forms of simple and affordable medicines and corresponding hygienic administration methods. (12)

Zinc supplements, containing zinc sulphate have been shown to reduce the duration of diarrhoea episodes by 25% and are also associated with a 30% reduction in stool volume (9). Zinc supplements are available in developing countries but require cup, bottle, or spoon delivery of the reconstituted suspension, limiting breastfeeding infant acceptability. Zinc sulphate, for which concentrations in HBM have not been conclusively shown to be impacted by maternal supplementation (13), constitutes therefore a relevant and important API to be delivered directly to neonates in an age appropriate manner.

One safety consideration raised and addressed in this work, is the potential maternal tolerability of a modified NSDS. Specifically assessment of dermatological impacts due to potential irritancy of a concentrated suspension/slurry of APIs or excipients in HBM on the mother's nipples is required. Presently the SMI assay was identified and explored as an *in vivo* assessment tool for this novel application. It was initially developed at the Laboratory of Pharmaceutical Technology at the University of Ghent to predict the mucosal irritation potency of pharmaceutical formulations and ingredients. (14, 15) The premise of the test is that slugs that are placed on an irritating substance will produce mucus. Tissue damage can be induced which results in the release of proteins and enzymes from the mucosal surface. Several studies have shown that the SMI assay is a useful tool for evaluating the local tolerance of pharmaceutical formulations and ingredients. (15, 16, 17, 18, 19, 20, 21, 22) A classification prediction model that distinguishes between irritation (mucus production) and tissue damage (release of proteins and enzymes) has been developed. The SMI study is proposed more acceptable and ethical in terms of the principles of reduction, refinement and replacement, compared to previous tests such as the Draize test (23), an invasive procedure which involves applying relatively large volumes (0.5 mL or 0.5 grams of a test substance to the eye or skin of a restrained, conscious mammal (usually a rabbit) and recording its effects. It is a simple yet efficient way, to assess mucosal tissue irritation without using large numbers of vertebrates such as mice, rabbits or non-human primates or using more complex reconstructed human epidermis 3D skin models.

The relevance of the SMI assay to reliably predict nasal irritation, stinging and burning sensations has been demonstrated in a clinical trial using several Over the Counter (OTC) liquid nasal formulations, isotonic, and hypertonic saline. (24) It has

also been shown that an increased mucus production with exposure to diluted shampoos was related with an increased incidence of stinging and burning sensations in the human eye irritation test. (25) The objective values obtained by means of the predictive SMI model for the mucus production, stinging, itching, or burning potential of the test blends can be estimated according to four categories (none, mild, moderate and severe). The limits for degree of discomfort on nasal and ocular mucosal surfaces are summarized in Table 1 (24, 25)

The purpose of the present study was to attempt to predict the stinging, itching or burning potential of a range of GRAS powder blends on the human nipple, some of which contain the model compound zinc sulphate, used in the treatment of diarrheal disease to support the development of a rapidly disintegrating tablet.

MATERIALS AND METHODS

Methods

Slugs

The parental slugs of *Arion lusitanicus* were collected in local gardens along Ghent and Aalter (Belgium) and bred in an acclimatized room at 18 - 20 °C. Test slugs were housed in plastic containers and fed with lettuce, cucumber, carrots, and commercial dog food. Slugs weighing between 3 and 6 g were isolated from the cultures two days before the start of an experiment. The body wall was inspected carefully for evidence of macroscopic injuries. Only slugs with clear tubercles and with a foot surface that showed no evidence of injuries were used for testing purposes. The slugs were placed in a plastic box lined with paper towel moistened with phosphate buffered saline (PBS) (0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride) (Sigma Aldrich, St. Louis, MO, USA) at pH 7.4 and were

kept at 18 – 20 °C. Daily, the body wall of the slugs was wetted with 300 µl PBS using a micropipette.

