J. Pharm. Pharmacol. 1993, 45: 126–130
Received March 11, 1992

Bromhexine plus Oxytetracycline: the Effect of Combined Administration Upon the Rheological Properties of Mucus from the Mini-pig

GARY P. MARTIN, BERNARD E. LOVEDAY AND CHRISTOPHER MARRIOTT

Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, UK

Abstract—Three adult mini-pigs were employed to assess the effects of a twice daily dosage (40 mg kg^{-1}) of oxytetracycline hydrochloride (OTC) and a combination of OTC with (0·5 mg kg^{-1}) bromhexine hydrochloride (BHC) on the rheological properties and wet weight of secreted tracheal mucus. Mucus was collected daily from open-ended tracheal pouches established surgically in the mini-pigs. After a five day control period, either OTC or OTC plus BHC was administered twice daily with the normal diet. Each study period was followed by a five day washout period when mucus was collected but no drug given. The viscoelastic properties of each mucus sample were determined using creep compliance analysis. OTC was shown to increase the residual shear viscosity ($P<0·01$) and increase the instantaneous compliance ($P<0·01$). An increase in the wet weight of the collected mucus occurred in one pig only ($P<0·01$). When BHC was co-administered with OTC, all of these changes were abolished. Evidence was obtained to suggest that BHC increased the concentration of OTC within the secreted mucus. BHC appeared to reverse the mucospissic activity of OTC in-vivo.

Antibiotic chemotherapy is routinely employed to control bacterial infections associated with acute exacerbations of chronic obstructive airways disease. Tetracycline antibiotics have been used extensively for this purpose in both man and animals when penicillin-resistant microorganisms have been isolated from sputum. However, Marriott & Kellaway (1975) found that the direct addition of a range of tetracyclines to human bronchial mucus in-vitro caused an increase in the viscosity and elasticity of the gel. Structural change was found to be induced by oxytetracycline hydrochloride (OTC) although the concentrations of tetracycline which induced significant rheological changes in this in-vitro study were unrealistically high when compared with those attained in bronchial secretions in-vivo (Campbell 1970; Bach & Leary 1972; Abdou & Kotzian 1975). However, should increased mucus viscosity and elasticity occur in-vivo as a consequence of systemic tetracycline delivery, then this would have obvious implications for the clearance of mucus from the infected airways of compromised patients.

Several previous studies have shown that the co-administration of a mucolytic with antibiotic can increase both the sputum and lung concentrations of the latter (Burgi & Stauffer 1971; Spatola et al 1987; Ricevuti et al 1988). Bromhexine, a drug which has been used clinically for a number of years as a mucolytic compound, has been shown to increase significantly the sputum concentration of OTC in bronchitic patients by between twice (Burgi et al 1968) and over three times (Bach & Leary 1972) compared with the antibiotic given alone. Bromhexine hydrochloride (BHC) has been shown to reduce significantly the viscosity and elasticity of tracheal mucus secreted by the mini-pig in-vivo (Martin et al 1990). Accordingly, the aim of the present study was to determine any changes which may occur in the rheological properties of mucus when OTC was administered systemically and also to examine the effect of co-administration of BHC. A second aim was to determine whether the combined administration of mucolytic and antibiotic resulted in higher mucus levels of antibiotic being attained than if the latter were administered alone. The study was undertaken using open-ended tracheal pouches established in mini-pigs (Martin et al 1990).

Correspondence: G. P. Martin, Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, UK.

Materials and Methods

Drugs

Bromhexine hydrochloride was provided by Boehringer Ingelheim Ltd, Bracknell, Berks, UK. Oxytetracycline hydrochloride was administered in a water-soluble powder base (Terramycin Soluble Powder; Pfizer Ltd, Sandwich, Kent, UK).

