Gastropods as an Evaluation Tool for Screening the Irritating Potency of Absorption Enhancers and Drugs

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Purpose. The objective of this study was to develop a simple alternative test using naked snails (slugs) for screening the irritating potency of chemicals on mucosal surfaces.

Methods. The effect of various absorption enhancers and two β-blocking agents on the mucosal tissue was determined from the total protein and lactate dehydrogenase released from the foot mucosa after treatment. Additionally, mucus production and reduction in body weight of the slugs caused by the treatment were measured.

Results. According to the effects on the mucosal epithelium of the slugs the following rank order of increasing toxicity was established: PBS, HP-β-CD (5%), β-CD (1.8%) and oxprenolol hydrochloride (1%) < DDPC (1%) < STDHF (1%) < BAC (1%), SDC (1%) and propranolol hydrochloride (1%). The results of the present study are in agreement with other studies using the same compounds on other models.

Conclusions. The results of this study indicated the mucosa of slugs can serve as a primary screening tool for the evaluation of chemicals on mucosal surfaces. By simply measuring mucus production and weight loss reliable toxicity information can be obtained. This demonstrates rapid screening tests can be carried out using simple toxicity endpoints.

KEY WORDS: mucosal toxicity; screening; absorption enhancers; drugs.

INTRODUCTION

There is a tremendous need for absorption enhancers to improve the absorption of especially hydrophilic compounds and especially for peptides and proteins. Penetration enhancers specially designed for improved mucosal absorption should be very helpful in that respect. Many of these absorption enhancers have the potential problem of causing irritation and membrane damage to the mucosal tissue (1,2). In order to study the toxicity of absorption enhancers various *in vivo* and *in vitro* models have been used (2,3,4). There is experimental evidence that small differences in molecular structures induce major mucosal damage as was seen, e.g., between propranolol hydrochloride and oxprenolol hydrochloride. Both drugs were included in this study.

Toxicity testing using vertebrate animals to evaluate the safety of xenobiotics to humans has been severely criticized based on ethical and financial considerations. The principal alternative to *in vivo* testing is *in vitro* testing. Many factors such as nervous control, systemic blood flow, reduced motility, and heterogeneous cell populations, however, are absent in simple cell culture models. In the workshop: "The three R's: The Way Forward" organized by the European Centre for the Validation of Alternative Methods (ECVAM) (Sheringham, Norfolk, UK, 1995) some replacement alternative methods and approaches were proposed. For example, the use of "lower" organisms with limited sentience and/or not protected by legislation controlling animal experiments, including invertebrates, plants, and microorganisms (5). The objective of this study was to develop a simple alternative test using non-vertebrates as a model organism for screening the toxicity of chemicals on a single-layered mucosal surface such as human intestinal, nasal, and respiratory mucosa, and other mucosal surfaces such as the vaginal and buccal mucosa. The naked snail (slug) *Arion lusitanicus* was used as a model organism. The foot of the slugs consists of a single-layered epithelium containing ciliated cells, cells with microvilli, and mucus secreting cells. Mucus secretion is necessary for the locomotion and the prevention of dehydration of the slug.

In this study the effect of the absorption enhancers and two β-blocking agents on the mucosal tissue was determined from the release of total protein and lactate dehydrogenase from the foot of the slug after treatment. Additionally, the mucus production and the reduction in body weight of the slugs were measured during the treatment period.

MATERIALS AND METHODS

Chemicals

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Janssen Biotech N.V. (Olen, Belgium), and β-cyclodextrin (β-CD) from Roquette (Lestrem, France). Didecanoyl-L-α-phosphatidylcholine (DDPC) was a gift of Rhône-Poulenc Rorer (Köln, Germany). Sodium tauro-24,25-dihydrofusidate (STDHF) was provided by Leo Pharmaceutical Products Ltd. A/S (Ballerup, Denmark). Sodium deoxycholate (SDC) and oxprenolol hydrochloride were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium). Propranolol hydrochloride was from Certa (Eigenbrakel, Belgium). All other reagents were of analytical grade.

Slugs

The animals were collected in the field and maintained in the laboratory (18–20°C) at a photoperiod of 12/12. The slugs were fed lettuce, cucumber, and commercial pig food.

