The Use of a Radiolabelled Saccharin Solution to Monitor the Effect of the Preservatives Thiomersal, Benzalkonium Chloride and EDTA on Human Nasal Clearance

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Abstract—The effect of thiomersal, benzalkonium chloride and ethylenediaminetetraacetic acid (EDTA) on the nasal mucociliary clearance of healthy volunteers has been investigated using a modified saccharin test and gamma scintigraphy concomitantly. A significant correlation was found between the two techniques. Using each subject as his/her own control, none of the preservatives significantly altered the rate of clearance or proportion cleared from the nasal cavity after the administration of a single dose. This result is at variance with some in-vitro findings.

Nasal mucociliary clearance is the mechanism by which inhaled particulate matter is cleared from the nasal cavity. In-vivo nasal mucociliary clearance may be evaluated by the observation, directly or indirectly, of the passage of a marker substance through the nasal cavity. Marker substances observed directly include dyes and particles (Yates 1924; Tremble 1948; Ewert 1965; Bang et al 1967). In addition, saccharin particles have been placed on the nasal mucosa and the time at which the subject reported a sweet taste recorded as a measure of nasal clearance time (Andersen et al 1974; Rutland & Cole 1981). Blue-stained saccharin particles have been used to verify the arrival of the particle at the pharynx by visual inspection (Hady et al 1983) and a saccharin and dye solution has also been employed (Van de Donk et al 1982). Indirect measurements of human nasal clearance have been made from the external monitoring of intranasally administered radioactive or radio-opaque markers. Such markers have consisted of radiolabelled suspensions (Proctor & Wagner 1965), a single radiolabelled particle (Quinlan et al 1969), radiolabelled solutions (Aoki & Crawley 1976) or radio-opaque Teflon discs (Yergin et al 1978) and barium sulphate particles (Hady et al 1983). The measurement of the transit of suspensions or solutions is particularly relevant when considering the fate of drugs or vaccines administered intranasally.

The effect of preservatives on the beat frequency of explants of ciliated tissue and transport rate of particles over a ciliated mucosa has been studied in-vitro (Greenwood et al 1946; Gallay 1960; Perrault et al 1978; Mostow et al 1979; Van de Donk et al 1980; Stanley et al 1985; Batts et al 1989, 1990). However, the results of such studies are equivocal and it appears that totally different results can be obtained depending upon whether mucus is present or not. Furthermore, there have been few investigations into the effect of preservatives on human nasal clearance. Van de Donk et al

(1982) reported a slowing of nasal mucociliary clearance after administration of benzalkonium chloride with ethylenediaminetetraacetic acid (EDTA), while benzalkonium chloride alone did not affect mucociliary clearance (Holmberg & Pipkorn 1986). The effect of thiomersal and EDTA alone on human nasal clearance has not been reported. Compromised mucociliary clearance can subject the sufferer to chronic respiratory infection due to the retention of hazardous substances within the respiratory tract (Wolff 1986). Since preservatives are present in most nasal drops and sprays to prevent growth of micro-organisms which might otherwise contaminate the product upon its repeated use, it is important to establish the effect of pharmaceutical preparations containing preservatives on mucociliary clearance in-vivo. Not only will this aid the selection of a suitable preservative for human use, but it will also help to resolve the question of which is the most predictive in-vitro model. This is the first report of a study that has been specifically designed to investigate the effect of preservatives on human nasal clearance using a modified saccharin test and gamma scintigraphy concomitantly.

Materials and Methods

Preservative solutions were prepared by dissolving the appropriate amount of preservative in 0.9% NaCl (saline) to give 0.01% w/v thiomersal, 0.01% w/v benzalkonium chloride and 0.1% w/v EDTA. Saline was used as the control solution. Preservative and control solutions were administered as drops sixty minutes before the administration of the radiolabelled nasal spray.

