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Classification of Cilio-Inhibiting Effects of Nasal Drugs

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Objective/Hypothesis: **Nasal drug formulations are widely used for a local therapeutic effect, but are also used for systemic drug delivery. In the development of new nasal drugs, the toxic effects on the mucociliary clearance and therefore on the ciliated tissue is of importance. In this study, the effect of nasal drugs and their excipients on the ciliary beat frequency (CBF) is investigated.** ***Study Design:*** **Experimental, in vitro.** ***Methods:*** **CBF is measured by a photograph–electric registration method. Excised ciliated chicken trachea tissue is incubated for 15 minutes in the formulation, followed by a reversibility test. To estimate the ciliostatic potential, a classification is given of all tested formulations. According to the CBF, after 60 minutes every drug or excipient could be classified as follows:** ***cilio-friendly*****: after 60 minutes the CBF has regained *75% or more* of its initial frequency;** ***cilio-inhibiting*****: after 60 minutes the CBF has regained *between 25% and 75%* of its initial frequency; or** ***ciliostatic*****: after 60 minutes the CBF has regained *25% or less* of its initial frequency.** ***Results:*** **Most formulations used are cilio-friendly or cilio-inhibiting. Only some are ciliostatic. Preservatives have a major role in the cilio-inhibiting effect of the drug. Also, other additives can contribute to the toxicity profile of nasal drug formulations.** ***Conclusion:*** **This classification of the cilio-inhibiting potential of nasal drug formulations is a valuable tool in the design of safe nasal drugs. The number of animal studies in vivo can be reduced substantially by using this in vitro screening technique. This study demonstrates that the effect on ciliary movement of most drug formulations is due to the preservatives and/or additives and mostly not to the drug itself.** ***Key Words:*** **Nasal drug, preservatives, ciliary beat frequency, ciliostatic, cilio-inhibiting, cilio-friendly.**

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INTRODUCTION

Nasal drug formulations, for instance, those containing decongestants and corticosteroids, are widely used for a local therapeutic effect. The nasal mucosa is also an attractive site for systemic drug absorption. It is an effective alternative for other routes of drug administration (oral, injection), for instance, in the case of antimigraine substances,[1,2] steroids,[3] and peptide and protein drugs.[4,5] Nasal drug absorption can be efficient because the nasal epithelium has a relatively large permeability and the subepithelial layers are highly vascularized.[6]

Nasal drug delivery has a number of clear advantages, including ease of administration, patient acceptability, and prevention of first-pass effect.[7] The relatively small surface area of the nasal cavity and the mucociliary clearance are drawbacks in nasal drug delivery. The residence time of a drug formulation in the nose is limited to only approximately 15 minutes, because of the nasal mucociliary clearance.[8–10] It is obvious that during acute or chronic nasal drug application, the drug itself and the formulation excipients should not disturb the nasal mucociliary clearance, because it is an extremely important defense mechanism of the respiratory tract. The mucociliary clearance remove bacteria viruses, allergens, and dust from the respiratory tract. Because ciliary movement is a major factor in mucociliary clearance, the influence of drug formulations on the ciliary beat frequency (CBF) is an important issue to establish the safety of nasally administered drugs and various formulation excipients such as preservatives[11–13] and absorption enhancing compounds.[13,14]

The aim of this study was to test the cilio-inhibiting effects of a number of drugs using ciliated chicken embryo tracheal tissue. Chicken trachea is a valid substitute for human material in studying ciliary activity in vitro.[15,16] Moreover, the reversibility of the observed effects was established after exposure of the ciliated tissue to the nasal drug formulations during 15 minutes, comparable to the situation in vivo. The evaluation of the influence on ciliary movement may offer a possibility to classify drugs and excipients according to their inhibiting effect.

MATERIALS AND METHODS

The nasal formulations selected for this study are widely prescribed drugs for local and systemic effects, some excipients,