Test Protocol

Slugs weighing between 3.5 g and 4.5 g were isolated from the cultures four days before the start of an experiment and placed in a vented plastic box lined with moist paper towel and placed at 95% relative humidity and 20°C.

The test substances were prepared in phosphate buffered saline (PBS, pH 7.4). HP-β-CD and β-CD and were studied at 5% (w/v) and 1.8% (w/v), respectively. All other absorption enhancers and β-blocking agents were studied at a 1% (w/v) level. Each experiment contained 3 negative control slugs (PBS), 3 positive control slugs (benzalkonium chloride, BAC, 1% w/v), and one or two series each containing 6 treatment slugs (absorption enhancer or β-blocking agent). In order to

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investigate the reproducibility of the test, the experiment with SDC 1% w/v was conducted twice independenlty on a separate occasion (SDC 1, n = 6 and SDC 2, n = 6). At the beginning of the experiment the slugs were placed individually on a membrane filter (cellulose acetate 0.45 μm, Sartorius AG, Germany) moistened with 2 ml of the test medium. After a 15 min incubation period the slugs were transferred to a fresh petridish containing 500 μL PBS. After 15 min the PBS was collected with a Pasteur pipette and the slugs were transferred to a fresh petridish containing 500 μL PBS. After 30 min the PBS was removed and the slugs were placed in a fresh petridish containing 500 μL PBS for 1 hr. This procedure was repeated until 6 hrs after the start of the experiment. The samples were immediately analyzed for the presence of proteins and lactate dehydrogenase released from the foot of the slug. The slugs were weighed separately before and after the 15 min during treatment. The change in weight caused by the treatment was calculated and expressed as % (w/w) of the body weight. The petridishes containing the test medium ware also weighed before and after the treatment. The weight of the mucus produced was calculated and expressed as % (w/w) of the body weight. 6 hrs after the start of the experiment the slugs were weighed again. During the night the slugs were placed in a petridish with a membrane filter containing 2 ml PBS and some lettuce. The petridishes were placed in the dark at 20°C ± 0.5°C and 95% relative humidity. This procedure was repeated during 5 successive days.

Analytical Procedures

Protein Content

The total protein concentration present in the PBS samples was determined with a NanoOrange™ protein quantitation kit (Molecular Probes, Leiden, The Netherlands) and expressed as mg/mL sample. The NanoOrange reagent allows accurate detection of proteins in solutions at concentrations between 10 ng and 10 μg/mL. The fluorescence measurements were carried out on a fluorometer (Kontron instruments SFM 25, Van Hopplynus, Brussel, Belgium) using excitation/emission wavelengths of 485/590 nm. Bovine serum albumin was used as a standard.

Lactate Dehydrogenase

The enzyme activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was measured with an enzyme kit (DG 1340 K, Sigma Diagnostica, Belgium) and expressed as U/L. LDH catalyzes the interconversion of lactate and pyruvate. 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 directly proportional to LDH activity in the sample. The LDH reagent measures the enzyme activity based on the optimized standard method recommended by the German Society for Clinical Chemistry (6). The LDH activity measurements were conducted on a Cobas Mira Plus analyzer (ABX, Brussel, Belgium). The detection limit was 5 U/L.

Statistics

Statistically significant differences between the negative control (PBS), the positive control (BAC), the absorption enhancers and the β-blocking agents 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 square root. To further compare the effects of the different treatments a multiple comparison among pairs of means was performed using a Tukey test with $p < 0.05$ as significance level. For all the statistical analysis the computer program SPSS version 7.5 was used.

RESULTS

Mucus Production and Weight Loss

Figure 1 illustrates the mucus production of the slugs for the 15 min contact period with different absorption enhancers and β-blocking agents for 5 consecutive days. The reduction in body weight caused by the different treatments is shown in Fig. 2. On first day of treatment all the slugs showed a reduction in body weight probably due to mechanical stress. Treatment with BAC, propranolol HCl, STDHF, and SDC resulted in high mucus secretion during the first contact period and decreased with repeated treatments, except for SDC. SDC caused the highest amount of mucus secretion during the second day of treatment. The body weight of slugs treated with PBS, HP-β-CD, β-CD, DDPC, and oxprenolol hydrochloride during 5 days fluctuated between 80% and 100% of the initial body weight. Treatment with PBS, HP-β-CD, β-CD, and DDPC resulted in a low amount of mucus secretions ranging between −5.2 and −1.92% of the body weight (Table I). These negative values can be due to the handling of the petridishes. When compared statistically, no significant differences ($p > 0.05$) could be