Animal model and drug administration

Open-ended tracheal pouches were surgically established in three adult pure-bred mini-pigs (*Suis scrofa domestica*, Gottingen strain) as described previously (Martin et al 1990). Catheters, linked to an isolated segment of five cartilage rings, were exteriorized on the back of the neck of the animal. Mucus samples were collected daily by flushing the pouches with sterile isotonic saline using gentle pressure applied via a syringe to one catheter and collecting via the second. The thirty day study period in each pig was divided into two fifteen day sections and compounds administered according to the regimen shown in Scheme 1. The first five days before

Day	Study period	Treatment
1–5	Control	None
6–10	Drug	Oxytetracycline hydrochloride (40 mg kg^{-1}) twice daily
11–15	Washout	None
16–20	Control	None
21–25	Drug	Oxytetracycline hydrochloride (40 mg kg^{-1}) plus bromhexine hydrochloride (0·5 mg kg^{-1}) both twice daily
26–30	Washout	None

SCHEME 1. Summary of the experimental protocol.

drug administration served as a control period. For the next five days, either OTC (40 mg kg^{-1}) or a combination of OTC (40 mg kg^{-1}) with BHC (0·5 mg kg^{-1}) was given orally, twice daily, mixed with the normal diet. Mucus was collected for another five days after drug administration had ceased, to provide a washout period. The saline washings from the pouch were immediately frozen and stored at −20°C until required. After thawing, the mucus gel was separated from the saline supernatant by low speed centrifugation.

Creep compliance analysis

Mucus samples were weighed and placed between the cone and plate of a variable stress rheometer (Petronics Viscoelastic Analyser, Integrated Petronics, London, UK). A small, constant stress was applied instantaneously to the material and the resultant strain measured by following the angular displacement of the cone with time (Martin et al 1973). Strain was converted to compliance by dividing by the applied stress and the resultant curve analysed in terms of a line spectrum (Barry 1974) to produce a residual shear viscosity (η_o) and an instantaneous shear compliance (J_o), the latter term being the reciprocal of elasticity.

Data representation and analysis

Values for the viscosity, compliance and wet weight of the mucus on days 2–5 and 17–20, days 7–10 and 22–25, and days 12–15 and 27–30 inclusive were taken as being representative of control, drug treatment and washout periods, respectively. This makes the assumption that the effects of the drugs are both manifest upon and eliminated from the mucus secretions within 24 h. Mean values for each parameter are shown in Table 1. Each untransformed data set was tested by three-way (drug treatment, animal, day number) analysis of variance. If the source of variation was attributable to drug effects, then mean values for each of the three treatment periods (control, drug and washout) were further analysed for statistical difference (Table 2) using Dunnet's multiple range test (Montgomery 1976).

There is a large inter- and intra-animal variation in the properties of the collected mucus (Table 1) and in order to represent graphically the effects of any drug treatment on three different animals, the technique of normalizing the data and plotting as four-day running means was employed, as described previously (Martin et al 1990). In brief, both rheological parameters (J_o and η_o) were normalized by finding the highest value in each data set (i.e. a period which consisted of 30 study days per pig), expressing this as 100% and calculating all other data points relative to this value. Four-day running means (n = 3) were then obtained through each 15 study day portion and graphs plotted of mean percentage in viscosity or compliance as a function of time (Figs 1, 2). Since changes in the wet weight were shown to be animal-dependent, mean percentage changes could not be calculated and therefore the percentage change for each of the three pigs was plotted separately (Fig. 3). It must be emphasized that depiction of the data in this manner (Figs 1–3) only enables clear trends, in response to drug treatment, to be visually represented; the statistical analysis of the original data enables the significance of any induced change to be assessed.

Assay of oxytetracycline

Both the mucus and saline portions of the daily samples were weighed and then assayed for OTC concentration employing an agar diffusion method using the punch hole technique and with *Bacillus cereus* (ATCC 11778) as test organism. Spore suspensions were prepared according to the method of Farrell & Williams (1978) and 1 mL quantities used to inoculate 250 mL molten agar.

A stock solution of OTC was prepared in sterile saline at a concentration of 1·8 g mL^{-1} and from this, standard solutions were prepared at concentrations of 0·9, 0·3 and 0·1 g mL^{-1} in either saline or mucus. The mucus standards were made by adding a known weight of drug-free mucus to an equal weight of the appropriate concentration of oxytetracycline stock solution and homogenizing by shaking and sonication.