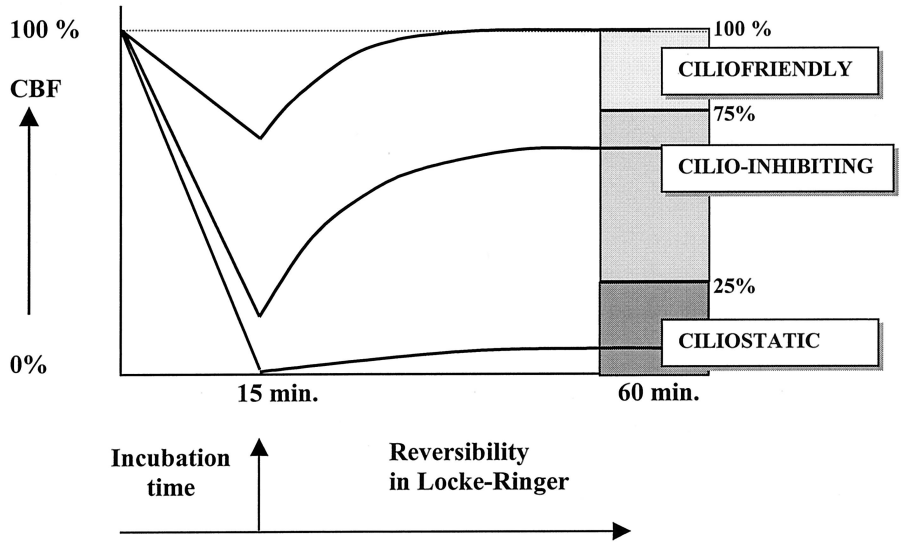

Fig. 1. Classification of the effect of nasal formulations on ciliary beat frequency (CBF). CBF is expressed as percentage of the initial frequency (100%). After 15 minutes incubation of the ciliated tissue in the nasal formulation, the reversibility of the CBF in Locke-Ringer solution is measured. At 60 minutes after the start of the incubation, the degree of reversibility is classified into three categories: cilio-friendly, cilio-inhibiting, or ciliostatic.

and investigational drug formulations indicated for systemic nasal drug absorption. Products have been selected that are available on the market in the United States and Europe, although brand names may sometimes differ.

Materials

Benzalkonium chloride (BAC; USP quality) was from Brocacef (Maarssen, The Netherlands), chlorobutanol was from Sigma-Chemie (Dreisenhofen, Germany), and sodium edetate (EDTA; PA quality) from Merck (Darmstadt, Germany). Randomly methylated β-cyclodextrin (RAMEB; degree of substitution 1.8) was obtained from Wacker (Burghausen, Germany). All other chemical compounds were from Sigma–Chemie (Dreisenhofen, Germany), and the drug substances were from Bufa (Uithoorn, The Netherlands).

The species of chickens used was Hubbard-Golden Comeet (Vossensteijn, Groenekan, The Netherlands).

(Non-)Prescription Nasal Drug Formulations

All nasal formulations selected for the present study are widely used prescription and non-prescription drugs for local or systemic effects, and were studied for their influence on ciliary

TABLE I.
The Effect of (Non-)Prescription Nasal Drug Formulations on Ciliary Beat Frequency (CBF) in vitro.

Nasal Product	Main Constituents	CBF t = 15 (SD)	CBF t = 60 (SD)	Classification
Aerodiol®	Estradiol, RAMEB	42 (7)	97 (8)	Cilio-friendly
Flixonase®	Fluticasone, BAC, phenylethylalcohol	9 (5)	62 (11)	Cilio-inhibiting
Imigran®	Sumatriptan, phosphate buffer	0 (0)	96 (14)	Cilio-friendly
Miacalcic®	Calcitonin, BAC	12 (9)	58 (20)	Cilio-inhibiting
Minrin®	Desmopressin, chlorobutanol	0 (0)	0 (0)	Ciliostatic
Nasacort®	Triamcinolone acetonide, BAC, EDTA	38 (7)	78 (8)	Cilio-friendly
Nasivin®	Oxymetazoline, BAC, EDTA	2 (5)	4 (10)	Ciliostatic
Nasivin® pur	Oxymetazoline	25 (4)	97 (13)	Cilio-friendly
Nasonex®	Mometasone fuorate, BAC, phenylethylalcohol	0 (0)	33 (19)	Cilio-inhibiting
Otriven®	Xylometazoline, citrate, glycerol	18 (5)	103 (6)	Cilio-friendly
Otrivin®	Xylometazoline, BAC, EDTA	21 (9)	36 (12)	Cilio-inhibiting
Rhinocort®	Budesonide, Sorbate, EDTA	25 (13)	98 (22)	Cilio-friendly
Sinex®	Oxymetazoline, BAC, chlorhexidine, EDTA, camphor, menthol, eucalyptol	0 (0)	0 (0)	Ciliostatic
Control				
Locke-Ringer (LR)		100 (3)	100 (4)	Cilio-friendly

CBF (% of initial frequency) after 15 min incubation in the test formulation (t = 15) and after reversibility testing in Locke-Ringer solution until 60 min (t = 60). Data are expressed as the mean (± standard deviation) of 6–8 experiments. Classification according to Figure 1.

