J. Pharm. Pharmacol. 1989, 41: 156–159
Received May 3, 1988

The Effect of Some Preservatives Used in Nasal Preparations on Mucociliary Clearance

A. H. BATTS, C. MARRIOTT, G. P. MARTIN AND S. W. BOND*

*Pharmaceutical Sciences Research Group, Department of Pharmacy, Brighton Polytechnic, Brighton, BN2 4GJ, UK, and *The Wellcome Foundation Ltd., Dartford, Kent, UK*

Abstract—The effect of methyl-*p*-hydroxybenzoate, propyl-*p*-hydroxybenzoate, chlorbutol, chlorocresol, EDTA, benzalkonium chloride, chlorhexidine, phenylmercuric nitrate and phenylmercuric borate on mucociliary transport rate of the frog palate has been examined. Following a variable number of applications all these preservatives halted transport, the first three reversibly. However, applications of thiomersal (0·01%) were well tolerated. The frog palate possesses a ciliated epithelium protected by mucus, since some of our findings are at variance with those previously reported results where the protective effect of mucus was negligible in the in-vitro model (usually trachea) employed, it would appear that the contribution of mucus to effective mucociliary clearance should not be underestimated.

Nasal drops and sprays are usually simple aqueous solutions containing substances with antiseptic, local analgesic and vasoconstrictor properties intended to act locally. However, the intra-nasal route may also be used to deliver compounds to the systemic circulation and provides a convenient and reliable means of self-administration which is likely to prove especially useful for those compounds which are difficult to deliver by the oral route. These include pharmacologically active polypeptides and proteins which are currently being developed, such as hormones and vaccines, which may fail to realize their full potential if the parenteral route is their sole means of administration.

The epithelium of the upper respiratory tract is covered by many hair-like cilia that beat in a co-ordinated manner within the periciliary fluid beneath a layer of viscoelastic mucus, the whole comprising the mucociliary apparatus. After inhalation, mucociliary clearance contributes to the body's primary non-specific defence mechanism by entrapping potentially hazardous substances such as dust and micro-organisms, allergens, carcinogens and cellular debris within the mucus blanket, which is then propelled by the claw-like tips of the underlying cilia towards the pharynx where it is swallowed or expectorated (Proctor 1977).

Compromised clearance may be associated with a variety of conditions including genetically misformed cilia (Afzelius 1976, 1979, 1981), cigarette smoking (Walker & Kiefer 1966) and bacterial or viral infection (Carson et al 1979; Iravani et al 1978). An alteration in the secreted mucus, such as is seen in cystic fibrosis, can also inhibit clearance (Di Sant Agnese & Davis 1976a,b,c; Wood et al 1976). Patients with such conditions suffer extensively from chronic respiratory infections such as bronchitis, sinusitis and rhinitis. These consequences of inhibited clearance emphasize that the constituents of preparations intended for nasal delivery should not adversely affect the clearance system.

Most nasal drops and sprays are presented as multi-dose preparations requiring a preservative to prevent the growth of micro-organisms upon repeated use. In studies investigating the effects of preservatives on the mucociliary clearance apparatus, ciliary beat frequency has been monitored using small portions of ciliated tissue usually from trachea (Greenwood et al 1946; Gallay 1960; Perrault et al 1978; Mostow et al 1979; Van de Donk et al 1980). We have examined the effect of a similar range of preservatives on mucociliary transport rate measured on the frog palate. This model has been shown to produce a good correlation with in-vivo tracheal clearance measured in mammals (Giordano et al 1977) and, since it possesses a ciliated epithelium protected by a visible and continuous layer of mucus (Morgan et al 1984), it might be expected to provide a better correlation with the human nasal epithelium than earlier models.

Correspondence to: A. H. Batts, Pharmaceutical Sciences Research Group, Department of Pharmacy, Brighton Polytechnic, Brighton, BN2 4GJ, UK.

Materials and Methods

Preparation of solutions

The control solution for most of the compounds was 0·9% w/v NaCl (May and Baker Ltd; UK) in distilled water. That for phenylmercuric nitrate was 1·3% w/v sodium nitrate or 5·07% w/v mannitol in distilled water. Chlorhexidine gluconate and phenylmercuric borate were also investigated using 5·07% mannitol as the control solution.

