J. Pharm. Pharmacol. 1993, 45: 925–927
Communicated January 20, 1993

Ciliotoxicity of methyl- and propyl-*p*-hydroxybenzoates: a dose-response and surface-response study

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Abstract—The effect of methyl-*p*-hydroxybenzoate (methyl paraben, MHB) and propyl-*p*-hydroxybenzoate (propyl paraben, PHB) on ciliary beat frequency was investigated using surface-response methodology. Both compounds are shown to be ciliotoxic at concentrations equal to or lower than those in use for preserving aqueous formulations. The dose-response curves show the typical sigmoidal pattern. Interaction by the two compounds is evidenced by the curved response surface for ciliotoxicity.

Screening of the parabens for ciliotoxicity is of interest, given the exploration of the nose as a potential site for the absorption of pharmacologically active peptides and proteins (Illum 1991; Zhou & Li Wan Po 1991), and the use of these antimicrobials as preservatives.

In an earlier study (Jian & Li Wan Po 1993) we showed that methyl-*p*-hydroxybenzoate (methyl paraben, MHB) and propyl-*p*-hydroxybenzoate (propyl paraben, PHB) interacted with each other in their ciliotoxic effects. The effect of each alkyl paraben depended on whether the other was present in the test solution. However, the interaction was only observed over certain concentration ranges. We therefore decided to investigate the interaction further using surface-response methodology (Box & Wilson 1951), a logical extension of the factorial experiments described earlier (Jian & Li Wan Po 1993).

Materials and methods

MHB and PHB were of analytical grade (Sigma Chemical Co.). Medium 199 enriched with Hank's 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 for 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. MHB and PHB combinations were prepared by appropriate dilutions with enriched medium 199.

Preparation of ciliated trachea. Each rat was killed by an experienced technician 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 again stored in the enriched medium until required.

The beat frequency of cilia was recorded and measured as described previously (Jian & Li Wan Po 1993). The area under the curve between the ciliary beat frequency decay curve and the time axis was calculated from 0 to 3600 s in the study of dose-ciliotoxic response and from 0 to 1800 s in the surface-response study, using the trapezoidal rule (Chan & Li Wan Po 1992). The statistical analyses were carried out using MINITAB. The complement of the area under the curve (CAUC) was used as an index of ciliotoxicity. CAUC is the difference between the area under the ciliary beat frequency vs time curve and the area between the line ciliary beat frequency at 100% and the abscissa over the time interval studied.

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Surface-response methodology has been extensively described elsewhere (Box & Wilson 1951). Essentially it consists of modelling functions of several variables using low-order polynomial functions for predictive purposes. Consider a response (e.g. some measure of toxicity, the complement of the area under the curve (CAUC) in this case, which is a function of two variables (concentrations of MHB (C_1) and PHB (C_2) in this study). This can be written as $CAUC = f(C_1, C_2)$. In a surface-response study, using a second order model, one attempts to describe the relationship between CAUC and C_1 and C_2 using a polynomial of the form:

$$CAUC = \beta_0 + \beta_1 C_1 + \beta_2 C_2 + \beta_3 C_1^2 + \beta_4 C_2^2 + \beta_5 C_1 C_2 \quad (1)$$

where the β_1 values are constant coefficients. Experimental CAUC observations at various levels of C_1 and C_2 can then be generated and the data fitted to the model. The goodness of the fit is assessed using analysis of variance. One or more of the β-coefficients may not be significantly different from zero and are therefore omitted from the fitted function to obtain the most parsimonious model. The term response surface is used because the response can be viewed as a surface, being a continuous function of the two continuous variables. When the response is a function of more than two variables it is referred to as a hypersurface.

In surface-response studies, one attempts to maximize or minimize the response (function) of interest in the region of interest. In the present example, the interest is in minimizing ciliotoxicity using CAUC as an index. Once the surface is defined, regions where the ciliotoxicity is below a given value can be defined. One may then combine this information with, for example, antimicrobial activity data (Gilliland et al 1992) to identify concentrations of the benzoates which can be used in the proposed formulations. No reference is made to the biological mechanisms or chemical stoichiometry involved since the model is empirical. Methods are also available for simultaneous multivariate response optimization.

The statistical design used is a central composite design consisting of a 2^2 factorial design augmented by additional axial points to ensure rotability ($\alpha = 1{\cdot}414$) (Box & Wilson 1951) and additional centre points to approximate uniform precision (Davies 1956). A replicate of the 13-point experiment was carried out to improve the precision of the estimates.

Results and discussion

All the beat frequencies were expressed as a percentage of the initial value. Fig. 1 shows typical ciliary beat frequency vs time curves when the cilia were exposed to different concentrations of the benzoates. CAUC is used as a summary measure, thereby bypassing the need to undertake multiple significance testing with the consequential increased risk of erroneously rejecting any null hypothesis put forward (Matthews et al 1990).