An 8 × 8 Latin-square design was used, incorporating eight replicates of each standard solution and eight replicates of each of five samples. Those plates with mucus samples contained standard solutions in saline alone. The plates were left for 30 min before incubation at 30°C for 18 h.

The diameters of the resulting zones of inhibition were measured to an accuracy of 0·1 mm. Unknown concentrations were interpolated from linear standard curves (log concentration being plotted as a function of zone diameter).

OTC detected in the saline washings was assumed to have been associated with the mucus secreted in the pouch. Accordingly, the total amount removed in the wash solution could be calculated, using the weight of the collected saline. This amount of OTC was then added to the total amount of OTC recovered from the mucus. This sum was divided by the weight of the collected mucus to obtain the reported mucus concentrations of antibiotic (Table 3).

Table 1. Mean values (s.d.) for the residual shear viscosity (η_o), instantaneous compliance (J_o) and wet weight for individual animals during each study period. On days 7–10, pigs were treated twice daily with 40 mg kg^{-1} oxytetracycline hydrochloride (OTC) and on days 22–25 with OTC plus 0·5 mg kg^{-1} bromhexine hydrochloride (BHC).

		η_o (N s m^{-2}) × 10^{-4}			J_o (m^2 N^{-1}) × 10^3			Wet wt (mg)		
Day	Study period	Pig 1	Pig 2	Pig 3	Pig 1	Pig 2	Pig 3	Pig 1	Pig 2	Pig 3
2–5	Control	7·27 (5·02)	2·54 (1·97)	2·31 (0·91)	1·00 (0·34)	2·50 (1·79)	2·55 (0·87)	441 (89)	695 (95)	877 (159)
7–10	Drug treatment (OTC)	32·86 (11·36)	10·69 (7·81)	8·04 (4·12)	0·47 (0·26)	1·70 (1·29)	1·10 (0·46)	2052 (961)	761 (42)	1018 (131)
12–15	Washout	15·98 (7·16)	2·61 (2·39)	1·90 (1·63)	0·71 (0·18)	3·01 (1·97)	3·76 (1·27)	634 (49)	932 (63)	870 (131)
17–20	Control	17·21 (5·92)	4·58 (2·29)	1·84 (0·45)	0·67 (0·49)	2·72 (0·95)	2·71 (0·06)	604 (231)	624 (208)	671 (296)
22–25	Drug treatment (OTC plus BHC)	19·69 (8·26)	1·65 (1·14)	2·24 (0·51)	0·55 (0·39)	4·80 (3·80)	2·09 (0·30)	948 (187)	617 (77)	942 (352)
27–30	Washout	17·79 (10·71)	4·09 (1·58)	4·01 (2·00)	0·74 (0·31)	4·02 (1·88)	2·66 (1·06)	831 (259)	578 (152)	587 (195)

Table 2. Significance levels for the differences in η_o and J_o obtained during the various periods of study.

Drug treatment	Oxytetracycline hydrochloride (40 mg kg^{-1}) twice daily		Oxytetracycline hydrochloride (40 mg kg^{-1}) plus bromhexine hydrochloride (0·5 mg kg^{-1}) twice daily	
	η_o	J_o	η_o	J_o
Control vs drug	0·01	0·01	NS	NS
Drug vs washout	0·01	0·01	NS	NS
Control vs washout	NS	NS	NS	NS

NS denotes not significant ($P > 0·05$).