Preservative solutions were made by dissolving the required amount in the appropriate control solution. The concentrations investigated were: methyl-*p*-hydroxybenzoate 0·02 and 0·15%, propyl-*p*-hydroxybenzoate 0·02%, benzalkonium chloride 0·01% (Sigma Chemical Company, UK); chlorbutol 0·5%, 4-chloro-*m*-cresol 0·05% and 0·10%, thiomersal 0·01%, phenylmercuric nitrate 0·002% (BDH Chemicals Ltd; UK); ethylenediaminetetraacetic acid, disodium salt, dihydride (EDTA) 0·1% (Aldrich Chemical Company Ltd., UK); phenylmercuric borate 0·002% (Zyma S.A., Switzerland); chlorhexidine gluconate 0·01% (ICI PLC, UK).

Transport rate was measured in-vitro using a modification of the frog palate preparation described by Sadé et al (1970). After being pithed, the frog (*Rana temporaria*) had its upper palate exposed. The frog was then introduced into a transparent chamber maintained at 20°C with a relative humidity of 100% and the palate surface observed through a

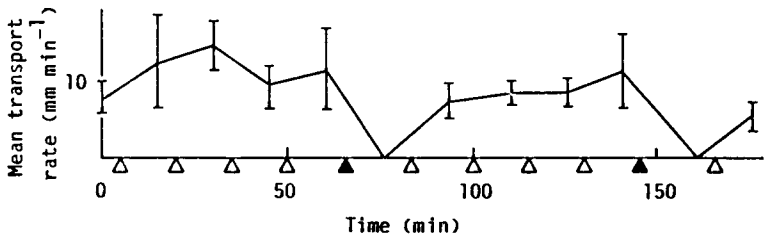

FIG. 1. The effect of propyl-*p*-hydroxybenzoate 0·02% w/v on mean transport rate. ▲; application of perservative, △; application of control solution.

stereo-microscope (Stereo Zoom 4, Bausch and Lomb UK Ltd), with a calibrated eyepiece (Bausch and Lomb UK Ltd 10× W.F. Stereo 31-15-71).

Control values were obtained for each experiment by applying 0·2–0·4 mL of the appropriate control solution to the palate, leaving it in contact for 10 min then draining it off. The transport rate was then measured by recording the time taken for graphite particles to travel a given distance, usually 0·3 cm, over the palate. This procedure was repeated three or four times before the test compound was similarly applied. If the preservative appeared to halt or decrease transport, the effect of rinsing the palate with the appropriate control solution was assessed. Each preservative was tested on at least six different palates.

Results

Figs 1–3 are examples of the results obtained for one of the six palates used. Values for mean transport rate are plotted with standard deviation bars. Methyl-*p*-hydroxybenzoate, propyl-*p*-hydroxybenzoate and chlorbutol halted transport, rinsing the palate reversed the toxic effect of methyl-*p*-hydroxybenzoate (0·02%) and propyl-*p*-hydroxybenzoate (Fig. 1), while reversal of the effect of methyl-*p*-hydroxybenzoate (0·15%) and chlorbutol was equivocal.

Chlorocresol (Fig. 2), benzalkonium chloride, EDTA and phenylmercuric nitrate halted transport irreversibly following one to two applications. Phenylmercuric borate in saline, halted transport after two to five applications, normally, but not always, irreversibly; in mannitol it caused transport to cease irreversibly, generally after a single application.

Chlorhexidine in 0·9% NaCl appeared to be well tolerated by the palate, and transport was still apparent after six applications. However, in a mannitol solution, one or two applications of chlorhexidine were generally sufficient to irreversibly halt transport.

Fig. 3 shows that activity could still be observed on the palate following nine applications of thiomersal, suggesting that in this model, this preservative is well tolerated.

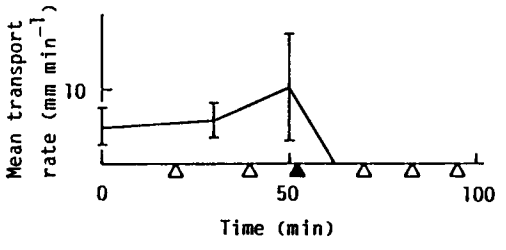

FIG. 2. The effect of chlorocresol 0·10% w/v on mean transport rate. ▲; application of perservative, △; application of control solution.