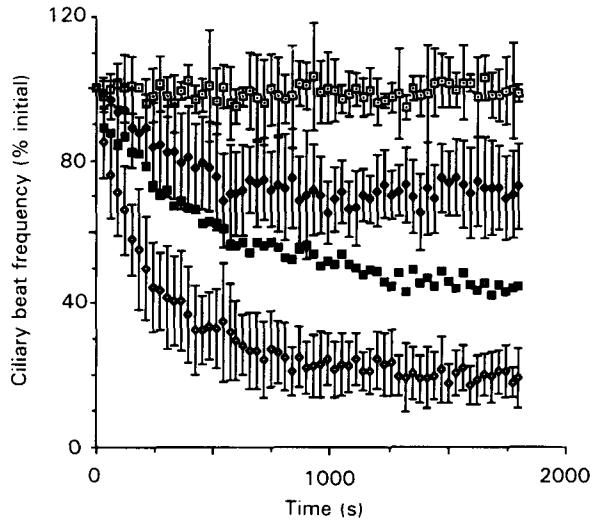

FIG. 1. Ciliotoxicity caused by methyl hydroxybenzoate (MHB) in combination with propyl hydroxybenzoate (PHB). □ Control; ◆ 0·22 mM MHB + 0·52 mM PHB; ■ 1·48 mM MHB + 0·52 mM PHB; ◇ 2·73 mM MHB + 0·52 mM PHB.

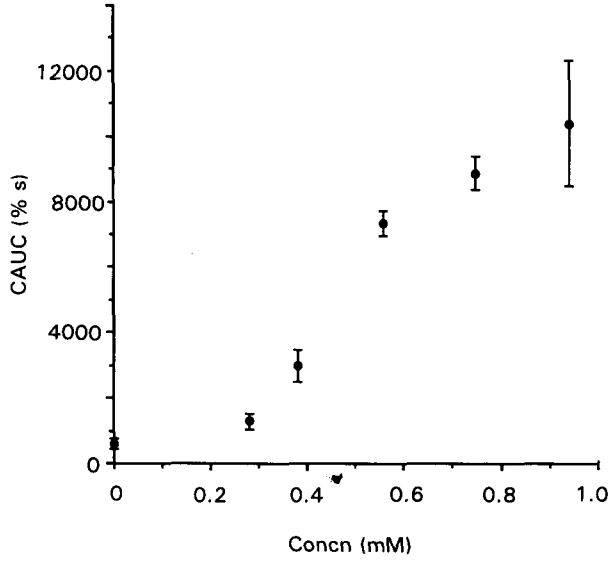

FIG. 2. Dose-response ciliotoxicity of methyl hydroxybenzoate for CAUC in rat trachea. CAUC is the complement of the area under the ciliary beat frequency vs time curve.

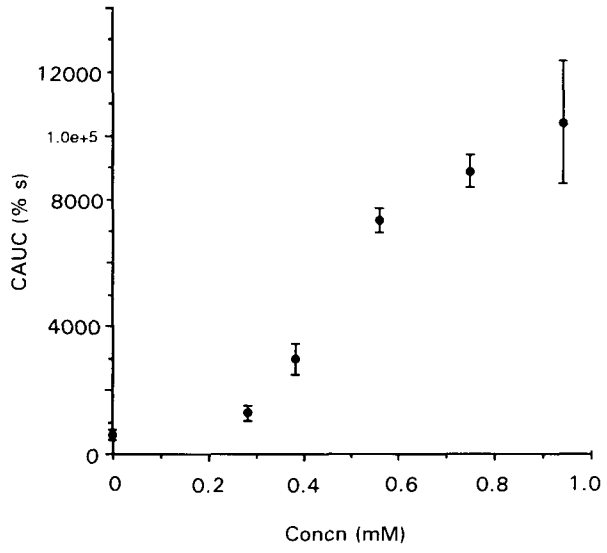

FIG. 3. Dose-response ciliotoxicity of propyl hydroxybenzoate for CAUC in rat trachea. CAUC is the complement of the area under the ciliary beat frequency vs time curve.

The dose-response relationships observed with MHB and PHB are shown in Figs 2 and 3, respectively. These show the typical sigmoidal pattern of dose-response relationships of pharmacologically active compounds. However, nonlinear fitting of the data to the logistic equation (eqn 2) yielded poor fits because of the high variance observed at some of the concentrations:

$$CAUC = a(1 + e^{b - dC}) \quad (2)$$

where a, b and d are constants and C is the concentration of the alkyl hydroxybenzoate.

Analysis of variance of the data showed that a quadratic surface model fitted the data well with the observed F values having significance probabilities of 0·0001 for the linear term and 0·0550 for the quadratic term. The lack of fit tests indicated that the linear and the quadratic terms (eqn 1) have significance probabilities of 0·0009 and 0·0021, thereby confirming the suitability of the second order model for describing the relationship between the response and the concentrations of the two alkyl hydroxybenzoates (Table 1). The assumption made of normally distributed data in the analysis of variance test is satisfactorily met.

Fig. 4 provides a three dimensional representation of the response surface. The curvature in the surface is obvious thereby

Table 1. Analysis of variance for the response-surface model.