The nasal spray solution contained 2 mg mL^{-1} saccharin in saline and was radiolabelled with ^{99}Tc-labelled diethylenetriaminepentaacetic acid ([^{99m}Tc]DTPA) prepared from a kit (Amerscan Pentetate II Technetium Agent, Amersham International plc, UK). The resultant solution contained approximately 10 MBq mL^{-1} ^{99m}Tc.

The spray was administered using spray applicator pumps (Perfect Valois Ltd, UK) having an ejection volume of 100–200 μL.

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In-vitro studies

Twenty two healthy volunteers, eighteen males and four females, aged 19–27 years, participated. None of the subjects had a history of chronic nasal or pulmonary disease or were suffering from any respiratory tract infection. The effect of the control solution on nasal clearance was assessed during the first session of the study and compared with the effect of the preservative solution, studied during the second session, at least 24 h later. Each volunteer acted as his or her own control. The study was approved by the local ethical committee and each volunteer gave informed written consent.

The appropriate preservative or control solution (0·3 mL) was administered, as drops, from an 0·5 mL plastic syringe into the entrance of one nostril while the subject was supine (Mygind 1979). The subject's head was tilted back during instillation of the drops and was then turned to the right and left and then returned to the original position before the subject sat up with the head tilted forward. Each position was held for 30 s. Such manoeuvres were carried out to ensure good coverage of the nasal cavity by the administered solution. The nasal spray device containing radiolabelled saccharin solution was primed by ejecting ten sprays. One hour after dosing with drops of preservative or control solution, the subject administered the spray by inserting the spray applicator tip approximately 1 cm into the same nostril and ejecting one spray dose. The subject was upright during the administration procedure. In addition, the design of the trial enabled the deposition site of the radiolabelled saccharin solution to be compared after administration as a nasal spray and as nasal drops, on separate occasions, in three of the subjects. This enabled a check on whether the radiolabelled spray was reaching the same area that had been treated with the preservative in drop form.

The deposition and subsequent clearance of the radiolabel was monitored using a gamma camera fitted with a low energy parallel-hole collimator and tuned to detect the peak γ-radiation of 140 keV in a 20% energy window of detection. The subject was seated with his or her head placed laterally against the vertical collimator surface with the nostril of interest nearest the detector. Subjects were consistently positioned with the aid of a nose support to allow accurate alignment of images. One min after administration of the radiolabelled saccharin spray, an image was recorded for 60 s. The subject was then imaged every 10 min for 2 h. All images were recorded and stored on magnetic tape for subsequent analysis. Stored images were recalled and displayed on a visual display unit and regions of interest were created defining the site of deposition, the turbinates, the glottis, the nasopharynx and the whole nasal cavity. The number of counts detected within each region was corrected for background counts and radioactive decay. Subjects were instructed to report the perception of any sweet taste, the time of which was recorded. The relative humidity was measured at each session using a whirling hygrometer (Casella, UK).

Results

For each volunteer, percentage activity at the site of deposition, turbinates and glottis was plotted against time.
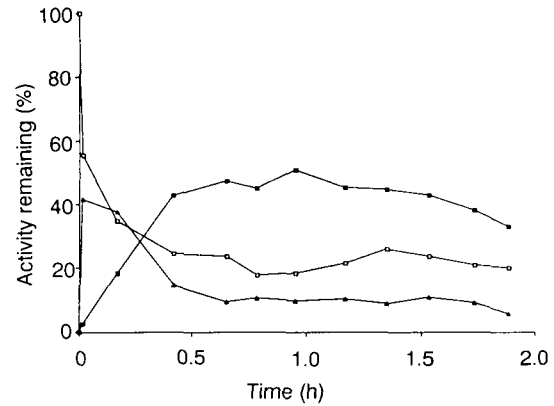

FIG. 1. Deposition and clearance profile after pre-dosing with saline. □ clearance from deposition site, ▲ clearance from turbinates, ■ clearance from glottis.

Fig. 1 shows an example from one volunteer after pre-dosing with saline. As radiolabel was cleared from the site of deposition the percentage activity increased correspondingly in the turbinates and then in the glottis. This general pattern of clearance was consistent for most volunteers. Data concerning clearance from the deposition site are presented in this paper since any inhibitory effect caused by a preservative would ultimately affect clearance from this site.