Discussion

The effects of methyl-*p*-hydroxybenzoate 0·15%, were similar to those published elsewhere (Gallay 1960; Perrault et al 1978; Mostow et al 1979; Van de Donk et al 1980). The reversibility of the observed effect appears to depend on the contact time and the concentration employed. This is substantiated by the observation that at the lower concentration (0·02%) the effect of methyl-*p*-hydroxybenzoate was always reversible, whereas at the higher concentration the reversal of the inhibiting effect was variable. Chlorbutol and propyl-*p*-hydroxybenzoate possessed similar properties to methyl-*p*-hydroxybenzoate, also in agreement with the results of Perrault et al (1978) and Van de Donk et al (1980). Methyl-*p*-hydroxybenzoate, propyl-*p*-hydroxybenzoate and chlorbutol are lipophilic and are believed to exert their toxic action upon micro-organisms following adsorption and subsequent diffusion through the lipophilic membrane.

Similar toxic effects may occur, following diffusion of the preservative across the ciliated epithelium, which halts transport. Subsequent rinsing may reverse the adsorption process and cause back-diffusion of the compounds from the cells of the mucous membrane, hence reversing the effect.

Chlorocresol, at both concentrations used, halted transport after a single application and this effect was irreversible. This could be explained by it possessing a higher oil-water partition coefficient than the previous three compounds such that rinsing with saline was insufficient to cause back-diffusion out of the cells. The oil-water partition coefficients of chlorocresol, methyl-*p*-hydroxybenzoate and propyl-*p*-hydroxybenzoate in vegetable oil are 117·0, 7·5 and 80·0, respectively, lending some support to this suggestion. It is also possible that transport over the frog palate was inhibited by an alteration of the well-defined viscoelastic properties the mucus gel must possess in order to flow on a ciliated epithelium (Litt et al 1976). Since this irreversibility contradicts the results of Van de Donk et al (1980) with trachea, it is likely that it is a consequence of the mucus layer covering the cilia of the frog palate.

In earlier studies the effect of preservatives has been assessed by monitoring ciliary beat frequency on isolated pieces of ciliated tissue, usually trachea, which were completely immersed in the test solution (Gallay 1960; Perrault et al 1978; Mostow et al 1979; Van de Donk et al 1980). Such immersion increases the likelihood of removal of any mucus layer that might be present, and in two of the studies mucus was deliberately removed (Gallay 1960; Mostow et al 1979).

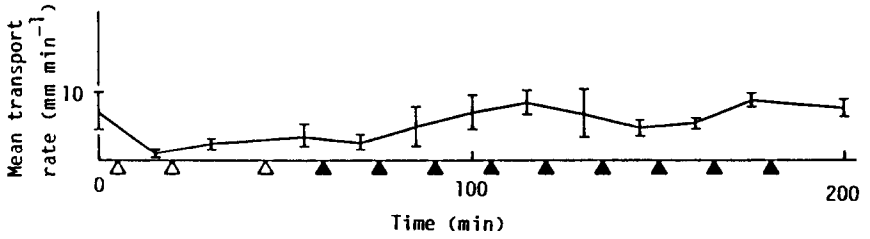

FIG. 3. The effect of thiomersal 0·01% w/v on mean transport rate. ▲; application of perservative, △; application of control solution.

With the tracheal preparation even if a mucus layer is present, the protection it affords the cilia is minimal since, when the ring is submerged in the test solution the compound is able to gain direct access to the cilia via the anterior and posterior (cut) surfaces of the ring. While such models supply useful information about the toxicity of compounds to the cilia, efficient mucociliary clearance is dependent upon the quantity and viscoelastic properties of the periciliary fluid and mucus as well as the number, beat frequency and co-ordination of the cilia (Mygind et al 1982).

The quaternary ammonium compound, benzalkonium chloride, halted transport irreversibly following one or two applications. Similar observations have been reported by Gallay (1960), Van de Donk et al (1980) who observed a slower onset of inhibition than we found with the frog palate. Benzalkonium chloride exhibits surfactant properties, it exerts its antibacterial action by damaging the external membrane of micro-organisms (Richards & Cavill 1976). Surface active compounds also have been shown to destroy the ciliary membrane (Summers & Gibbons 1971) and thus benzalkonium chloride might be expected to be toxic to cilia. Moreover, surfactants are likely to alter the viscoelastic nature of the mucus gel (Martin et al 1978). This dual effect could explain the faster onset of action we observed.