Results

The values obtained for η_o, J_o and wet weight for each pig at each stage of the study are shown in Table 1. Consistent with a previous investigation (Martin et al 1990), a large inter- and intra-animal variation was found in the rheological properties of the collected mucus. It is, however, apparent from the results that the administration of OTC increased the viscosity (η_o) of the secreted mucus in all three pigs (Table 1). This effect was found to commence within 24 h of administering the first dose of OTC, when the mucus which had accumulated in the pouches in the intervening time was collected and subjected to rheological analysis. The increase in η_o of mucus was accompanied by a decrease in J_o (Table 1) and both of these changes were shown to be significant ($P < 0·01$, Table 2). After OTC was withdrawn, the viscosity (Fig. 1) and compliance (Fig. 2) returned to control values, there being no significant difference in J_o and η_o of mucus collected during control and washout periods (Table 2). There was a general increase in the wet weight of the collected mucus in response to OTC treatment in all three pigs (Table 1; Fig. 3); however, only for one animal (pig 1) was this increase shown to be significant ($P < 0·01$). Concurrent with the administration of OTC to pig 1, a large increase in mucus wet weight occurred whilst when the drug was withdrawn, the weight of the collected mucus decreased (Fig. 3). In pig 2, there was an increase in wet weight after OTC was given, but this was less marked than in pig 1 and the weight of mucus collected continued to increase after drug treatment had ceased. The wet weight of the collected mucus increased immediately OTC was administered to pig 3, but again this response was much less than that found in pig 1. The mean concentration of OTC in the mucus samples collected during the drug treatment period was also animal-dependent (Table 3). The OTC concentration was less than 0·1 μg g^{-1} in two of the pigs and was not detected by the assay employed, whereas in pig 1 the mean mucus concentration drug treatment with the antibiotic was 0·42 μg g^{-1}.

Table 3. Mean mucus concentration (s.d.) of oxytetracycline hydrochloride (μg g^{-1}) during treatment twice daily with 40 mg kg^{-1} oxytetracycline hydrochloride alone (OTC) or OTC plus 0·5 mg kg^{-1} bromhexine hydrochloride (BHC).

	OTC	OTC + BHC
Pig 1	0·42 (0·23)	1·83 (0·77)
Pig 2	ND	ND
Pig 3	ND	0·16 (0·32)

ND denotes not detected (concentration of OTC $< 0·1$ μg g^{-1}).

When BHC was co-administered with OTC the changes in viscosity and compliance which occurred in response to OTC

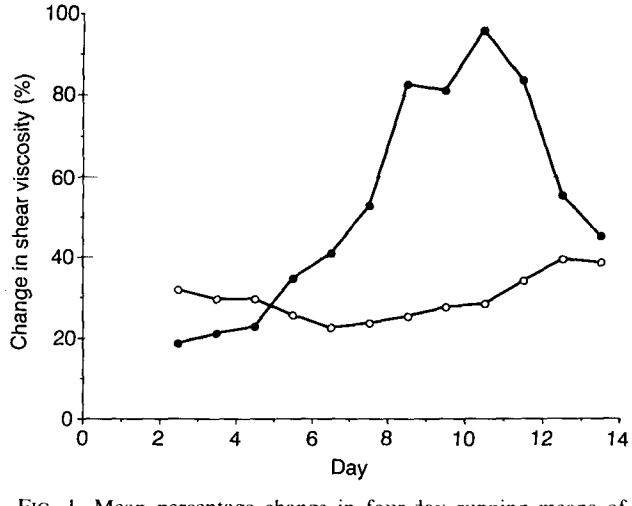

FIG. 1. Mean percentage change in four-day running means of residual shear viscosity (η_o). ● OTC alone, ○ OTC plus BHC.

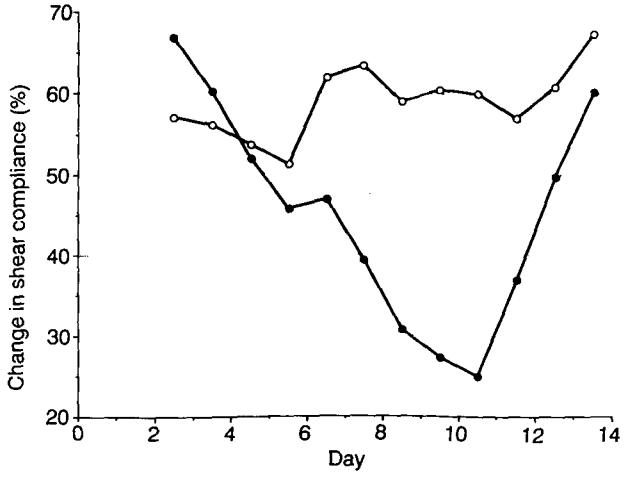

FIG. 2. Mean percentage change in four-day running means of instantaneous shear compliance, normalized to the highest value. ● OTC alone, ○ OTC plus BHC.

