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Kinetic Evaluation of the Ciliotoxicity of Methyl- and Propyl-*p*-hydroxybenzoates Using Factorial Experiments

L. JIAN AND A. LI WAN PO

The Drug Delivery Research Group, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK

Abstract—The ciliotoxicity of methyl-*p*-hydroxybenzoate (methyl paraben, MHB) and propyl hydroxybenzoate (propyl paraben, PHB) was investigated. It is shown that at the concentrations used (0·28 and 0·38 mM) PHB exerted only mild toxicity. The MHB solutions used (1·18 and 2·36 mM) were much more ciliotoxic. There was, however, an order of magnitude difference in the concentrations used as a result of constraints imposed by their differing aqueous solubilities. There was no evidence of synergism in the ciliotoxicity of the two compounds when the MHB concentration was raised from 1·18 to 2·36 mM and the PHB concentration was raised from 0·28 to 0·38 mM in a 2^2 factorial experiment. At those levels the two compounds showed additive effects. On the other hand, clear synergism was evidenced by the fact that the ciliotoxicity of both MHB and PHB was dependent on whether the cilia were exposed to each hydroxybenzoate singly or in combination. The results combined with those of an earlier study, indicate that it is not possible to improve the selectivity of antimicrobial activity without also increasing ciliotoxicity.

The *p*-hydroxybenzoates are used widely as preservative agents in pharmaceutical formulations. Very often, combinations of methyl and propyl hydroxybenzoate (MHB and PHB) are added to aqueous formulations because of their claimed synergistic effects. In a recent paper (Gilliland et al 1992), a factorial design was used to demonstrate that the two alkyl hydroxybenzoates exert additive rather than synergistic antimicrobial effects as defined by Berenbaum (1977). The additive effects provide the necessary validated rationale for the use of combinations of the hydroxybenzoate in product formulation. Thus it was shown that combinations of PHB and MHB at concentrations which slow down or inhibit bacterial growth when used singly, produced definite kill as a result of their additive effects. In this report, we describe work which was aimed at elucidating whether the additive antimicrobial effects of the hydroxybenzoates were accompanied by reduced, additive or synergistic ciliotoxic effects. The results are important because of the potential use of the hydroxybenzoates as preservatives for drug formulations intended for application to the nose. There has been particularly wide interest in this subject recently because of the potential use of the nose as a portal for the systemic delivery of peptide drugs (Davis et al 1986; Zhou & Li Wan Po 1991). Most of the commonly used preservative agents are ciliotoxic (Van de Donk et al 1980, 1982) and there is need for effective and safer alternatives for intranasal use.

Material and Methods

MHB and PHB were of analytical grade (Sigma Chemical Co.). Medium 199 enriched with Hanks' salts was purchased from Gibco, UK. Adult male Wistar rats, 350–450 g, were used in the study.

The enriched medium 199 was used as the control solution in all cases. MHB and PHB solutions were prepared separately by shaking the appropriate amounts in the enriched medium 199 at room temperature (21°C). For MHB, the stock solution was 1800 mg L^{-1} (11·83 mM) while for PHB, the solution was 170 mg in 100 mL (0·94 mM). Complete dissolution was checked by UV spectrophotometry (Philips PU 8720 UV/VIS scanning spectrophotometer) and the solutions were stored at 4°C. Before use, the solutions were brought to room temperature and shaken to equilibrium as verified by UV spectrophotometry. The MHB and PHB combinations were prepared by appropriate dilutions with enriched medium 199. For example, to make a solution referred to as containing 10% MHB and 30% PHB, 10 mL of the stock MHB solution and 30 mL of the stock PHB solution were pipetted into a 100 mL volumetric flask and made up to volume with enriched medium 199. The percentage composition therefore refers to the volume composition of the final solutions with respect to the stock solutions. The same applies to the solutions referred to as 10% MHB+40% PHB, 20% MHB+30% PHB and 20% MHB+40% PHB solutions. Ten percent MHB as defined above is equivalent to 1·18 mM and 20% to 2·36 mM. Likewise, 30% PHB is equivalent to 0·28 mM and 40% to 0·38 mM.

Preparation of ciliated trachea

Each rat was killed humanely by an experienced technician with a single sharp blow on the head and the trachea immediately removed and incubated at 37°C in enriched medium 199. Ring segments of about 1 mm were cut from the trachea and also stored in the enriched medium until required.

Recording of ciliary beat frequency

Before each measurement, a tracheal ring with healthy ciliated epithelium was placed in the microscope slide chamber. The correct position for viewing the ciliated epithelium was obtained using the microscope and a video monitor (Hitachi).