BAC = benzalkonium chloride; EDTA = sodium edetate; RAMEB = randomly methylated β-cyclodextrin.

TABLE II.
The Effect of Investigational Nasal Formulations on Ciliary Beat Frequency (CBF) in vitro.

Investigational Products	Main Constituents	CBF t = 15 (SD)	CBF t = 60 (SD)	Classification
Hydroxocobalamin 2.0%	Hydroxocobalamin, Locke-Ringer	90 (13)	88 (5)	Cilio-friendly
Hydroxocobalamin 1.2%	Hydroxocobalamin, acetate buffer	0 (0)	79 (12)	Cilio-friendly
Melatonin 0.05%	Melatonin, Locke-Ringer	80 (12)	99 (4)	Cilio-friendly
Melatonin 0.2%	Melatonin, β-Cyclodextrin	42 (5)	102 (3)	Cilio-friendly
Midazolam 3.1%	Midazolam, benzylalcohol, propylene glycol	0 (0)	0 (0)	Ciliostatic
Propranolol 1.0%	Propranolol, Locke-Ringer	0 (0)	0 (0)	Ciliostatic

CBF (% of initial frequency) after 15 min incubation in the test formulation (t = 15) and after reversibility testing in Locke-Ringer solution until 60 min (t = 60). Data are expressed as the mean (± standard deviation) of 6–8 experiments. Classification according to Figure 1.
BAC = benzalkonium chloride; EDTA = sodium edetate; RAMEB = randomly methylated β-cyclodextrin.

beating in undiluted form. The following formulations were investigated: estradiol (Aerodiol®; Servier, Paris, France) 0.2% w/v, containing randomly methylated β-cyclodextrin (RAMEB) 2.0% w/v; fluticasone (Flixonase®; Glaxo Wellcome B.V., Zeist, The Netherlands) 0.05% w/v, containing BAC 0.02% w/v and phenylethylalcohol 0.25% w/v; sumatriptan (Imigran®; Glaxo Wellcome B.V.) 20% w/v in a phosphate buffer pH 5.4; salmon calcitonin (Miacalcic®; Novartis Farmaceutica, Barcelona, Spain) 2200 IU/mL, containing benzalkonium chloride (BAC) 0.01% w/v; desmopressin (Minrin®; Ferring, Malmö, Sweden) 0.01% w/v, containing chlorobutanol 0.5% w/v; triamcinolone acetonide (Nasacort®; Rhône Poulenc Rorer B.V., Amstelveen, The Netherlands) 0.05% w/v, containing cellulose, sodium carboxymethylcellulose, polysorbate 80, BAC, and EDTA; oxymetazoline (Nasivin®; Merck, Darmstadt, Germany) 0.05% w/v, containing BAC and EDTA; oxymetazoline (Nasivin® pur; Merck) 0.05% w/v, preservative-free; mometasone fuorate (Nasonex®; Schering-Plough B.V., Maarssen, The Netherlands) 0.05% w/v, containing BAC, polysorbate 80 and phenylethylalcohol; xylometazoline (Otriven®; Novartis Consumer Health, Munich, Germany) 0.1% w/v, containing citric acid, sodium citrate and glycerol, preservative-free; xylometazoline (Otrivin®; Novartis Consumer Health, Breda, The Netherlands) 0.1% w/v, containing BAC and EDTA; budesonide (Rhinocort®; Astra Pharmaceutica, Zoetermeer, The Netherlands) 0.1% w/v, containing potassium sorbate and sodium edetate (EDTA); and oxymetazoline (Sinex®; Richardson Vicks B.V., Rotterdam, The Netherlands) 0.05% w/v, containing BAC 0.02% w/v, chlorhexidine digluconate, EDTA 0.01% w/v, and also menthol, camphor, eucalyptol, and tyloxapol.