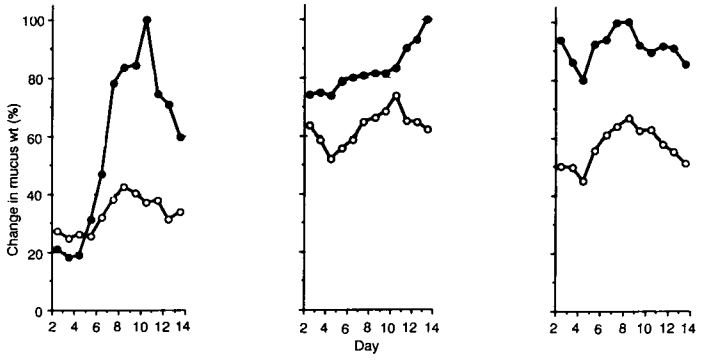

FIG. 3. Percentage change in four-day running means of wet weight of mucus collected from each of three pigs, normalized to the highest value. ● OTC alone, o OTC plus BHC.

treatment were abolished (Table 1, Figs 1, 2). There were no significant differences ($P > 0{\cdot}05$) in J_o and η_o values of mucus collected during either the control, drug or washout phases of the study (Table 2). Although in all three pigs there was again an apparent increase in wet weight of the mucus collected when the combination of drugs was administered (Fig. 3), the increase was shown not to be significant ($P > 0{\cdot}05$). The mean concentration of OTC in the mucus samples when OTC was given in combination with BHC was again animal-dependent (Table 3). The concentration of OTC was over fourfold higher in pig 1 when BHC was co-administered with the antibiotic, and became detectable in pig 3. In pig 2, however, any OTC was still below the limit of detection.

Discussion

The dose of OTC employed in the present study was high in comparison with a previous study carried out in piglets when a total daily dose of 25 mg kg^{-1} was employed (Abdou & Kotzian 1975). In this earlier investigation, the mean concentration of OTC detected in a pancreatin extract of bronchial mucus was found to be 2·1 $\mu g\ g^{-1}$ of extract, which is markedly greater than the levels detected in the three pigs in this study (Table 3). Indeed, the concentrations of OTC detected in the mucus secreted in the tracheal pouches were also lower than those found in the bronchial trees of calves (Kotzian et al 1977) and in expectorated human sputum after lower doses had been administered (Campbell 1970; MacCulloch et al 1974). The study of MacCulloch et al (1974) emphasized the erratic secretion of OTC into the mucus of bronchiectasis patients, administered 1 g per day of the antibiotic, with two patients out of six exhibiting undetectable levels ($< 0{\cdot}05\ \mu g\ g^{-1}$) whilst one achieved a concentration of 0·7 $\mu g\ g^{-1}$. Similar variability in OTC concentrations in sputum has also been reported (Brogan et al 1972). Interspecies variation and different methods of mucus collection make inter-study comparison of antibiotic levels difficult. Despite the low concentrations of OTC detected in the mucus recovered from the tracheal pouches, significant changes in the rheological properties of the gel were induced by antibiotic treatment. Structure was clearly promoted in the tracheal mucus, as reflected by the increase in viscosity and decrease in compliance (Figs 1, 2). This in-vivo response to OTC administration has apparently not been reported previously, although treatment of mucus in-vitro with much larger concentrations of tetracycline (1–10 $\mu g\ g^{-1}$) has been demonstrated to produce a similar effect (Marriott & Kellaway 1975). Since such a relatively large concentration of tetracycline is required to induce a measurable change in rheology of mucus in-vitro, it is highly unlikely that this effect alone will account for the mucospissic activity found in this study. It is possible, therefore, that OTC affects mucus structure before its secretion by the tracheal epithelium. Interestingly, the OTC mucus concentration was found to be highest in pig 1, where the greatest increase in wet weight of the mucus also occurred (Fig. 3). The co-administration of BHC with OTC eliminated the changes in rheological properties and wet weight which were induced when the latter drug was given alone. BHC is classified as a mucolytic agent and is used in the treatment of respiratory disorders associated with viscid mucus. Several clinical trials have been performed to assess the efficacy of BHC in chronic obstructive airways disease, but despite the general observations of increased sputum volume in patients (Aylward 1973) and an improvement in symptoms such as cough and ease of expectoration (Hamilton et al 1970), most workers have been unable to show significant improvements in lung function. Rheological studies have indicated that BHC has little direct effect on the viscosity of bronchitic sputum in-vitro but that increased elasticity can result (Takishima et al 1980). Recently, Martin et al (1990) have demonstrated that BHC at twice daily doses of 0·5, 1·0 and 2·0 mg kg^{-1} for five days induced significant reductions in viscosity and elasticity of mucus collected from the pig tracheal pouch using identical collection and analysis procedures to the present study. The elimination of the mucospissic action of OTC by BHC would appear to support the findings of an earlier study which demonstrated that BHC possessed mucolytic activity (Martin et al 1990). One compound may antagonize the action of the other either at a receptor level or during synthesis of the glycoprotein. The concomitant use of BHC and OTC did cause a generalized increase in the weight of the collected mucus (Fig. 3), although it must be emphasized that this increase was not significant. It is perhaps worth noting, however, that the pigs in which there was the greatest increase in wet weight (pigs 1 and 3) were also the two pigs in which OTC was detected.