The deposition and clearance of the ^{99m}Tc-DTPA solution administered as a nasal spray was compared with the deposition and clearance of the same solution administered as drops. The area covered by the drops was larger than that of the spray and, within the constraints imposed by viewing the nasal cavity laterally, appeared to include the area of deposition of the radiolabelled spray. The images indicated the presence of 25–30% of the radiolabelled saccharin solution at the nasopharynx almost immediately after dosing as drops. This was confirmed by the subjects reporting a sweet taste. Rates of clearance of the nasal spray from the deposition site were found to be biphasic. The first phase was faster than the second and rarely lasted longer than 20 min.

In total eighteen saccharin detection times were obtained from the study (of a possible 44). These were plotted against the clearance half-time of the first phase of clearance from the deposition site (Fig. 2) and percentage activity at the
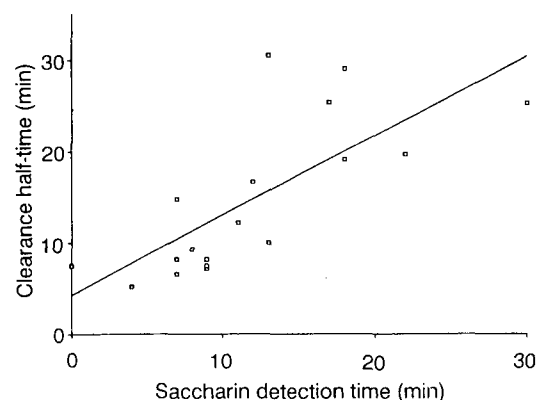

FIG. 2. Relationship between the clearance half-time of the first phase of clearance from the deposition site and saccharin detection time. Correlation coefficient = 0·74. P < 0·001.
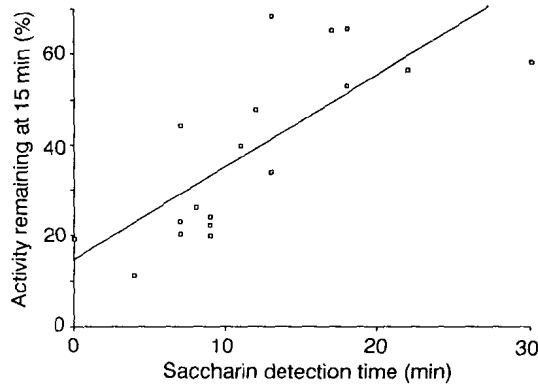
FIG. 3. Relationship between % activity remaining at the deposition site after 15 min and saccharin detection time. Correlation coefficient = 0·76. $P < 0·001$.

deposition site after 15 min (Fig. 3). A positive correlation (t-test) was found to exist between saccharin detection time and both these parameters.

Of the 22 subjects participating in the study, only 10 reported perceiving a sweet taste following pre-dosing with both placebo and preservative. Of these, three were excluded from the analysis because the tracer solution was administered as a spray on one occasion and as drops on the other, so the results were not comparable. Of the remaining seven subjects, five had received EDTA. Table 1 shows the time, following administration of the radiolabelled saccharin spray, when each subject reported the perception of a sweet taste (saccharin detection time) following dosing with saline or EDTA. The Table also shows the corresponding percentage activity at the deposition site at t = 15 min and half-times of the first phase of clearance. The change in these parameters following pre-dosing with EDTA illustrates an increase in clearance but statistical testing using Wilcoxon's matched-pairs signed-ranks test indicated the differences were not significant ($P > 0·05$).

Figs 4–6 show plots comparing the mean clearance (± s.d.)

Table 1. Saccharin detection times, percentage activity at deposition site at t = 15 min and clearance half-times of the first phase of clearance after pre-dosing with saline or EDTA 0·1%.