	Sum of squares	Degrees of freedom	Mean square	F value	*P* value
Source					
Linear	$1{\cdot}796 \times 10^{10}$	2	$8{\cdot}980 \times 10^{9}$	133·1	0·0001
Quadratic	$4{\cdot}814 \times 10^{8}$	3	$1{\cdot}605 \times 10^{8}$	3·0	0·0550
Error	$1{\cdot}071 \times 10^{9}$	20	$5{\cdot}353 \times 10^{7}$		
Corrected total	$1{\cdot}951 \times 10^{10}$	25			
Model					
Linear	$1{\cdot}090 \times 10^{9}$	6	$1{\cdot}816 \times 10^{8}$	6·675	0·0009
Quadratic	$6{\cdot}082 \times 10^{8}$	3	$2{\cdot}027 \times 10^{8}$	7·451	0·0021
Pure error	$4{\cdot}625 \times 10^{8}$	17	$2{\cdot}721 \times 10^{7}$		

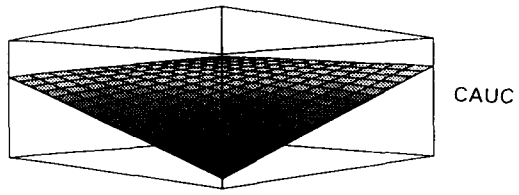

FIG. 4. Response-surface plot for ciliotoxicity of methyl and propyl hydroxybenzoates. CAUC is the complement of the area under the curve (% s).

demonstrating visually why a quadratic model provides a better description of the response than does a model which includes only linear terms.

The surface-response study provides a means for quantitatively defining the range of acceptable concentrations of the two alkyl hydroxybenzoates for preserving nasal formulations. In practice the combination with the highest preservative concentrations consistent with absence of ciliotoxicity would be chosen. On intra-nasal application, dilution with nasal secretions would reduce the preservative concentrations and hence reduce ciliotoxicity. Our results suggest that a combination of the data reported here with data on the antimicrobial kinetics of the two compounds would enable optimization of their use with respect to both efficacy and toxicity.

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J. Pharm. Pharmacol. 1993, 45: 927–928
Communicated December 18, 1992

The purely chronotropic effects of relaxin in the rat isolated heart

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Abstract—The endogenously occurring protein relaxin was evaluated for its cardiac effects in the rat isolated heart. Relaxin caused a dose-dependent tachycardia without positive inotropic effects in intact preparations and in preparations where the atria had been removed. It is concluded that relaxin acts on both the atrial and ventricular pacemakers to increase heart rate, but the precise mechanism of action remains unknown.

Binding sites for relaxin, a heterodimeric member of the insulin family of proteins, have been identified in the rat atrium (Osheroff et al 1992). Following these observations, subsequent studies have shown that relaxin can induce both inotropic and chronotropic effects on rat isolated atrial preparations (Kakouris et al 1992; Ward et al 1992). However, it is not clear whether relaxin has effects on other excitable cardiac tissue. This study was conducted to elucidate further the cardiac effects of relaxin. This was accomplished using isolated hearts where the effects on atrial and ventricular pacemaker tissue could be studied separately.

Materials and methods

Male Sprague-Dawley rats, 300–350 g (Charles River, Portage, IN, USA), were anaesthetized with 60 mg kg^{-1} intraperitoneal pentobarbitone sodium (Fort Dodge Laboratories Inc., Fort Dodge, IA, USA). Following thoracotomy the hearts were removed and placed in 4°C Krebs–Henseleit solution, containing (mM): NaCl 118, KCl 4·7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHCO_3$ 25, KH_2PO_4 1·2 and glucose 11, and gassed with 95% O_2–5% CO_2. Any associated lung and thymic tissue was trimmed and the ascending aorta cannulated. The heart was then perfused at 10 mL min^{-1} with Krebs–Henseleit solution at 37°C. A small incision was made in the left atrium and a saline-filled balloon (Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a pressure transducer (Gould Electronics, Valley View, OH, USA) was placed into the ventricle for the measurement of left ventricular developed pressure (LVDP). LVDP was taken as the maximal pulse pressure in mmHg developed during each systole. From this pressure recording, heart rate was electronically derived on a Grass polygraph (Quincy, MA, USA).

In six experiments, after a 15–20 min stabilization period, the atria were removed. The heart rate was then allowed to stabilize at its new rate before the administration of relaxin. In all other experiments the atria were left intact and drugs administered after a 20-min stabilization period.

Relaxin was administered as 50-μL bolus injections into the perfusion fluid immediately before it entered the heart. Cumulative dose-response curves were constructed using human recombinant gene-2 relaxin (Genentech Inc., South San Francisco, CA, USA). Adrenaline bitartrate (Sigma Chemical Co., St Louis, MO, USA) was dissolved as 1 mg mL^{-1} stock solution in 0·9% NaCl (saline) containing 1 mg mL^{-1} sodium ascorbate and administered into the perfusate as 50- or 150-μL bolus injections. Serial dilutions of both drugs were made using saline. All doses were given at 15-min intervals. Relaxin and adrenaline were tested in separate preparations.

Statistical significance was determined by a two-tailed Student's unpaired t-test and $P < 0.05$ taken as significant.

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