When OTC and BHC were coadministered, the concentration of OTC recovered in the pouch increased in two of the three pigs compared with OTC given alone (Table 3). This tends to support the findings from previous investigations in which it was shown that when BHC was given with OTC, higher antibiotic levels were achieved in mucous secretions (Burgi et al 1968; Bach & Leary 1972; Offermeier et al 1972; Abdou & Kotzian 1975; Kotzian et al 1977). More recently, potentiation of OTC antimicrobial activity against vaginal microorganisms by concurrent administration of BHC has been demonstrated in-vivo (Malhi et al 1988) and attributed to effects of BHC on vaginal mucus. The mechanism underlying the increase in OTC concentration in response to concurrent treatment with BHC is not known. Tetracyclines are highly bound to plasma proteins and have also been shown to bind to mucus glycoproteins (Brown et al 1983; Kearney & Marriott 1987). BHC may compete for the same binding sites on glycoprotein or other mucus components thereby displacing OTC and leading to higher free concentrations of antibiotic. Displacement of OTC may also reduce the mucospissic activity, which in turn would reduce the diffusional resistance of mucus to more antibiotic diffusing from the epithelium towards the axis of the pouch. Whatever the prevalent mechanism of action, however, it can be concluded that, on the basis of the results from this study, BHC reverses the mucospissic activity of OTC in-vivo.

This is a novel and important finding which could be of relevance to the use of tetracycline antibiotics during exacerbations in chronic obstructive airways disease.

Acknowledgements

The technical skill and expertise of Dr N. A. Hodges and Dr G. W. Hanlon, Department of Pharmacy, University of Brighton, who undertook the antibiotic assays, is gratefully acknowledged. The authors also wish to thank Mrs E. Cheek for advice on the statistical analysis of the results.

References

Abdou, M. A.-F., Kotzian, J. (1975) Erhozhung des Oxytetracyclin-Spiegels in der Bronchialschleimhaut beim Schwein durch gleichzeitige Verabreichung von Bromhexin (Bisolvon). Tierarztl. Umschau 30: 565–566

Aylward, M. (1973) A between patient double blind comparison of S-carboxymethyl-cysteine and bromhexine in chronic obstructive bronchitis. Curr. Med. Res. Opin. 1: 219–227

Bach, P. H. Leary, W. P. P. (1972) The effects of bromhexine on oxytetracycline penetrance into sputum. S. Afr. Med. J. 46:1512–1514