Investigational Nasal Formulations

The investigational hydroxocobalamin formulation consisted of hydroxocobalamin 1.2% w/v and NaCl 0.7% w/v in 20 mmol/L sodium acetate buffer of pH 4.5. Melatonin nasal prepa-

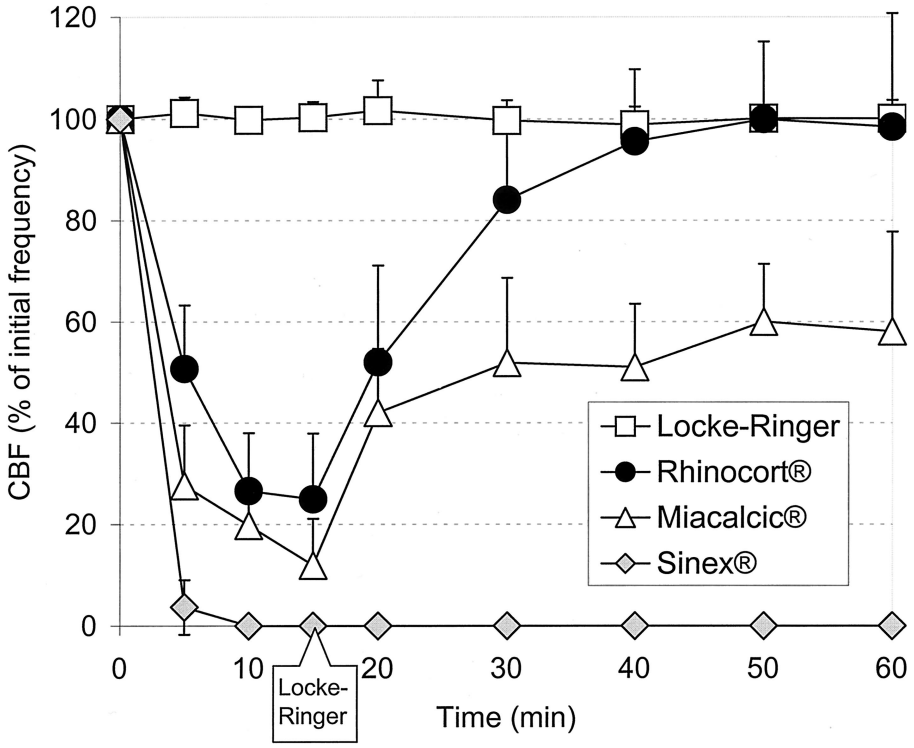

Fig. 2. The effect of three nasal products on CBF. After 15 minutes incubation of the ciliated tissue in the nasal formulation, the reversibility of the CBF in Locke-Ringer solution was measured. The effect, after reversibility testing at 60 minutes, of Rhinocort® (●) is classified as cilio-friendly, that of Miacalcic® (△) as cilio-inhibiting, and that of Sinex® (◇) as ciliostatic. Locke Ringer (□), the control solution, has no cilio-inhibiting influence. CBF is expressed as percentage of the initial frequency (100%) and data are mean + standard deviation.

