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Factorial designs in ophthalmic formulation development of Enalkiren

Jitendra P. Patel, Kennan Marsh, Linda Carr and George Nequist

Abbott Laboratories, Pharmaceutical Products Division, 1401 Sheridan Road, North Chicago, IL 60064 (U.S.A.)

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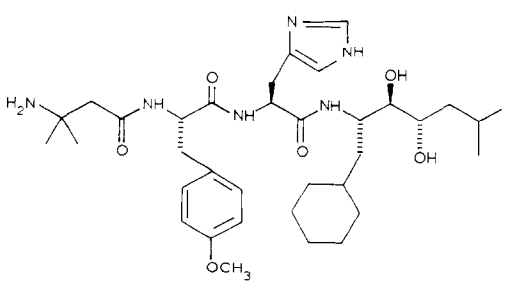
Summary

A formulation development approach for ophthalmic solutions using a 2^3 factorial design is presented. Enalkiren (ABBOTT-64662), a renin inhibitor, has been shown to lower intraocular pressure in rabbits and monkeys. The influence of pH, presence of EDTA and the addition of PVP K90 to solutions of Enalkiren for ophthalmic use was investigated in rabbit. Results indicated that pH had a significant effect on the aqueous humor levels of Enalkiren after application to the rabbit eye. Additionally, interaction effects between PVP and pH were identified. It was demonstrated that aqueous humor levels of Enalkiren could be increased by an average of 50% by adding 4% PVP K90 to a pH 7 formulation. The application of a factorial design study may be useful in the evaluation of variables that influence the in vivo performance of ophthalmic formulations.

Introduction

Corneal application of Enalkiren (ABBOTT-64662), (2*S*,3*R*,4*S*)-2-[3′-amino-3′-methylbutyryl-*O*-methyl-L-tyrosyl-L-histidylamino]-1-cyclohexyl-3,4-dihydroxy-6-methylheptane, a renin inhibitor, has been shown to lower intraocular pressure (Giardina et al., 1989; Stein et al., 1989) in unanesthetized rabbits and anesthetized monkeys. Several factors influence the penetration of topically applied drugs into the eye. These formulation variables include pH and buffer capacity (Ahmed and Patton, 1984), the nature of the buffer species (Ahmed and Chaudhuri, 1988), osmotic pressure (Barendsen et al., 1979), type and concentration of antimicrobial preservative (Camber and Edman, 1987), and viscosity (Grass and Robinson, 1984; Ludwig and Van Ooterghem, 1988).



Correspondence: J.P. Patel, Abbott Laboratories, Pharmaceutical Products Division, 1401 Sheridan Road, North Chicago, IL 60064, U.S.A.

The factorial design provides a means to evaluate simultaneously the influence of the individual formulation variables and their interactions at several levels with a minimum of experiments. The application of factorial experimental design has been reported for stability studies in solids (Ahlneck and Waltersson, 1986), solutions (Bolton et al., 1984, Gupta et al., 1988, Hung et al., 1988) and suspensions (Waltersson, 1986). Factorial experimental designs have been employed to optimize chemical stability of drugs in solution (Devay et al., 1985) and for process optimization of granule and tablet processes (Malinowski and Smith, 1975). Factorial experimental designs have also been recently used in the evaluation of preservative efficacy (Karabit et al., 1989). The application of factorial experimental design has been reported by Nath and Shingbal (1974) in the study of the effects of several polymers on the in vitro release of atropine sulfate and procaine hydrochloride from eye drops using a stationary dialysis technique.

In the present investigation a factorial approach has been employed in the experimental design and statistical analysis of aqueous humor drug levels in rabbit for the development of an ophthalmic formulation for the renin inhibitor peptide Enalkiren.

Materials and Methods

Drugs, chemicals and reagents

Enalkiren was obtained within Abbott Laboratories. Chemicals and reagents used to prepare the ophthalmic formulations were pharmacopeial grade (USP). All other chemicals were analytical reagent grade.

Factorial design

In this study a 2^3 factorial experimental design was used. The effect of three factors on the aqueous humor levels of Enalkiren in the rabbit eye was studied at two levels. These factors were pH (A), concentration of EDTA (sodium salt) (B), and the presence of polyvinylpyrrolidone (PVP K90) (C). The factorial study experimental design and the values for these factors at the two levels are summarized in Table 1.

TABLE 1

2^3 Factorial study design and experimental conditions

Trial number [a]	Factors		
	A	B	C
1 (1)	–	–	–
2 (a)	+	–	–
3 (b)	–	+	–
4 (ab)	+	+	–
5 (c)	–	–	+
6 (ac)	+	–	+
7 (bc)	–	+	+
8 (abc)	+	+	+
Control	–	–	–

[a] For the three factors A, B and C, the eight combinations (Trials) are designated as: 1, a, b, ab, c, ac, bc and abc, where 1 refers to all factors at their low level; if factor A is at its high level, and B and C are at their low level, the combination is denoted as a, etc. (Bolton, 1984a).