Barry, B. W. (1974) Rheology of pharmaceutical and cosmetic semisolids. Adv. Pharm. Sci. 4: 1–72

Brogan, T. D., Allen, L., Hutt, H., Ryley, H. C. (1972) Effect of bromhexine therapy on concentrations of oxytetracycline in the sol phase of sputum. Br. J. Clin. Pract. 26: 555–558

Brown, D. T., Marriott, C., Beeson, M. F. (1983) Antibiotic binding to purified mucus glycoproteins. J. Pharm. Pharmacol. 35 (Suppl.): 80P

Burgi, H., Stauffer, H. U. (1971) Der Einfluss von Bromhexine auf den Sulfonamidgehalt des Sputums bei chronischet Bronchitis. Arzneim. Forsch. 9: 299–301

Burgi, H., Kleber, A., Regli, J., Gent, M. (1968) Antibiotics in sinus secretions. Lancet ii: 406

Campbell, M. J. (1970) Tetracycline levels in bronchial secretions. J. Clin. Path. 23: 427–434

Farrell, W., Williams, J. D. (1978) Tetracyclines. In: Reeves, D. S., Phillips, I., Williams, J. D., Wise, R. (eds) Laboratory Methods in Antimicrobial Chemotherapy. Churchill Livingstone, Edinburgh, pp 237–238

Hamilton, W. F. D., Palmer, K. N. Y., Gent, M. (1970) Expectorant action of bromhexine in chronic obstructive bronchitis. Br. Med. J. 1: 260–261

Kearney, P., Marriott, C. (1987) The effects of mucus glycoprotein on the availability of tetracycline. II Binding. Int. J. Pharm. 35: 211–217

Kotzian, J., Abdou, M. A.-F., Salamon, E. (1977) Steigerung des Oxytetracyclinspiegels im Bronchialscheim bei Kalbern nach gleichzeitige Verabreichung von Bisolvon. Tierarztl. Umschau 31: 132–134

MacCulloch, D., Richardson, R. A., Allwood, G. K. (1974) The penetration of doxycycline, oxytetracycline and minocycline into sputum. N Z Med. J. 80: 300–302

Malhi, J. S., High, A.-J., Hanlon, G. W., Gard, P. R., Marriott, C. (1988) Potentiation of oxytetracycline antimicrobial activity in-vivo by concurrent administration of bromhexine. Int. J. Pharm. 43: 77–81

Marriott, C., Kellaway, I. W. (1975) The effect of tetracyclines on the viscoelastic properties of bronchial mucus. Biorheology 12: 391–395

Martin, G. P., Marriott, C., Kellaway, I. W. (1973) Direct effect of bile salts and phospholipids on the physical properties of mucus. Gut 19: 103–107

Martin, G. P., Loveday, B. E., Marriott, C. (1990) The effect of bromhexine hydrochloride on the viscoelastic properties of mucus in the mini-pig. Eur. Resp. J. 3: 392–396

Montgomery, D. C. (1976) In: Design and Analysis of Experiments. Wiley and Sons, Chichester, pp 48–49

Offermeier, J., Miller, R., Brandt, H. D. (1972) The effect of bromhexine on the concentration of oxytetracycline in nasal mucus. S. Afr. Med. J. 46: 1509–1511

Ricevuti, G., Mezzone, A., Uccelli, E., Gazzani, G., Fregnan, G. B. (1988) Influence of endosteine, a mucolytic agent, on amoxycillin penetration into sputum in patients with an infective exacerbation of chronic bronchitis. Thorax 43: 585–590

Spatola, J., Poderoso, J. J., Wiemeyer, J. C. M., Fernandez, M., Guerreiro, R. B., Corazza, C. (1987) Influence of Ambroxol on lung tissue penetration of amoxicillin. Arzneim. Forsch. 37: 965–968

Takishima, T., Sato, S., Aoki, T., Maeda, S. (1980) The effect of mucolytic agents and stirring on sputum viscoelasticity. Tohoku J. Exp. Med. 131: 103–117