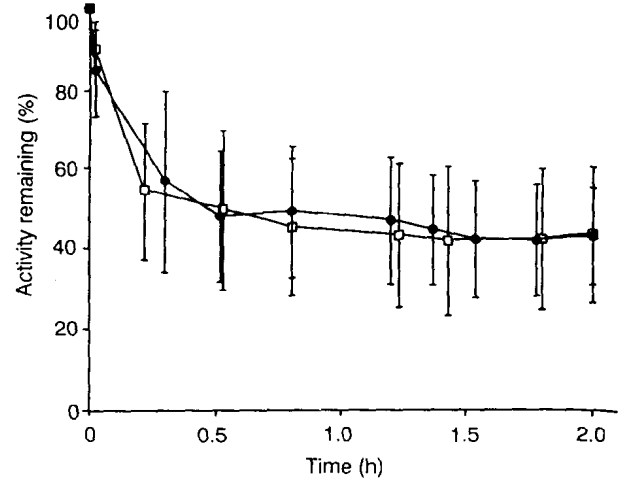| Subject	Saline	EDTA 0·1%
Saccharin detection time (min)		
1	11	9
2	22	9
3	8	7
4	18	8
5	18	7
% Activity (t = 15 min)		
1	39·8	19·7
2	56·5	24·0
3	25·9	23·1
4	65·4	66·8
5	53·1	44·2
Clearance half-time (min)		
1	12·2	7·2
2	19·8	7·5
3	9·3	8·3
4	29·2	39·1
5	19·2	14·8

FIG. 4. The effect of thiomersal on mean clearance from the deposition site. □ Normal saline, ● thiomersal. Mean ± s.d. of measurements made from 5 subjects.
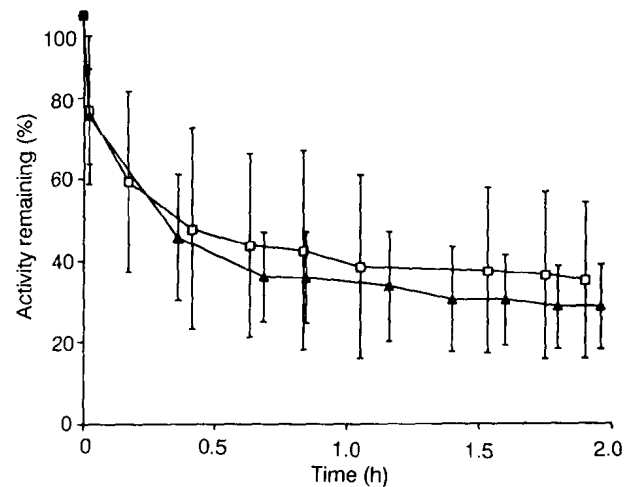

FIG. 5. The effect of benzalkonium chloride on mean clearance from the deposition site. □ Normal saline, ▲ benzalkonium chloride. Mean ± s.d. of measurements made from 6 subjects.
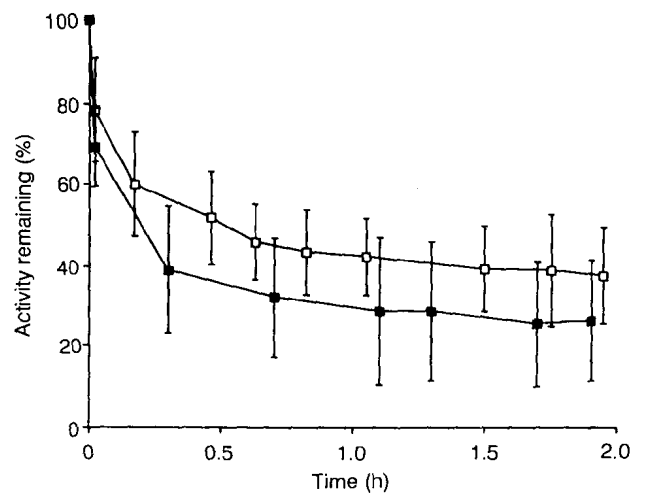

FIG. 6. The effect of EDTA on mean clearance from the deposition site. □ Normal saline, ■ EDTA. Mean ± s.d. of measurements made from 5 subjects.