For each solution, measurements of the beat frequency were taken at three different sites on each of three tracheal

Correspondence: A. Li Wan Po, The Drug Delivery Research Group, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK.

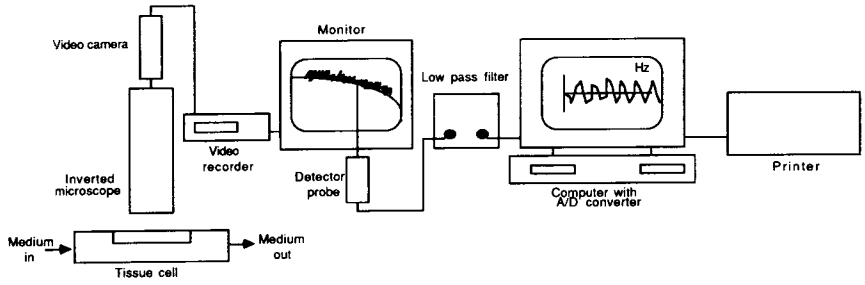

FIG. 1. Data acquisition and processing unit for measuring ciliary beat frequency.

explants from three different rats. All the observations were completely randomized. The initial beat frequency was initially measured at room temperature in drug-free solution. The reference solution was then removed and replaced with drug solution. The beat frequency was measured again and expressed as a percentage of the initial value. The tracheal rings were each monitored over 60 min. New tracheal explants were used for each solution and the slide chamber was rinsed with control medium several times before introducing a new drug solution.

The equipment used for recording ciliary beat frequency consisted of an inverted binocular microscope (Olympus CK2-TRP) fitted with a $\times 20$ object and a $\times 10$ ocular lense. A video camera (Hitachi KP-143) was attached to the phototube behind the eyepieces thereby enabling the image of the cilia to be displayed on a video monitor (Hitachi) and to be recorded on a video cassette recorder (VCR) (Akai VS-425 EK).

The movements of the cilia recorded on the video tape were detected by a data acquisition unit (Fig. 1) based on equipment described by Teichtahl et al (1986). The data acquisition unit consists of a probe, a low pass filter and an Opus PC III computer with on-board A/D converter. The probe picks up an optical signal through its photo-cell, converts it to an electrical signal which is amplified to between 0 and +300 mV. The filter circuit takes the signal from the probe, filters out frequencies above 30 Hz, and adjusts the signal to 0–5 V.

A sampling period of 10 ms, corresponding to a sampling rate of 100 Hz was used. The highest frequency detectable was 50 Hz. The signal derived from the video monitor was converted by fast Fourier transform into a frequency spectrum using software written in C to run on an Opus PC III computer. Each sample consists of 256 data points corresponding to 256 ms.

The average result is given for the group. A program called HARDCOPY written by us is used to allow graphics dump from the screen to the printer/plotter.

Statistics

The area under the curve (AUC) values were calculated using the trapezoidal rule and a Lotus 1-2-3 Macro (Chan & Li Wan Po 1993). Minitab was used for carrying out the statistical analyses.

Results

Effect of MHB and PHB on their own

Fig. 2 shows the decrease in ciliary beat frequency as a function of time for cilia exposed to 20% solutions of MHB; logarithmic transformation indicated the decay was not exponential. In order to avoid multiple point testing and the associated problems (Matthews et al 1990), the area under the curve (AUC) between the decay curve and the line $y = 100\%$ was used as the summary statistic for this and similar experiments with 30 and 40% solutions of PHB and 10% solutions of MHB (data not shown).

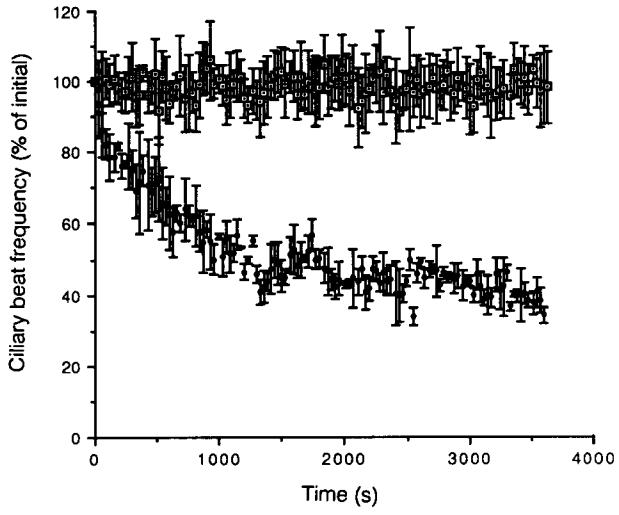

FIG. 2. Ciliary beat frequency as a function of time for cilia exposed to control (□) and methyl hydroxybenzoate (●).