Human breast milk

Anonymised HBM samples were obtained from approximately 20 healthy donors from the Queen Charlotte's and Chelsea Hospital Milk Bank (Imperial College Healthcare NHS Trust). The donors had all consented for their milk to be used for research as it was not able to be used for donation. They were screened for HIV 1 and 2, HTLV I and II, hepatitis B and C, and syphilis, and ethical approval for use was obtained from the University of Cambridge (Cambridge Human Biology Research Ethics Committee (REC), University of Cambridge (REC number HBREC.2012.01). Milk from 10 donors was pooled for the experiment, half of which was centrifuged at 5411 g (5500 RPM) using a Sigma 3 –16 PK centrifuge (Sigma–Zentrifugen, Osterode, Germany) for 15 min. A fat layer obtained at the top of the flask was then carefully removed using a curved face spatula, and the remaining milk was pooled into a single flask. This fat layer, fat-free milk layer and the milk not centrifuged were then combined in this study to achieve a standardised fat content of 4.6 % wt. Samples were then placed in 50 mL centrifuge tubes and stored at –80 °C to be used thawed prior making the slurries.

Test slurries

The following materials were sourced for the manufacture of blends A - I: zinc sulphate monohydrate (Sigma, Sigma-Aldrich, Dorset, UK); lactose monohydrate (direct compression grade, DFE Pharma Goch, Germany); sodium starch glycolate (Explotab, Mendell GmbH Volklingen, Germany); sodium croscarmellose (FMC Biopolymer, Girvan, UK); microcrystalline cellulose (MCC) (Avicel PH102, FMC Biopolymer, UK); magnesium stearate (Sigma-Aldrich, Dorset, UK); crospovidone

(Polypasdone XL, Ashland, UK) and sodium stearyl fumarate (Alubra PG-100, FMC Biopolymer, UK).

The following 330 mg blends, corresponding to the composition of one NSDS tablet containing 20 mg elemental zinc (6% w/w) based on blend A and I, detailed in Table 2, were prepared by hand filling the individual components in to 6.5 mL glass scintillation vials followed by blending in a Turbula mixer at 44 rpm for 5 minutes. Blend A was comprised of a mixture of zinc sulphate and lactose (filler or bulking agent) in combination with functional excipients at levels commonly used in the formulation design of a rapidly disintegrating immediate release solid oral dosage form, namely: microcrystalline cellulose (compression aid), sodium starch glycolate (disintegrant), croscarmellose sodium (disintegrant) and magnesium stearate (lubricant). The composition of the blends E, F, G and H were chosen based on variations of the lead platform formulation (A) to comprise lactose and each of the functional excipients in blend A individually. An alternative disintegrant (crospovidone, blend B) and lubricant (sodium stearyl fumarate, blend C) were also evaluated. Blends D and I acted as controls for zinc sulphate, and comprised lactose with all of the functional excipients and zinc sulphate respectively. The blends were reconstituted into an homogenous slurry with the aid of vortex mixing until visually suspended, using 1 mL HBM. This volume was chosen to mimic a scenario where burst release of the API and excipients occurred, thus yielding potentially a worst case concentration for the compounds. The final slurries were designed to represent the most concentrated suspension that would be in contact with the mother's nipple assuming near-instant disintegration of the tablet during breastfeeding.

Methods

The SMI assay

Before a test was considered valid, the following criteria was met: the negative control (PBS) generated a total mucus production less than 5.5% of initial body weight to be classified as causing no stinging, itching, or burning; the positive control (1 w/v% benzalkonium chloride (BAC) (Sigma Aldrich, St. Louis, MO, USA)) in PBS generated a total mucus production above 17.5% of initial body weight to be classified as causing severe stinging, itching, and burning.

The stinging, itching, or burning (SIB) potency of the test items and the negative and positive controls were evaluated by placing 3 slugs per treatment group 3 times a day on 100 µl of test slurry in a Petri dish. After each 15-min contact period (CP) the slugs were transferred for 60 min into a fresh Petri dish on paper towel moistened with 1 ml PBS to prevent desiccation. After the third CP the slugs were placed in a Petri dish on a membrane filter (cellulose acetate 0.45 µm, 90 mm diameter, Sartorius AG, Goettingen, Germany) moistened with 2 ml PBS until the next day. The overview of the test procedure is illustrated in figure 2.