EDTA is an accepted preservative potentiator often used in conjunction with benzalkonium chloride. It is thought to aid penetration of the preservative into the bacterial cell by damaging certain outer layers of the cell envelope and, in some instances, to affect internal sites (Richards & Cavill 1976). It is possible that the irreversible cessation of transport caused by EDTA could be explained by a similar disruption of ciliated epithelium, since, via its chelating ability, it causes expansion of the intercellular spaces and therefore permits an increase in the permeability of the tissue to various molecules including EDTA itself (Grass & Robinson 1988). Calcium is believed to play a vital role in the regulation of ciliary activity. It is currently believed that two mechanisms exist for generating motility in cilia and eukaryotic flagella: a Mg^{2+}-dependent sliding of microtubules and a Ca^{2+}-sensitive system controlling the sliding and bending (Satir 1982). Hence, inhibition of mucus transport is likely to be related to decreased ciliary beating caused by the sequestration of Ca^{2+} and/or Mg^{2+} by EDTA. It has been stated that the calcium present in the mucus of the nose should be sufficient to counteract completely the effect of EDTA and render it of negligible toxicity (Van de Donk et al 1980). Our results suggest that this may not be the case and it should be noted that not all Ca^{2+} present in mucus is available for chelation since a proportion is bound to the glycoprotein molecule.

The cessation of transport resulting from applying phenylmercuric borate, in 0·9% NaCl, to the palate, was slower in onset than the other compounds. Although no precipitate was observed, the low solubility of the halide salts of phenylmercuric compounds is well documented therefore the experiments were repeated with mannitol as solvent. This rapidly halted transport, implying that not all the preservative was available from the saline solution.

Chlorhexidine, with its detergent-like properties, might be expected to be toxic to cilia. It is capable of thickening cervical mucus (U.S. Patent 4,590,070) which might also impair mucus transport. We found that transport was still observable after six, 10 min applications of chlorhexidine dissolved in 0·9% NaCl, however, in mannitol it halted transport after one or two 10 min applications. In saline it is likely that the dihydrochloride was formed, this has decreased solubility compared with the gluconate which is likely to explain the decreased activity observed. Van de Donk et al (1980) found chlorhexidine in Locke-Ringer solution to halt tracheal ciliary beat irreversibly after 80 min. The abundance of chloride ions in that solution might have decreased the activity.

Of the compounds investigated, thiomersal appeared the least toxic which contradicts former studies where irreversible ciliotoxicity in the absence of mucus was observed (Perrault et al 1978; Van de Donk et al 1980). This suggests that the cilia are protected by the mucus.

In comparing our results with earlier work, a number of differences emerge which may be attributed to the different models used. Since mucociliary clearance is a complex function of the physical properties of the mucus (Gelman & Meyer 1979), coupled to appropriately functioning cilia (Sleigh 1981), it is important that neither aspect is overlooked when choosing an in-vitro model. In possessing a ciliated epithelium protected by an intact layer of mucus the frog palate provides such a model.

A knowledge of the effect of preservatives on cilia protected by mucus as well as on unprotected cilia should lead to a greater understanding of the mode of action of compounds in such a system and should ultimately lead to the best choice of preservative for use in future nasal preparations.

Our results show that within the constraints of formulation and packing requirements, thiomersal should be preferentially included in nasal drops and sprays.

There are instances where a transient slowing or halt of mucus transport is desirable to increase the contact time between the active principle and nasal mucosa. Microspheres have been used as a nasal drug delivery system to increase the half life of nasal clearance (Illum et al 1987) and perhaps

those preservatives halting transport reversibly might be useful in a similar manner. It remains to be determined, however, whether a compound with a gradual but irreversibly toxic effect upon the mucociliary system is more, or less appropriate, in the long term, than a compound appearing immediately but reversibly toxic.

References

Afzelius, B. A. (1976) A human syndrome caused by immotile cilia. Science 193: 317–319

Afzelius, B. A. (1979) The immotile-cilia syndrome and other ciliary diseases. Int. Rev. Exp. Cytol. 19: 1–43

Afzelius, B. A. (1981) in: Schweiger H. G. (ed.) International Cell Biology. Springer-Verlag, Berlin, pp 440–447

Carson, J., Collier, A., Clyde, W. (1979) Ciliary membrane alterations occurring in experimental *Mycoplasma pneumoniae* infection. Science 206: 349–351

Di Sant Agnese, P. A., Davis, P. B. (1976a) Research into cystic fibrosis (first of three parts). N. Engl. J. Med. 295: 481–485

Di Sant Agnese, P. A., Davis, P. B. (1976b) Research into cystic fibrosis (second of three parts). Ibid. 295: 534–541