The AUC values were subjected to one-way analysis of variance with the single factor being hydroxybenzoate preparation rather than to two-factor analysis of variance with concentration and alkyl hydroxybenzoate as two separate factors (Table 1). Since the results showed a significant difference in the effect of the four different preparations, further analysis was carried out using Tukey's multiple range test because of its good performance in Monte Carlo studies (Dunnett 1980). This showed that the AUC

Table 1. Analysis of variance table for effect of exposure to different hydroxybenzoate preparations on ciliary beat frequency.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F	*P*
Hydroxybenzoate	83·93	3	27·98	252	<0·001
Error	3·55	32	0·111		
Total	87·49	35			

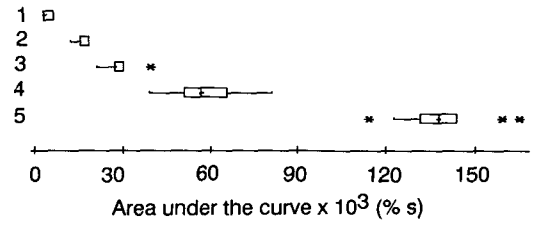

FIG. 3. Boxplot of ciliotoxicity of alkyl hydroxybenzoate solutions. 1. Control; 2. 30% propyl hydroxybenzoate; 3. 40% propyl hydroxybenzoate; 4. 10% methyl hydroxybenzoate and 5. 20% hydroxybenzoate. *Are outlier values. AUC are in % s.

values for the different solutions were different from each other (Fig. 3).

Factorial experiment

To investigate whether MHB and PHB interact in their ciliotoxic effects, the rat cilia were exposed to four different combinations. Fig. 4 shows the decay in ciliary beat frequency. The AUC values were calculated and subjected to analysis of variance appropriate for a 2×2 factorial design.

The results (Table 2) indicate a significant concentration effect with MHB ($P < 0.05$) but a less significant effect with PHB ($P = 0.082$) when their respective concentrations were raised from 10 to 20% and 30 to 40% in the 2^2 factorial experiment. There were, however, clear additive effects (Fig. 4).

Additional trials were carried out with the cilia exposed to enriched medium 199 only and the areas under the decay curves recorded as before. When these results are combined with those obtained with the cilia exposed to 10% MHB, 30% PHB and the combination 10% MHB and 30% PHB, the data set can be regarded as originating from a separate 2^2 factorial experiment. The two levels of MHB are hence 0 and 10% and the two levels of PHB are 0 and 30%.

Analysis of variance of the AUC values showed that the ciliotoxicity of the two hydroxybenzoates depended on whether they were present singly or in combination (Table 3). In particular, the results show that 10% MHB was significantly more ciliotoxic in the presence of PHB than in its absence. Likewise 30% PHB was significantly more toxic in the presence of 10% MHB. Figs 5 and 6 show clearly that the two compounds exert synergistic ciliotoxic effects under those conditions.

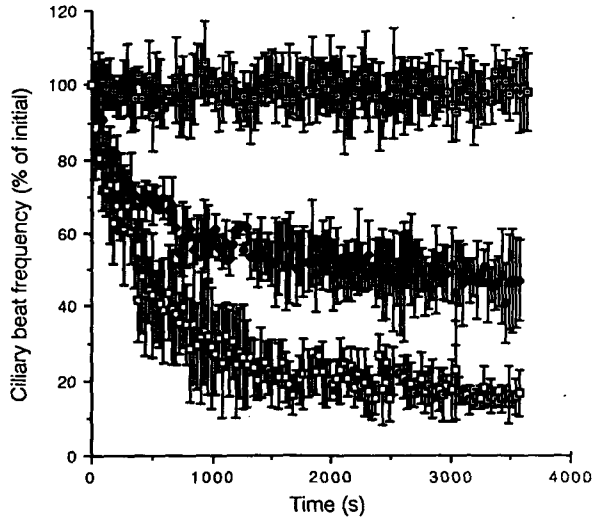

FIG. 4. Effects of combinations on the beat frequency of cilia in rat trachea. □ Control; ● 10% methyl hydroxybenzoate + 30% propyl hydroxybenzoate; □ 20% methyl hydroxybenzoate + 40% propyl hydroxybenzoate.

Discussion

In the only published study of MHB ciliotoxicity that we can identify (Mostow et al 1979), the method used consisted of visual counting of beating cilia in cultured tracheal rings of ferrets. Although the results were not subjected to any statistical analysis, it was evident that exposure to MHB inhibited ciliary activity in a dose-dependent manner. Batts et al (1990) and van de Donk et al (1980) also reported that MHB and PHB were ciliotoxic. The latter showed that the cilia inhibitory effects were reversible.