The amount of mucus produced during each contact period was measured by weighing the Petri dishes with the test item before and after each 15 minute CP. The mucus production was expressed as the % of the body weight. The slugs were weighed before and after each 15 minute CP and 24 hours after the first CP. The total mucus was calculated for each slug and then the mean per treatment group was calculated.

Based on the endpoints of the SMI assay the stinging, itching, or burning potency of the test item(s), as defined in Table 1, was estimated using a classification prediction

model. Mortality was documented for slugs exposed to each of the sample slurries, including the controls and HBM 24 hours after the third CP.

RESULTS AND DISCUSSION

The primary aim of this research was to evaluate the potential of commonly used tablet excipients and zinc sulphate used as an antidiarrheal model compound to cause irritation of the human nipple through extrapolation of the SMI assay. This utilizes the terrestrial slug *Arion lusitanicus*. The body wall of the slugs is layered and has a mucosal surface. The outer single-layered columnar epithelium contains cells with cilia and micro-villi. Mucus secreting cells cover the subepithelial connective tissue. This micro-anatomy is similar to that of the lactiferous ducts of the mother's nipple which are lined by a columnar epithelium supported by myoepithelial cells. Hence the SMI was hypothesized to be a potential predictive model for nipple irritancy during drug administration via the NSDS. It was assumed that the results of this study would represent a worst case assessment for irritation, since ocular/nasal surfaces are more sensitive than skin.

The average amount of mucus produced during each 15 minute CP and total MP is presented in Table 3, Figure 3..

According to the classification prediction model of the SMI test, the negative control (PBS) did not induce reactions in the slugs (total MP < 3-5.5%). The positive control on the other hand (BAC 1% w/v) induced, as expected, a high mucus production during each contact period (total MP \geq 17.5%) resulting in a classification corresponding to severe stinging, itching, and burning (SIB) reactions. The acceptance criteria were met and the experiment was considered valid. Graphical summaries of the data are presented in Figures 3 and 4.

The total mucus production values for the reconstituted blends are bracketed by the positive and negative controls. Slugs treated with HBM did not produce an increased amount of mucus compared to the negative control. Similarly the blends that did not contain zinc sulphate did not increase mucus to production to a level at which it could be classified as “mildly irritant” (Table 1), with the exception of blend H containing lactose and magnesium stearate, which only just exceeded by 0.1% the limit for “no irritation” as classified using the ocular classification only (Table 1 and Table 2). . The total MP for HBM is only slightly negative, and it is therefore concluded that HBM was tolerated very well, resulting in a classification as not causing discomfort. The negative MP been induced ($< -0.7\%$) may have also resulted in tissue damage, but this was not the case.

Slugs treated with blends B, C, D, E, F, and G produced only a slightly increased amount of mucus during each contact period, compared to the negative control. All placebo blends resulted in a total MP <3 and 5.5% , corresponding with the classification “no SIB reactions”, suggesting acceptable tolerability. For all placebo blends the first contact induced the highest MP, but was much lower during the second and third CP. The slightly negative mucus production that was observed for the negative control in the third CP was hypothesised to be due to the fact that the slugs produced only a minimal amount of mucus during each contact period and also that only a minimal amount of the test substance remains on the body wall of the slugs.

Slugs treated with blends A and I, both containing the API zinc sulphate at a concentration of approximately 6% w/w, produced a higher amount of mucus during each CP in comparison with the negative control and all other blends tested. For blend A, the total MP also increased for the first two CPs; in the third CP it was lower

than during the first CP. For blend I, MP was comparable during the first and second CPs, but increased substantially during the third contact period. This is however within the accepted limits. Similar reactions were observed in other experiments, where there is a certain tolerance for the the first two CPs, and then an overreaction in the third CP. Although it can be interesting to look at the results of the three CPs separately, it is the total MP over the three CPs together that is used for the classification, as there can be quite some variability in the slugs' reactions. Both blends induced a total MP between 10% and 17.5% and were therefore classified as causing moderate SIB reactions.