Table 2. Mean clearance half-times (min) of the first and terminal phase of clearance following the administration of saline or preservative (±s.d.).

Test solution	First phase	Terminal phase
Saline	16·5 (±8·0)	441 (±100)
Thiomersal	21·2 (±15·5)	352 (±107)
Saline	17·9 (±7·7)	321 (±314)
EDTA	15·4 (±13·6)	231 (±120)
Saline	21·9 (±25·4)	274 (±105)
Benzalkonium chloride	19·7 (±10·7)	317 (±365)

from the deposition site after administration of thiomersal, benzalkonium chloride and EDTA (to 5, 6 and 5 subjects, respectively) compared with the same parameter determined after the administration of saline. Each subject acted as his or her own control. The pattern of deposition and clearance following the administration of saline and each preservative was the same, and followed biphasic clearance on logarithmic plots (not shown). Clearance rates, expressed as clearance half-times in minutes calculated by the least squares fit of regression lines constructed for the first and terminal phase of each curve are given in Table 2. The clearance rate of the [^{99m}Tc]DTPA/saccharin solution was not significantly altered ($P > 0.05$) by any of the preservatives (Wilcoxon's matched-pairs signed-ranks test). In addition there was no significant difference ($P > 0.05$) in the proportion of radiolabelled nasal spray cleared from the site of deposition at 10, 20 30, 60 and 90 min after administration, following treatment with saline or preservative (Mann-Whitney U-test).

Procedures were carried out at a temperature of 26·4 ± 0·4°C and a relative humidity of 64·6 ± 3·3%.

Discussion

A control pattern of deposition and clearance of [^{99m}Tc]DTPA/saccharin nasal spray was determined 1 h after pre-dosing with drops of saline. The results suggest that within 20 min of instillation an average of approximately 50% of the dose from the nasal spray reaches, and is subsequently cleared from, the ciliated zones in the main nasal passage where it would be required for topical therapy and where rapid absorption of compounds into the systemic circulation is likely to occur (Parr 1983). During the first phase of clearance, only rapidly absorbed compounds are likely to achieve therapeutic concentrations in the systemic circulation. This includes small lipophilic molecules which are rapidly absorbed (Hirai et al 1981), but may also include low molecular weight polar compounds which give a high degree of absorption if permitted a sufficient contact time of 10–20 min (McMartin et al 1987). If it is assumed that most of the remaining nasal spray is cleared to the nasopharynx at the slower rate of the second clearance phase, then the nasal route may be exploited for drugs with an even longer absorption half-life.

A surprisingly high number of volunteers were unable to detect a sweet taste on one or both occasions following administration of the radiolabelled saccharin solution. Of these, gamma scintigraphy showed that some volunteers were not clearing the nasal spray which was retained at the

site of deposition. Others, however, were unable to detect the saccharin even though they appeared to be clearing normally and had been able to detect saccharin on another occasion. Those saccharin detection times reported were in agreement with those of earlier studies (Andersen et al 1974; Yergin et al 1978; Puchelle et al 1981; Sakakura et al 1983) and ranged from zero to 30 min with a mean of 12 min.

The main techniques of assessing nasal mucociliary clearance in man are the saccharin test and the use of radiolabelled or radio-opaque markers. These have been compared, although rarely simultaneously, with varying degrees of correlation (Andersen et al 1974; Yergin et al 1978; Puchelle et al 1981; Hady et al 1983; Brondeel et al 1983; Sakakura et al 1983). In the present study a modified saccharin test and the technique of gamma scintigraphy have been combined to determine which, if any, is most efficient at detecting the ciliostatic effect of preservatives. Comparison of the transport of radiolabelled or radio-opaque particles with that of saccharin particles suffers from the problem of the solubility and clearance of saccharin in the periciliary fluid as well as the mucus (Passali et al 1984). It has been shown (Lucas & Douglas 1934) that transport may occur in the periciliary fluid when the overlying gel, and therefore deposited particles, is stationary. By presenting the saccharin and radiolabel to the nasal epithelium in the same solution this problem appears to have been avoided which probably contributed to the strong correlation between the two techniques. However, a recent study (Cheema et al 1988) suggested that [^{99m}Tc]DTPA binds avidly to respiratory tract mucus glycoprotein, a result which implies that the radiolabel may still have been associated with the gel layer, while the saccharin was free to diffuse into the periciliary fluid.