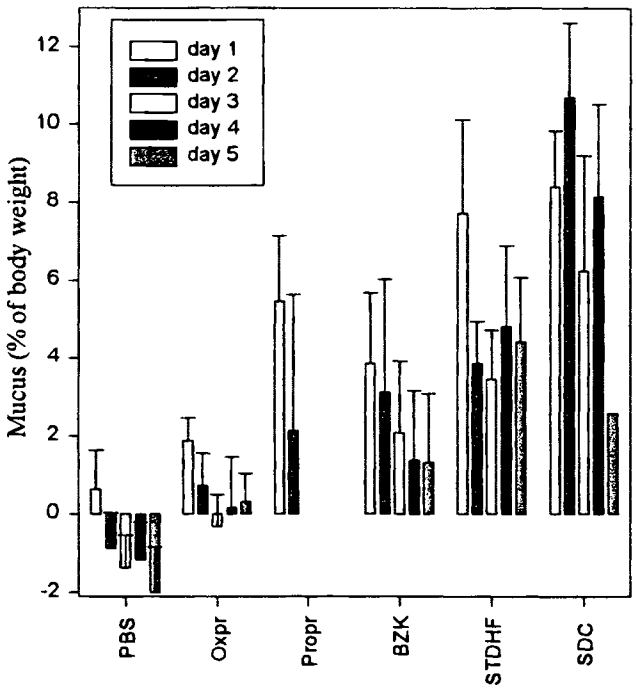

Fig. 1. Mucus secreted by the slugs during a 15 min contact period. Data are presented as the mean ± S.D. (n = 6–15).

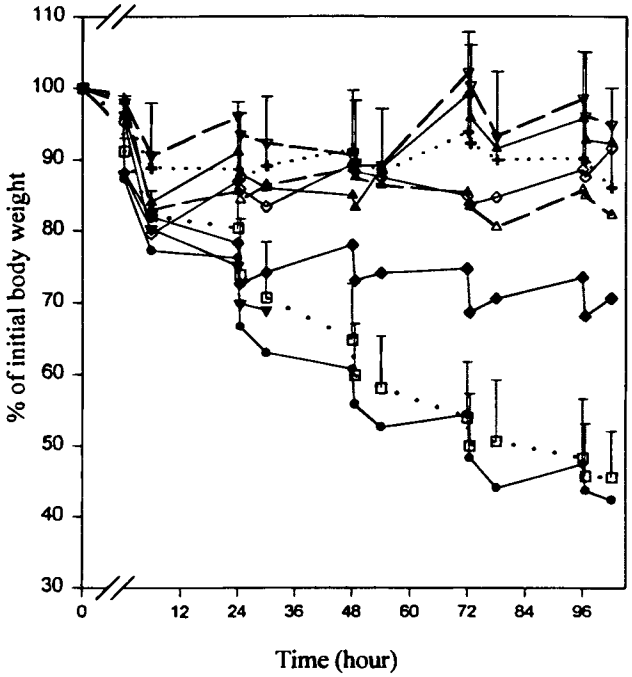

Fig. 2. Reduction in body weight caused by a treatment with PBS (+), BAC (□), HP-β-CD (▽), β-CD (Δ), STDHF (♦), SDC (●), DDPC (◇), oxprenolol hydrochloride (▲) and propranolol hydrochloride (▼). Data are presented as the mean (n = 6–15). S.D. bars are indicated for PBS and BAC.