With an n of 3 for each of the reconstituted slurries, considered alongside formulation (e.g. suspension homogeneity, which was not evaluated) and *in vivo* variability, it is not possible to assign statistical similarities or differences between the formulations in terms of mucus production, with a large degree of confidence. However, the data suggests that the zinc sulphate is the major contributing factor to mucosal irritancy, and that the lack of response to the GRAS excipients evaluated promotes a large formulation design space. It is to be noted that the concentrations tested represent a higher concentration than would be expected to be released using the NSDS, since it would likely take more than 1 mL of HBM to disintegrate a tablet during use. (10) Therefore, zinc sulfate may not cause as much irritancy as implied by this worst case study.

No mortality was observed immediately following the three CPs, however 24 hours after the final CP, two out of three slugs had died in the cohort exposed to the positive controls as well as formulations A and I (both containing zinc sulphate). The observed mortality in the slugs exposed to the positive control, was not surprising. Slugs treated with benzalkonium chloride using a similar protocol (30 min exposure

to irritant each day for 5 days), often indicate tissue damage after the first contact period. (15, 16) This damage accumulates over time, inducing mortality of the slugs. In the current study, damage also occurred after exposure to benzalkonium chloride, although this was not quantified using microscopy, resulting in the observed mortality.

Further, zinc salts (including sulphate) have been previously demonstrated to be highly toxic to freshwater fish and invertebrates and metal sulphates have been incorporated into proprietary slug and snail control products. (26) Exposure to elemental zinc in food (albeit at much higher concentrations) has been shown to have negative developmental impact on slugs. (27, 28)

The relationship between API chemical structure and the endpoints mortality and MP, has previously been studied for the antimicrobial agent, benzalkonium chloride, and it was concluded that the activity and toxicity of different analogues depend on the alkyl chain length of the bactericidal molecule. (16) The slug mortality and increase in MP of the slugs exposed to slurries containing zinc sulphate appears to be due to the API and possibly related to its chemical structure; further investigations around API structure / SMI assay activity may help to better validate the model for different applications. While the SMI data generated in this study suggest a possibility of local irritation on the human skin, the dermatological impact may be less than implied with this relatively non-invasive testing method, when nasal and ocular classification scales are used, as these are likely to be more sensitive than human skin.

Further validation of the model is required to build an *in vivo* / *in vivo* correlation between the SMI model and irritancy of the breastfeeding nipple during administration of a rapidly disintegrating immediate release tablet via the NSDS.

Such a correlation would be a useful pre-formulation tool to assess risk during early formulation development. Additional opportunities to improve the predictiveness of the model for the selection of APIs for use with the NSDS include gaining a better understanding of similarities and differences of the histology of the breastfeeding nipple and the slug mucosa, considering species other than *Arion lusitanicus* for the assay, and essentially, optimising exposure time of the slugs to the concentrated slurries to correlate to an average breast feed. Since the average HBM mass delivered per feed is estimated to be 50-80g of milk over a 7 to 10 minute period, the study of the tablet components at lower concentrations, correlating to slower release from the NSDS could be tested for irritancy potential. (29, 30, 31)

CONCLUSIONS

This was the first SMI study to be conducted evaluating excipients used in the manufacture of solid oral dosage forms, slurried in HBM. The existing SMI model has indicated a potential for local dermatological irritation when zinc sulphate at a potentially worst case concentration is delivered via the NSDS, with further investigation warranted. Mild irritation is suggested by the change in MP, and body weight of the slugs exposed to slurries of lactose and functional GRAS excipients that are used to induce rapid release of API from solid oral dosage forms. This implies these excipients could be used within functional levels as part of a flexible formulation design space that could facilitate the incorporation of a wide range of non-irritant APIs, with different physicochemical properties, into tablets for use with the NSDS. This could potentially broaden the utility of this novel drug delivery platform for the clinical treatment of neonates.