In the absence of the occurrence of ciliostasis, the highly significant correlation ($P < 0.001$) between the saccharin detection times and the first-phase clearance half-time from the deposition site and the percentage activity at the deposition site at 15 min suggests that the technique of gamma scintigraphy and the modified saccharin test are equally suitable for assessing ciliotoxicity in-vivo. The greater simplicity and reduced cost of the saccharin test might overcome its disadvantage associated with the number of volunteers giving false results by an inability to detect saccharin.

The preservatives investigated in this study were chosen on the basis of their effects in-vitro. From the results of studies employing the frog palate, where the ciliated epithelium is protected by a layer of mucus, benzalkonium chloride and EDTA might have been expected to inhibit or halt nasal mucociliary clearance whereas thiomersal should have been well tolerated (Batts et al 1989). In contrast, the results of in-vitro studies where the protective effect of mucus is negligible suggest that benzalkonium chloride and thiomersal should inhibit or halt nasal clearance (Greenwood et al 1946; Gallay 1960; Perrault et al 1978; Van de Donk et al 1980; Stanley et al 1985; Batts et al 1990). The effect of EDTA would have been difficult to predict from the variety of in-vitro results (Lee & Verdugo 1976; Van de Donk et al 1980; Stanley et al 1985; Batts et al 1990). In-vitro studies have reported ciliostasis in the presence of benzalkonium chloride (0·01%) after as little as 5 min (Stanley et al 1985) or as long as 45 min (Gallay 1960). Therefore a time of 60 min between appli-

cation of preservatives and monitoring of nasal clearance was chosen to allow the preservatives time to exert an effect. Most in-vitro studies have shown any cilio-inhibitory effects of the preservatives studied to be irreversible. Despite the in-vitro predictions, this study showed that 0·3 mL thiomersal (0·01%), benzalkonium chloride (0·01%) and EDTA (0·1%), administered as drops 1 h before clearance measurement, was well tolerated by the nasal epithelium and did not significantly alter the rate of nasal spray clearance. It is possible that any differences caused by the preservatives may have been masked by the wide variations in clearance rates measured, since each preservative was administered to only five or six volunteers. However our findings for the effect of benzalkonium chloride on clearance support those previously reported by Holmberg & Pipkorn (1986) and it would appear that these compounds do not alter nasal clearance in-vivo.

A variety of reasons may explain the discrepancies between the in-vitro and in-vivo observations. Dilution of the preservative by nasal secretion might be expected to reduce the ciliotoxic effect of a preservative in comparison with in-vitro studies. The exact volume of nasal secretion is unknown but has been approximated to be 0·4 mL (Stanley et al 1985). In the present study this would halve the concentration of preservative applied. Dilutions far in excess of this seem to be necessary to prevent the cilio-inhibitory effects of preservatives in-vitro (Stanley et al 1985; Batts 1989), so while dilution may reduce the toxicity of the preservative it is unlikely to be the sole factor in abolishing it.

Cilia in-situ may have a higher resistance than cilia in-vitro or the cilia of the nasal mucosa may be protected by the mucus blanket. The barrier function of mucus is highlighted by the ciliotoxicity of thiomersal in the absence of mucus (Perrault et al 1978; Van de Donk et al 1980; Stanley et al 1985; Batts et al 1990) in contrast with its innocuity when applied to the frog palate (Batts 1989). It seems possible that mucus, whether lining the frog palate or the human nasal cavity, may prevent thiomersal from interacting with the underlying epithelium. By way of contradiction, diffusion experiments have shown that, after a considerable lag time, thiomersal is able to cross a 1 mm thick layer of purified mucus glycoprotein and is, therefore, likely to gain access to the cilia of the palate or nasal cavity (Batts 1989). Any delay in the time taken for thiomersal to reach the cilia, in combination with mucociliary clearance, might prevent sufficient contact time for the compound to exert its ciliotoxic effect.