detected between PBS, HP-β-CD, β-CD, and DDPC, indicating comparable effects. Treatment with oxprenolol hydrochloride resulted in a slight, but significant increase in mucus production in comparison with the negative control slugs (Table I). Reduction in body weight due to the production of mucus was shown for STDHF. During the 15 min incubation period with STDHF, the mucus production ranged between 3.5% and 7.7%, and the weight loss between 6.3 and 11.9% of the body weight. 6 hrs after the incubation period the body weight increased slightly. The body weight of slugs treated with STDHF during 5 days decreased to 70% of the initial body weight. Substances that caused severe epithelial damage resulted in the leakage of blood through the mucosa of the foot thereby further reducing the body weight. This was clearly demonstrated for slugs treated with SDC, BAC and propranolol hydrochloride. The mucus excreted by the slugs treated with propranolol hydrochloride was yellow, slugs treated with BAC, STDHF, and SDC produced dark orange colored mucus whereas normal mucus is colorless. During the 15 min incubation period with BAC, SDC, and propranolol hydrochloride the mucus production ranged between 2.1% and 10.6% and the weight loss between 1.7% and 15.7% of the body weight. During the subsequent sampling period and night the body weight further decreased. After the second day of treatment with propranolol hydrochloride all the slugs died and at that time the body weight was reduced to 68% of the initial body weight. After the third day of treatment with SDC, 50% of the slugs died and only one slug survived until the fifth day of treatment. The slugs treated during the 5 day treatment period with SDC and BAC showed a reduction of the initial body weight to 45% and 43%, respectively. ANOVA testing resulted in no significant differences ($p > 0.05$) for the total mucus production and the total reduction in body weight for both the experiments with SDC.

Protein Release

Figure 3 illustrates the protein released by the foot mucosa of the slugs after the 15 min contact period during 5 consecutive days for the different treatments. The samples taken 15 and 30 min after the first incubation period showed high protein levels (between 0.2–0.8 mg/mL) for all treatments. Then the protein levels decreased below 0.2 mg/mL except for slugs treated with propranolol hydrochloride. From the second day on, treatment with BAC, SDC, and propranolol hydrochloride caused a significantly ($p < 0.05$) higher protein release compared to the negative control slugs; the highest levels being reached just after the 15 min incubation period. The protein levels for the negative control slugs were below 0.07 mg/mL. HP-β-CD, β-CD, STDHF, and oxprenolol hydrochloride showed low protein levels that were comparable with the negative control slugs. A

Table 1. Total Weight Loss and Total Amount of Mucus Production for the 15′ Contact Period; Total Protein and Total LDH Released During 5 Consecutive Days.

Treatment	Mucus[c]	Weight loss[c]	Protein (mg/mL)	LDH (U/L)	N
PBS	−4.18 ± 2.43	8.45 ± 3.00	2.14 ± 0.89	—	14
BAC (1%)	11.13 ± 4.83[a]	34.08 ± 7.20[a]	8.14 ± 2.96[a]	451.44 ± 265.80	15
HP-β-CD (5%)	−5.20 ± 1.81	6.64 ± 2.93	2.78 ± 1.00	—	6
β-CD (1.8%)	−1.92 ± 2.41	10.60 ± 4.54	2.52 ± 1.01	—	6
DDPC (1%)	−4.12 ± 1.72	9.71 ± 2.88	2.53 ± 0.90	31.63 ± 14.08[b]	6
STDHF (1%)	24.22 ± 6.83[a]	41.05 ± 7.61[a]	2.53 ± 0.87	33.18 ± 19.22[b]	6
SDC (1%) (1)	31.14 ± 6.52[a]	45.59 ± 6.16[a]	8.75 ± 1.32[a]	364.74 ± 175.07	6
SDC (1%) (2)	28.03 ± 6.83[a]	40.38 ± 10.28[a]	9.98 ± 2.56[a]	320.08 ± 116.58	6
Oxpr HCl (1%)	2.73 ± 2.61[a]	14.79 ± 4.37	2.15 ± 1.01	—	6
Propr HCl (1%)	7.29 ± 2.79[a]	19.23 ± 4.10[a]	7.38 ± 1.94[a]	136.47 ± 75.33[b]	6

Note: Data are presented as the mean ± S.D. (n = 6–15).
[a] Significantly different from the negative control slugs.
[b] Significantly different from the postive control slugs.
[c] In % (w/w) of the initial body weight at the start of the contact period.