ACKNOWLEDGEMENTS AND DISCLOSURES

This work was made possible through the support of the Saving Lives at Birth partners: the United States Agency for International Development (USAID), the Government of Norway, the Bill & Melinda Gates Foundation, Grand Challenges Canada, and the UK Department for International Development (DFID). Additional support was provided by the Gates Cambridge Trust.

Many thanks go to Gillian Weaver, manager of the Queen Charlotte's and Chelsea Hospital Milk Bank (Imperial College Healthcare NHS Trust) for coordinating access to the HBM samples. These samples were provided by the Imperial College Healthcare NHS Trust Tissue Bank. Other investigators may have received samples from these same tissues. The research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Stephen Gerrard is an inventor of the nipple shield delivery system (US patent 8357117 B2. See <http://justmilk.org>).

REFERENCES

1. Samardzic J, Turner MA, Bax R, Allegaert K. Neonatal medicines research: challenges and opportunities. Expert opinion on drug metabolism & toxicology. 2015 Jul 3;11(7):1041-52.
2. Beggs SA, Cranswick NE, Reed MD. Improving drug use for children in the developing world. Archives of disease in childhood. 2005 Oct 1;90(10):1091-3.

3. UNICEF, WHO. 2010. Sources and prices of selected medicines for children. Including therapeutic food, dietary vitamin and mineral supplementation. 2nd ed. April 2010 Accessed 2016 March 03 .Available from http://www.who.int/medicines/publications/essentialmedicines/Sources_Prices_2010.pdf
4. WHO. 2007. Promoting safety of medicines for children. Accessed 2016 March 3. Available from: http://www.who.int/medicines/publications/essentialmedicines/Promotion_safe_med_childrens.pdf
5. Walsh J, Cram A, Woertz K, Breikreutz J, Winzenburg G, Turner R, Tuleu C.. Playing hide and seek with poorly tasting paediatric medicines: do not forget the excipients. *Advanced drug delivery reviews*. 2014 Jun 30;73:14-33.
6. Pandolfini C, Bonati M. A literature review on off-label drug use in children. *European journal of pediatrics*. 2005 Sep 1;164(9):552-8.
7. Stoltenberg I, Winzenburg G, Breikreutz J. Solid oral dosage forms for children—formulations, excipients and acceptance issues. *J. Appl. Ther. Res*. 2010;7:141-6.
8. WHO, Revised WHO Classification and Treatment of Pneumonia in Children at Health Facilities: Evidence Summaries. 2014. Accessed 2016 March 3. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK264163/>
9. WHO. Diarrhoeal disease. Fact sheet N°330, April 2013 2016 March 3. Available from: <http://www.who.int/mediacentre/factsheets/fs330/en/>
10. Gerrard SE, Orlu-Gul M, Tuleu C, Slater NK. Modelling the physiological factors that affect drug delivery from a nipple shield delivery system to

- breastfeeding infants. Journal of pharmaceutical sciences. 2013 Oct 1;102(10):3773-83.
11. JustMilk (2015), Last Accessed 2016 March 3. Available from: <http://www.justmilk.org/>
 12. WHO, 2015. Global Health Observatory (GHO) data on Under-five. Accessed 2016 March 3. Available from http://www.who.int/gho/child_health/mortality/mortality_under_five_text/en/
 13. Emmett PM, Rogers IS. Properties of human milk and their relationship with maternal nutrition. Early human development. 1997 Oct 29;49:S7-28.
 14. Adriaens E, Bytheway H, De Wever B, Eschrich D, Guest R, Hansen E, Vanparys P, Schoeters G, Warren N, Weltens R, Whittingham A. Successful prevalidation of the slug mucosal irritation test to assess the eye irritation potency of chemicals. Toxicology in Vitro. 2008 Aug 31;22(5):1285-96.
 15. Adriaens E and Remon JP. 1999. Gastropods as an evaluation tool for screening the irritating potency of absorption enhancers and drugs. *Pharm. Res.* 16: 1240-1244
 16. Adriaens E, Dierckens K, Bauters TG, Nelis HJ, Van Goethem F, Vanparys P, Remon JP. The mucosal toxicity of different benzalkonium chloride analogues evaluated with an alternative test using slugs. Pharmaceutical research. 2001 Jul 1;18(7):937-42.
 17. Adriaens E, Ameye D, Dhondt MM, Foreman P, Remon JP. Evaluation of the mucosal irritation potency of co-spray dried Amioca®/poly (acrylic acid) and Amioca®/Carbopol® 974P mixtures. Journal of controlled release. 2003 Mar 26;88(3):393-9.