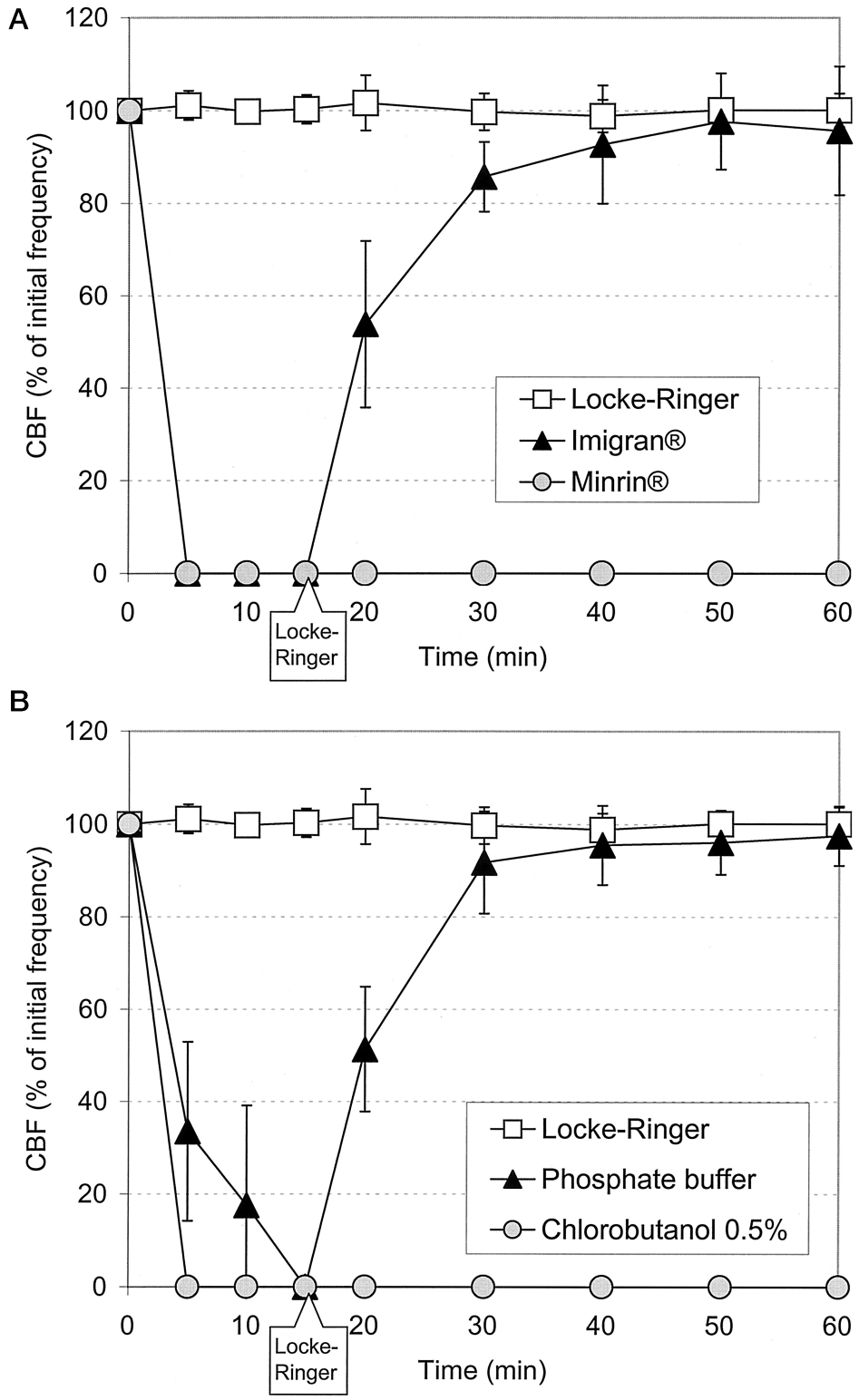

Fig. 3. (**A** and **B**) Effects of Imigran® and Minrin® on CBF: contribution of formulation constituents. Its constituents can explain effects of both nasal products. The effect, after reversibility testing, of Imigran® (containing a phosphate buffer) (▲, **A**) is probably the result of the buffer solution (▲, **B**). The ciliostatic effect of Minrin® (○, **A**) is caused by its preservative chlorobutanol 0.5% (○, **B**). CBF is expressed as percentage of the initial frequency (100%) and data are mean ± standard deviation.

rations contained melatonin 0.2% w/v, NaCl 0.9% w/v, and the solubilizer β-cyclodextrin 0.75% w/v in water. The midazolam formulation consisted of midazolam hydrochloride 3.1% w/v, benzylalcohol 1% v/v, and propylene glycol 25% v/v in water. Propranolol hydrochloride 1.0% w/v was dissolved in Locke-Ringer.

Excipients

A number of excipients used in the (non-)prescription and investigational nasal drug formulations were measured for their effect on ciliary beat frequency, after dissolving these substances in Locke-Ringer solution: the solubilizer/absorption enhancer

TABLE III.
The Effect of Excipients on Ciliary Beat Frequency (CBF) in vitro.

Excipient	CBF t = 15 (SD)	CBF t = 60 (SD)	Classification
NaCl 0.9%	74 (12)	95 (8)	Cilio-friendly
BAC 0.01%	54 (22)	70 (11)	Cilio-inhibiting
BAC 0.02%	52 (27)	20 (19)	Ciliostatic
BAC 0.01%/EDTA 0.1%	35 (14)	43 (23)	Cilio-inhibiting
Benzylalcohol 1%/propylene glycol 25%	0 (0)	0 (0)	Ciliostatic
Chlorobutanol 0.5%	0 (0)	0 (0)	Ciliostatic
Phenylethylalcohol 0.5%	0 (0)	97 (12)	Cilio-friendly
Phosphate buffer (120 mM; pH 5.4)	0 (0)	98 (6)	Cilio-friendly
Potassium sorbate 0.2%/EDTA 0.1%	62 (9)	99 (5)	Cilio-friendly
RAMEB 2.0%	61 (17)	93 (6)	Cilio-friendly
Sodium acetate buffer (20 mM; pH 4.5)	0 (0)	88 (15)	Cilio-friendly

CBF (% of initial frequency) after 15 min incubation in the test formulation (t = 15) and after reversibility testing in Locke-Ringer solution until 60 min (t = 60). Data are expressed as the mean (± standard deviation) of 6–8 experiments. Classification according to Figure 1.