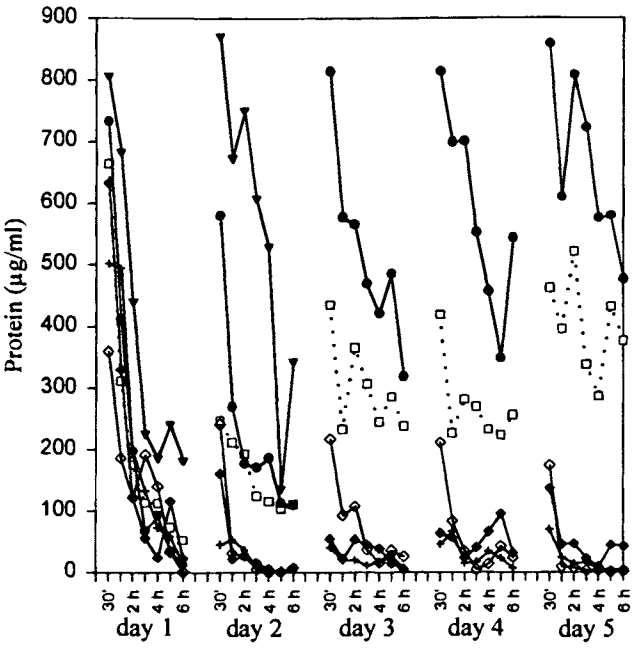

Fig. 3. Protein released from the mucosa after a treatment with PBS (+), BAC (□), STDHF (♦), SDC (●), DDPC (◇) and propranolol hydrochloride (▼). Data are presented as the mean (n = 6–15).

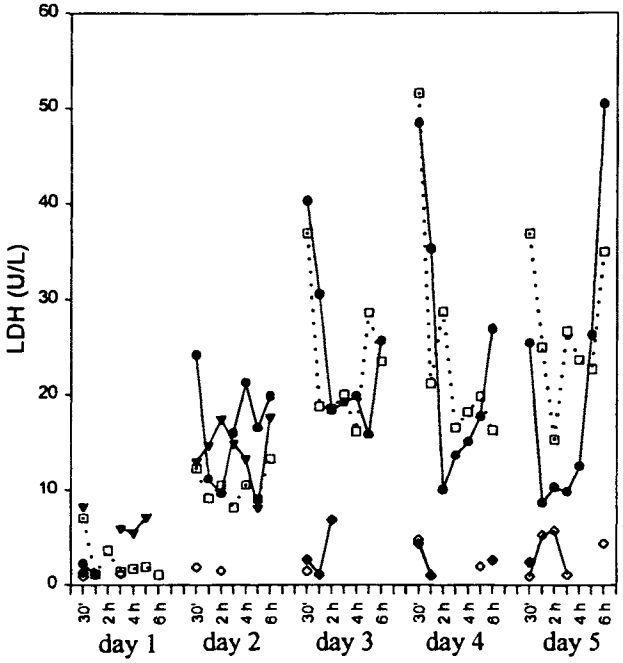

Fig. 4. LDH concentration in the PBS samples after a treatment with STDHF (♦), DDPC (◇), BAC (□), SDC (●) and propranolol hydrochloride (▼). Data are presented as mean values (n = 6–15).

slight increase in protein release was detected just after the treatment with DDPC, although not statistically different in comparison with the negative control slugs. Significant differences were not seen among the two experiments with SDC. Moreover, both experiments showed similar protein concentration profiles. Multiple comparison suggested the following subsets: (1) PBS, oxprenolol hydrochloride, β-CD, DDPC, STDHF, and HP-β-CD; and (2) propranolol hydrochloride, BAC, SDC1, and SDC2.

LDH Release

The release of the cytosolic enzyme LDH from the foot mucosa of the slugs is an indication of cell damage. For the slugs treated with PBS, HP-β-CD, β-CD, and oxprenolol hydrochloride the release of this enzyme was below the detection limit. Figure 4 shows the LDH release after incubation with DDPC, STDHF, SDC, propranolol hydrochloride, and BAC. The following subsets were determined: (1) STDHF, DDPC, and propranolol hydrochloride; (2) SDC and BAC.