Factors	Factor levels	
	Low (–)	High (+)
(A) pH of solution	5.6	7.0
(B) EDTA concentration	0%	0.1%
(C) PVP K90	0%	4%

The calculation matrix which can be used for a 2^3 factorial design has been discussed in detail by Bolton (1984a). The main effects and interaction terms can be calculated from the totals of the individual treatment combinations by means of a table of signs (Yates, 1937; Bolton, 1984a). Theoretical and statistical considerations in the application of factorial designs to pharmaceutical stability studies with an example of data analysis have been reported (Bolton, 1983). In the present study, the calculations of these values was performed by use of a statistical software package (Statgraphics® STSC, Rockville, MD, U.S.A.) on a desk-top computer (Hewlett Packard Vectra QS/165). The resulting sum of squares, subsequent significance tests and the calculation of the average values were also obtained using this software package.

Formulations for factorial screening study

The two levels of the three formulation variables, which yield the eight (trial) formulation combinations, are listed in Table 1. This table also lists the variable levels for a control formulation to be used in this factorial study. The following procedure was followed to prepare the formulations according to the composition indicated in Table 1.

Glycerin (1.1 g) was weighed into beakers calibrated to 100 ml volume. 17 ml of 1% acetic acid was added, followed by the addition of 1 ml of a 1% solution of benzalkonium chloride (BAK). 70 ml of Water for Injection (WFI) was added and the contents mixed using a magnetic stir bar. Enalkiren (1 g) was added and the stirring was continued until all the solutions were clear. PVP K90 (4 g), if needed, was slowly added to these solutions with continued mixing to avoid formation of clumps of PVP. The solutions were mixed to dissolve the PVP and the final volumes adjusted to 100 ml with WFI. pH values were measured and adjusted, if necessary, to values designated in Table 1 using a 2% sodium hydroxide solution. All formulations were filtered using a 0.2 μm nylon filter (Pall Corp., Cortland, NY), prior to studies in rabbit.

Evaluation in rabbit

According to the experimental design (Table 1), 25 μl of the experimental formulation was instilled in either the left or right (randomly assigned) eye of a New Zealand white rabbit (2–4 kg, Mohican Valley Rabbitry, Loudenville, OH) using a glass microliter syringe (Hamilton, Reno, NV). An equal volume of the control formulation was instilled in the opposite eye of the same animal. The 25 μl dose was instilled just above the cornea and allowed to spread over the cornea into the pocket formed by the lower eyelid and glove. The left eye of each animal was dosed first; dosing in the right eye occurred within 30 s of the left. During the study the animals were maintained in a normal upright position in a rabbit restrainer to prevent irritation of the eye by rubbing or scratching. Each rabbit was killed 60 min after dosing (T-61 solution, Hoechst Rousell). The cornea and sclera were rinsed with normal saline and gently blotted with gauze. A sample of aqueous humor (about 150–200 μl) was withdrawn from the anterior chamber of each eye using a glass syringe with a 25 gauge needle. The aqueous humor was placed directly in a limited volume autosampler vial for the reverse-phase HPLC analysis.

Chromatography

Parent compound was separated from aqueous humor contaminants on a 5 cm $\times$ 4.6 mm 3 μm Spherisorb ODS2 column (Regis, Morton Grove, IL) with a mobile phase consisting of acetonitrile : methanol : 0.01 M tetramethylammonium perchlorate in 0.1% trifluoroacetic acid (35 : 5 : 60 v/v) at a flow rate of 0.7 ml/min with low wavelength UV quantitation at 205 nm. The chromatographic system was composed of a high-efficiency pump (Kratos SF400 Ramsey, NJ), an automatic sample injector (Waters WISP 712, Milford, MA) and a variable-wavelength UV detector (Kratos SF783) with computer-controlled data collection and integration (DS650, Kratos). The autoinjector was programmed to inject a methanol wash every two samples to maintain the integrity of the separation. Under these conditions, the retention time for Enalkiren was about 8 min. The peak area of spiked standards (external) vs concentration was linear (correlation coefficient > 0.998) over the concentration range 0–800 ng/ml with a mean percent standard deviation of 7% for triplicate standards and an estimated quantitation limit of about 20 ng/ml. The variation between multiple HPLC runs was estimated to be less than 7%.