BAC = benzalkonium chloride; EDTA = sodium edetate; RAMEB = randomly methylated β-cyclodextrin.

RAMEB in concentrations of 2.0% w/v, the preservative BAC in concentrations of 0.01% and 0.02% w/v, and the preservatives phenylethylalcohol and chlorobutanol in concentrations of 0.5% w/v. Additionally, combination preparations of the preservative BAC 0.01% and potassium sorbate 0.2% with EDTA 0.1% w/v in Locke-Ringer were tested. Three vehicle solutions were investigated: 120 mmol/L phosphate buffer (adjusted to pH 5.4), 20 mmol/L sodium acetate buffer containing NaCl 0.9% w/v (adjusted to pH 4.5), and benzylalcohol 1% v/v with propylene glycol 25% v/v in water.

Locke-Ringer (Control Solution)

Locke-Ringer (LR) is an isotonic solution of the following composition per liter of water: NaCl, 7.72 g (132 mmol); KCl, 0.42 g (5.63 mmol); $CaCl_20.2H_2O$, 0.16 g (1.24 mmol); $NaHCO_3$, 0.15 g (1.79 mmol); glucose, 1.00 g (5.55 mmol). Locke-Ringer solution was prepared using Millipore-deionized water, and the solution was subsequently sterilized for 20 minutes at 120°C. The pH of the Locke-Ringer solution was established at 7.4.

Ciliary Beat Frequency Measurements

Ciliary beat frequency (CBF) measurements were performed on the ciliated epithelium of isolated chicken embryo trachea as described previously.[13,17] Briefly, the chicken embryo trachea was dissected from the embryo and sliced into small rings of approximately 1 mm thickness. The trachea slices were placed in stainless steel supporting rings, and were allowed to recover for 30 minutes in Locke-Ringer solution. Thereafter, the tissue samples were put in a well containing 1.0 mL of the test solution, and placed under an Olympus BH-2 light microscope. The microscope table was connected with a thermostat to maintain a temperature of 33°C. The CBF was subsequently monitored using a photograph–electric registration device. A light beam was transmitted through the moving cilia, and after magnification by the microscope the flickering light was projected to a photocell. The electrical signal generated by this photocell was visualized with a computer monitor. The frequency of the signal was calculated electronically by Fast-Fourier transform algorithm and displayed as a frequency distribution.

After starting the incubation, the CBF was measured at 5, 10, and 15 minutes. Thereafter, to test the reversibility of CBF, the trachea slices were washed by shaking them vigorously in a tube with 3 mL Locke-Ringer. Then the slices were replaced in pure Locke-Ringer and CBF was measured again every 5 to 10 minutes until 60 minutes after the start of the incubation. Every formulation has been tested using tissue samples of at least six different chickens.

CBF data were calculated as the relative frequency of the initial frequency measured in Locke-Ringer solution at the start of the experiment, the latter being expressed as 100%.

Classification of Effects on CBF

The influence of the studied nasal drug formulations and excipients on CBF was classified into the following three categories (Fig. 1):

1) Cilio-friendly: after 60 minutes the CBF has regained *75% or more* of its initial frequency.
2) Cilio-inhibiting: after 60 minutes the CBF has regained *between 25 and 75%* of its initial frequency.
3) Ciliostatic: after 60 minutes the CBF has regained *25% or less* of its initial frequency.

RESULTS

A summary of the results is shown in Tables I, II, and III. The CBF of the control solution (Locke-Ringer) remained 100% of the initial frequency for at least 1 hour in all experiments (Table I).

Nasal Products

Imigran®, Rhinocort®, Nasacort®, and Aerodiol® reduce CBF, and this effect is reversible. Imigran® arrested the ciliary beating within 5 minutes, but the mean CBF recovered to 96% of the initial frequency at completion of the reversibility test. Rhinocort® (Fig. 2), Nasacort®, and Aerodiol® resulted in mild effects on the CBF after 15 minutes incubation: the mean CBF decreased to 25%, 38%, and 42%, respectively. In the subsequent reversibility test CBF increased to 98%, 78%, and 97%, respectively, of their initial frequency.

Miacalcic® (Fig. 2) and Flixonase® appeared to have almost identical effects on CBF. Their initial frequency dropped to 12% and 9%, respectively, after 15 minutes

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