DISCUSSION

The objective of the study was to develop an alternative in vivo test using non-vertebrates for screening the toxicity of absorption enhancers on mucosal surfaces. The terrestrial slug *A. lusitanicus* was used as the test organism because the body wall of slugs consists of an outer single-layered epithelium composed of epithelial and mucus gland cells overlying connective tissue. The connective tissue has a spongy appearance due to ramifications of the haemocoel (7). The body wall of slugs consists of mucus secreting cells and mucous glands that are interspersed among ciliated and microvillous cells (8). The body wall is particularly vulnerable to mechanical or chemical damage, and mucus secretions serve to lubricate and to protect the skin against damage (9). Slugs treated with irritating substances produce mucus in order to protect the body wall, thereby reducing the body weight. Severe damage causes leakage of blood through the mucosa of the foot followed by a further reduction of the body weight. When the single-layered epithelium is seriously damaged, blood can flow out from the ramifications of the haemocoel into the connective tissue. The samples of the slugs treated with propranolol hydrochloride, BAC, and SDC were slightly yellow, indicating the leakage of blood. This was also confirmed by the significant higher protein and LDH release. Roach (10) estimated the mean protein content of *A. ater* blood at 10.55 mg/ml although the protein content was very variable. According to Runham and Hunter (11), the main blood protein is haemocyanin.

According to their effects on the mucosal epithelium of the slugs the absorption enhancers and β-blocking agents can be classified into four different categories: (1) PBS, HP-β-CD, β-CD and oxprenolol hydrochloride; (2) DDPC; (3) STDHF; and (4) propranolol hydrochloride, BAC, and SDC. The effect of the first category was comparable with the negative control slugs. Treatment with DDPC probably resulted in a quick repair of the mucosal tissue as the protein and LDH released were only slightly increased just after the 15 min contact period followed by a decrease to the normal values. Since no significant influence was detected on the total mucus production, the reduction in body weight and the total protein release, the cytotoxicity caused by DDPC might be reversible. The irritating potential of STDHF was shown by the significant reduction in body weight and the mucus production during the treatment. However, no significant increase in protein release and only a low LDH release were detected indicating the mucus excreted by the slugs probably protected the body wall. Propranolol hydrochloride, SDC, and BAC belong to the most damaging agents

causing severe and irreversible toxicity. Slugs exposed to these molecules showed a significantly increased mucus production, a significant reduction in body weight, and a significant protein and LDH release. We also estimated the release of the membrane bound enzyme alkaline phosphatase in this study, however these results showed a very low reproducibility and are therefore not reported.

The results of the present study are in agreement with other studies using the same compounds but on other models. Administration of 5% HP-β-CD to the rat nasal mucosa resulted in only minimal removal of epithelial membrane proteins and total absence of LDH activity (12). 5% HP-β-CD and 1.8% β-CD showed only minor effects on the ciliary beat frequency of chicken embryonal trachea tissue (13). Intermodel differences appear to exist for the evaluation of the cytotoxic potential of DDPC. 1% DDPC had no effect on the mucociliary transport rate in the frog palate model (3). Low concentrations of DDPC were found to reversibly decrease the electrical properties of rabbit nasal mucosa, whereas 2% DDPC concentrations decreased these parameters in a toxic way (14). Treatment of human red blood cells with 0.027% DDPC resulted in 100% hemolyses after 30 min. The authors assumed that incorporation of DDPC into cellular membranes happens very quickly, resulting in membrane disorders that might be responsible for the absorption enhancing and the cytotoxic effect (15). Based on the release of marker compounds in the nasal cavity of the rat the following rank order of increasing toxicity was established: 5% HP-β-CD < 1% STDHF < 1% SDC (2). Treatment of the rat nasal mucosa with 0.5% and 1% STDHF resulted in intermediate scores (2–3) and 1% SDC resulted in the highest scores (4–5) of membrane damage (4).

The toxicity of BAC to different cell types is well documented. Human corneal epithelial cells were strongly affected after a treatment with BAC (16). The ciliary beat activities in rabbit tracheas were completely halted by 0.05% BAC after 10 min (17). Treatment of the human buccal mucosa with propranolol hydrochloride caused ulcerations that took up to weeks to heal (18). Propranolol hydrochloride resulted in the highest macroscopic and histological scores in the Carlborg-Densert cat esophagus model, the Alphin-Droppleman cat gastric mucosa model and the rabbit colon (19).

In conclusion, the results of this study indicate the mucosa of slugs can serve as a primary screening tool for the evaluation of chemicals on mucosal surfaces. By simply measuring mucus production and weight loss reliable toxicity information can be obtained. This demonstrates rapid screening tests can be carried out using simpler toxicity endpoints, which have the advantage of not requiring complex test equipment, sophisticated chemical analysis, or a long time to be completed.

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