Di Sant Agnese, P. A., Davis, P. B. (1976c) Research into cystic fibrosis (third of three parts). Ibid. 295: 597–602

Gallay, C. (1960) Essays physiologuiques des gouttes nasales. Pharm. Acta Helv. 35: 358–374

Gelman, R. A., Meyer, F. A. (1979) Mucociliary Transference Rate and Mucus Viscoelasticity Dependence on Dynamic Storage and Loss Modulus. Am. Rev. Respir. Dis. 120: 553–557

Giordano, A. M., Shih, C. K., Holsclaw, D. S., Khan, M. A., Litt, M. (1977) Mucus clearance: *in vivo* canine tracheal vs. *in vitro* bullfrog palate studies. J. Appl. Physiol. 42: 761–766

Grass, G. M., Robinson, J. R. (1988) Mechanisms of corneal drug penetration I: *In vivo* and *in vitro* kinetics. J. Pharm. Sçi. 77: 3–14

Greenwood, G., Pittenger, R. E., Constant, G. A., Ivy, A. C. (1946) Effect of zephiran chloride, tyrothricin, penicillin and streptomycin on ciliary action. Arch. Otolaryngol. 43: 623–628

Illum, L., Davis, S. S., Faraj, N. (1987) Microspheres as a nasal drug delivery system. J. Pharm. Sci. 76: (11) S74

Iravani, J., Melville, G. N., Horstmann, G. (1978) Tracheobronchial clearance in health and disease: with special reference to interciliary fluid. In Respiratory Tract Mucus. Ciba Fndn. Symp., 54 Elsevier/North Holland, New York pp 235–252

Litt, M., Khan, M. A., Wolf, D. P. (1976) Mucus rheology: Relation to structure and function. Biorheology 13: 37

Martin, G. P., Marriott, C., Kellaway, I. W. (1978) Direct effect of bile salts and phospholipids on the physical properties of mucus. Gut 19: 103–107

Morgan, K. T., Patterson, D. L., Gross, E. A. (1984) Frog Palate Mucociliary Apparatus: Structure, Function and Response to Formaldehyde Gas. Fundam. Appl. Toxicol. 4: 58–68

Mostow, S. R., Dreisen, R. B., Manawadu, B. R., La Force, F. M. (1979) Adverse effects of lidocaine and methylparaben on tracheal ciliary activity. Laryngoscope 89: 1697–1701

Mygind, N., Pedersen, M., Nielsen, M. H. (1982) in: Procter D. F. and Andersen I. (eds) The nose, upper airway physiology and the atmospheric environment. Elsevier Biomedical Press, Amsterdam, p. 71

Perrault, G., Dreyfuss, M. F., Saunie, R. (1978) Etude de la fonction ciliaire. Application aux essais de tolérance des gouttes nasales. Sci. Techn. Pharm. 7: 383–388

Proctor, D. F. (1977) The upper airways. I. Nasal physiology and defence of the lungs. Am. Rev. Respir. Dis. 115: 97–129

Richards, R. M. E., Cavill, R. H. (1976) Electron microscope study of effect of benzalkonium chloride and edetate disodium on cell envelope of *Pseudomonas aeruginosa*. J. Pharm. Sci. 65: 76–80

Sadé, J., Eliezer, N., Silberberg, A., Nevo, A. C. (1970) The role of mucus in transport by cilia. Am. Rev. Respir. Dis. 102: 48–52

Satir, P. (1982) Mechanisms and controls of microtubule sliding in cilia. Symp. Soc. Exp. Biol. 35: 179–201

Sleigh, M. A. (1981) Ciliary function in mucus transport. Chest (suppl) 80: 791–795

Summers, K. E., Gibbons, I. R. (1971) Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. Proc. Natl. Acad. Sci. USA 68: 3092–3096.

U.S. Patent 4,590,070 (Chantler et al., assigned to I.C.I.)

Van de Donk, H. J. M., Muller-Plantema, I. P., Zuidema, J., Merkus, F. W. H. M. (1980) The effects of preservatives on the ciliary beat frequency of chicken embryo tracheas. Rhinol. 18: 119–133

Walker, T. R., Kiefer, J. E. (1966) Ciliostatic components in the gas phase of cigarette smoke. Science, 153: 1248–1250

Wood, R. E., Boat, T. F., Doershuk, C. F. (1976) State of art: cystic fibrosis. Am. Rev. Respir. Dis. 113: 833–878