18. Callens C, Adriaens E, Dierckens K, Remon JP. Toxicological evaluation of a bioadhesive nasal powder containing a starch and Carbopol® 974 P on rabbit nasal mucosa and slug mucosa. *Journal of Controlled Release*. 2001 Sep 11;76(1):81-91.
19. Ceulemans J, Vermeire A, Adriaens E, Remon JP, Ludwig A. Evaluation of a mucoadhesive tablet for ocular use. *Journal of controlled release*. 2001 Dec 13;77(3):333-44.
20. Dhondt MM, Adriaens E, Remon JP. The evaluation of the local tolerance of vaginal formulations, with or without nonoxynol-9, using the slug mucosal irritation test. *Sexually transmitted diseases*. 2004 Apr 1;31(4):229-35.
21. Dhondt MM, Adriaens E, Roey JV, Remon JP. The evaluation of the local tolerance of vaginal formulations containing dapivirine using the Slug Mucosal Irritation test and the rabbit vaginal irritation test. *Eur J Pharm Biopharm*. 2005 Aug;60(3):419-25
22. Weyenberg W, Vermeire A, Dhondt MM, Adriaens E, Kestelyn P, Remon JP, Ludwig A. Ocular bioerodible minitablets as strategy for the management of microbial keratitis. *Investigative ophthalmology & visual science*. 2004 Sep 1;45(9):3229-33.
23. Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of pharmacology and Experimental Therapeutics*. 1944 Nov 1;82(3):377-90.

24. Lenoir J, Adriaens E, Remon JP. New aspects of the Slug Mucosal Irritation assay: predicting nasal stinging, itching and burning sensations. *Journal of Applied Toxicology*. 2011 Oct 1;31(7):640-8.
25. Lenoir J, Claerhout I, Kestelyn P, Klomp A, Remon JP, Adriaens E. The slug mucosal irritation (SMI) assay: development of a screening tool for the evaluation of ocular discomfort caused by shampoos. *Toxicology in Vitro*. 2011 Dec 31;25(8):1919-25.
26. Boone C.; Bond C.; Buhl K.; Stone D. 2012. *Zinc Sulfate General Fact Sheet*, National Pesticide Information Center, Oregon State University Extension Services. Last Accessed 2016 March 3. Available from: <http://npic.orst.edu/factsheets/zns04gen.html>
27. Schüder I, Port G, Bennison J. Barriers, repellents and antifeedants for slug and snail control. *Crop Protection*. 2003 Sep 30;22(8):1033-8.
28. Marigomez JA, Angulo E, Saez V. FEEDING AND GROWTH RESPONSES TO COPPER, ZINC, MERCURY AND LEAD IN THE TERRESTRIAL GASTROPOD ARIONATER (LINNE). *Journal of molluscan studies*. 1986 May 15;52(1):68-78.
29. Geddes DT, Kent JC, Mitoulas LR, Hartmann PE. Tongue movement and intra-oral vacuum in breastfeeding infants. *Early human development*. 2008 Jul 31;84(7):471-7.
30. Kent JC, Mitoulas LR, Cregan MD, Ramsay DT, Doherty DA, Hartmann PE. Volume and frequency of breastfeedings and fat content of breast milk throughout the day. *Pediatrics*. 2006 Mar 1;117(3):e387-95.

31. Prieto CR, Cárdenas H, Salvatierra AM, Boza C, Montes CG, Croxatto HB.
Sucking pressure and its relationship to milk transfer during breastfeeding in
humans. *Journal of reproduction and fertility*. 1996 Sep 1;108(1):69-74.