Table 2. Analysis of variance table for effect of exposure to hydroxybenzoate combinations on ciliary beat frequency in rat trachea.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P
Methyl hydroxybenzoate	6916·42	1	6916·42	165·98	<0·001
Propyl hydroxybenzoate	134·58	1	134·58	3·23	0·082
Interaction	26·36	1	26·36	0·63	0·432
Error	1333·41	32	41·67		
Total	8410·77	35			

Table 3. Analysis of variance table for effect of exposure to methyl and propyl hydroxybenzoates singly or in combination, on ciliary beat frequency in the rat trachea.

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F value	P
Methyl hydroxybenzoate	893·86	1	893·86	499	<0·001
Propyl hydroxybenzoate	280·61	1	280·61	157	<0·001
Interaction	178·06	1	178·06	99	<0·001
Error	57·23	32	1·79		
Total	1409·76				

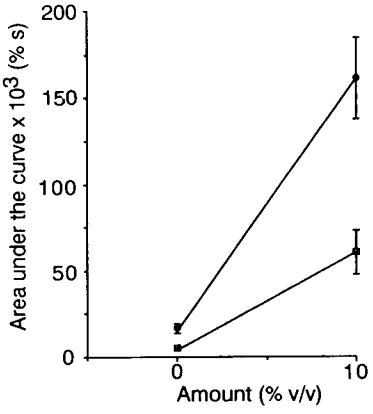

FIG. 5. Effect of increasing methyl hydroxybenzoate concentration in the presence (●) and absence (□) of propyl hydroxybenzoate. Error bars refer to standard error (n = 9). See text for definition of % and AUC.

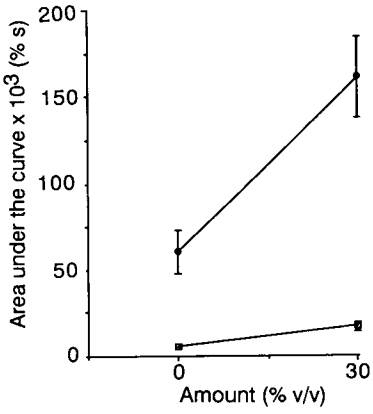

FIG. 6. Effect of increasing propyl hydroxybenzoate concentration in the absence (□) and presence (●) of methyl hydroxybenzoate. Error bars refer to standard error (n = 9). See text for definition of % and AUC.

In the present study the results of the factorial experiments show that the two hydroxybenzoates exerted obvious synergism in their ciliotoxicity. The ciliotoxicity of the 10% MHB was significantly more pronounced in the presence of 30% PHB than in its absence. The increase in ciliotoxicity was significantly higher than can be explained by additive effects. However, no synergism was apparent when the MHB concentration was increased from 10 to 20% in the presence of 30 or 40% PHB.

Those results are surprising because the two hydroxybenzoates are structurally similar, differing only in alkyl chain length. Therefore one would have expected the same mode of action and hence additive effects. Moreover the synergism did not extend across the whole concentration range studied. In particular, additive effects only were observed in the MHB concentration range 10–20%. The basis for the synergistic effects observed at the lower concentrations is not known to us at present but the effects appear real. Each graphical point was generated using three observations on three different segments of trachea from three different rats. In particular, adsorptive losses could not be detected even at the lowest concentrations used. Combinations of solutions of MHB and PHB are widely used in the belief that they exert synergistic antimicrobial effects. In an earlier study from our laboratory no such synergism was observable when *Escherichia coli* were exposed to mixtures of the two hydroxybenzoates (Gilliland et al 1992). However, we did not obtain satisfactory data at the lower concentrations used in this study. At those levels the hydroxybenzoates exerted mild antimicrobial effects which were of the same magnitude as the errors associated with the viable count method used. Therefore, strict comparison of the antimicrobial effects and the ciliotoxicity of the compounds cannot be made. It may be possible that different sites on the cilia, subject to the ciliotoxicity of the hydroxybenzoates, have different hydrophobicities. Nonetheless, the results of the present study and those of the earlier study (Gilliland et al 1992) suggest that it is not possible to maximize selective antimicrobial activity while at the same time minimize ciliotoxicity. In choosing the concentrations of the esters to be used we were largely driven by the ciliotoxicity of the solutions used. Coincident solutions, with respect to saturation, led to either very low or very high ciliotoxicity. In both those cases the variance in the measurements increase to levels higher than those associated with the experimental effect. Use of such solutions are therefore inappropriate. Nonetheless, the results presented herein suggest that a more extensive study of the dose-response relationship, perhaps using surface response methodology, of the effects observed would be appropriate.

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