In-vitro studies of the effect of EDTA on ciliary beat frequency in the absence of mucus present contradictory results. Those studies reporting ciliotoxicity suggest that a lack of extracellular calcium may be responsible. EDTA was observed to halt transport rate over the frog palate after one or two applications, although in the presence of calcium, in excess of that required to saturate the chelation sites of EDTA, it was well tolerated (Batts et al 1989). The frequency at which cilia beat is modulated by intracellular Ca^{2+} concentration (Girard & Kennedy 1986) and cultured tracheal cilia from the rabbit have been shown to increase their beat frequency via an increase of intracellular calcium ions, in response to mechanical stimulation (Sanderson & Dirksen

1986). Mechanical stimulation is required to initiate ciliary beating and therefore mucociliary transport over the frog palate (Spungin & Silberberg 1984). Therefore, depletion of extracellular calcium by EDTA may be expected to be of greater significance to the ciliary activity of the frog palate, which will need to increase and maintain intracellular calcium concentration in order to respond to stimulation, than to cilia of higher vertebrates which maintain a baseline activity in the absence of stimulation. Such a hypothesis might explain why EDTA did not appear to affect nasal mucociliary clearance in-vivo despite its effect on transport over a frog palate and implies that the frog palate may possess certain drawbacks as an in-vitro model of the human nasal mucosa when assessing drugs that interact with calcium ions. There are of course other possible explanations for the lack of toxicity of EDTA in-vivo. The concentration of calcium in human nasal secretions has been reported to be $8·1 \pm 2·0$ mequiv L^{-1} (Lorin et al 1972), which may reduce the ciliotoxicity of EDTA in-vivo, through complex formation. In addition, [^{14}C]EDTA has been shown to bind avidly to respiratory mucus glycoprotein and it is unlikely that appreciable amounts of EDTA are able to cross mucus layers of physiological thickness over periods of 4–5 h (Cheema et al 1988). During the course of the in-vivo investigation, it is improbable therefore that a toxic amount of EDTA diffused through the mucus layer to interact with the cilia. Both of these mechanisms are likely to become saturated when applying EDTA to the frog palate indicating that EDTA may appear ciliotoxic in-vitro but not in-vivo.

From the findings in the present study, it is concluded that the preservatives thiomersal, benzalkonium chloride and EDTA do not affect nasal mucociliary clearance. Although the study does not reflect the clinical situation in which subchronic administration is more usual, the lack of effect suggests that the preservatives investigated would not exert a significant ciliotoxic action in-vivo. In-vitro studies on explants of ciliated epithelium from various species did not predict this and must be considered limited in their usefulness. Studies on the frog palate, employed as a model of the human nasal epithelium, predicted the non-toxicity of thiomersal, but not benzalkonium chloride or EDTA (Batts et al 1989). However, it should be recognized that in-vitro conditions used (0·3 mL of preservative applied to the small area of the palate for 10 min every 15–20 min) were likely to present a more severe challenge to the protective effect of the mucus than a single application of a similar volume to the human nasal epithelium. The frog palate might provide a more useful model of the nasal mucosa if compounds were applied at reduced concentrations, in line with the dilution capacity of nasal secretion. The use of smaller volumes might also be appropriate although transport over the palate is still likely to occur in the absence of a full complement of working cilia, as it is in the nasal cavity, since the physical properties of the mucus enables small unciliated areas of epithelium to be bridged (Lucas 1933). Such modifications might be necessary if other animal models were employed and the ease of access to the frog palate with minimal trauma to the ciliated epithelium suggests that, with modification, its use as an in-vitro model of human mucociliary clearance is preferable.

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