Results and Discussion

Each of the experimental formulations containing 1% Enalkiren (as the diacetate) was randomly instilled only in one eye of the rabbit while the other eye received a control formulation. The eight experimental formulations and the control formulation were evaluated as one study group. The evaluation was repeated for a total of four study groups (four replicates) each time employing a new randomization schedule. All animals were dosed on the same day and the analysis of all the

TABLE 2

Enalkiren percent aqueous humor ratio (experimental eye/control eye) in rabbits

Trial no.	Percent aqueous humour ratio				
	Expt 1 [a]	Expt 2	Expt 3	Expt 4	Mean (SD)
1	171	121	57	138	122 (48)
2	86	190	156	33	116 (70)
3	212	74	165	71	131 (70)
4	219	97	71	120	127 (65)
5	194	83	77	[b]	118 (66)
6	143	174	191	284	198 (60)
7	80	58	116	[b]	85 (29)
8	133	428	152	224	234 (135)

[a] Expt 1–4 refers to study groups (replicates) 1–4.
[b] Not determined. Formulations showed trace (< 20 ng/ml) of A-64662.

aqueous humor samples was also done in a single HPLC analytical run. The ratio (experimental formulation/control formulation) of the aqueous humor levels for each rabbit at 60 min, expressed in percent, was used as a response for the statistical analysis of the data. In this manner, using one eye as a 'control', it is believed that the intra-animal variability could be minimized. Unpublished independent experiments had demonstrated that administration of [^{14}C]Enalkiren in one eye of rabbit did not show detectable levels in the other eye. Additionally, preliminary ophthalmic formulation development experiments and drug distribution studies (unpublished results) had indicated that Enalkiren aqueous humor levels remained unchanged over a period of 2 h after instillation and therefore, 60 min was selected as an appropriate sampling time to evaluate the in vivo performance of Enalkiren formulations. The percent aqueous humor level ratios of Enalkiren for trials 1–8 were calculated for each rabbit in the four replicate experiments and the data are presented in Table 2.

The three formulation factors in this study were selected to ascertain whether the transport of Enalkiren into the rabbit aqueous humor can be influenced by potentially different mechanisms. Ophthalmic solutions, which are not at physiological pH when instilled into the eye, have been reported to increase the drainage rate due to reflex tear production (Ahmed and Patton, 1984) resulting in a large reduction in the drug concentration available for absorption in the precorneal area. In this study, pH was examined as an experimental variable at values of 5.6 and 7. In vitro concentrations of EDTA above 0.01% have been reported (Grass and Robinson, 1988) to increase permeability of rabbit cornea to glycerol due to an increase in the size of the intercellular spaces of both the epithelium and the endothelium. EDTA at a concentration of 0.1% was included as a formulation variable to determine its effects on the permeability of Enalkiren into the aqueous humor. PVP K90 at 4% was included as a formulation variable to evaluate the effect of the viscosity-inducing agent on the penetration of Enalkiren into the aqueous humor. The addition of 4% PVP K90 increased the average viscosity (25 °C) of the formulations in Table 1 from a value of about 1 cs (no PVP) to 18 cs (4% PVP K90). The contact time of ophthalmic formulations on the surface of the eye can be increased by the addition of viscolysers (Ludwig and Van Ooteghem, 1989). The average tonicity of all the formulations was 190 mosmol/kg (range 175–205). It is recognized that several other variables may also influence the penetration of Enalkiren into the eye. These include the type and concentration of buffer (Ahmed and Patton, 1984; Ahmed and Chaudhuri, 1988), the osmotic value of the formulation (Barendsen et al., 1979) and the concentration of the antimicrobial preservative benzalkonium chloride (Camber and Edman, 1987). These variables have not been evaluated in this study.

The effects of pH, EDTA concentration and the presence of 4% PVP K90 on the aqueous humor ratio gave results (Table 2) that were statistically evaluated using an analysis of variance (ANOVA) calculation. This ANOVA indicated that among the main factor effects only pH was marginally significant ($p < 0.1$), but of greater statistical significance ($p < 0.05$) was the PVP-pH two-factor interaction. Other main effects (PVP and EDTA), two-way interactions (pH-EDTA and PVP-EDTA) and three-way interaction (pH-PVP-EDTA) were not statistically significant. In the absence of significant interactions, the effect of pH describes the average change in percent aque-

ous humor ratio when going from a pH value of 5.6 to 7. However, a significant PVP-pH interaction in this study indicates that the effect of pH depends on the level of PVP in the formulations. An inspection of the average percent aqueous humor ratio values and the associated 95% confidence intervals calculated for pH and the PVP-pH two-factor interaction (Table 3) indicates that the effect of pH in increasing the aqueous humor levels of Enalkiren is significant only when PVP is present at the 4% level. These calculations show that in the absence of PVP, the average aqueous humor ratio values at pH 5.6 and 7 are not statistically different.

Statistically significant pH-PVP interaction suggests that the ophthalmic formulation should be prepared at a neutral pH to enhance the partitioning of the drug into the eye and simultaneously the residence (contact) time must be increased by the addition of PVP in order to improve the Enalkiren aqueous humor level. From the results of this factorial experiment it is not possible to distinguish between a general viscosity effect and a specific enhancement by PVP. Concentrations of PVP other than 4% were not evaluated in this study.

The results of the 2^3 factorial screening study demonstrated that the addition of 4% PVP K90 to a formulation adjusted to pH 7 should lead to significantly increased Enalkiren aqueous humor levels when compared to the control formulation.

TABLE 3

Average percent aqueous humor ratios calculated for significant main effects and two factor interactions

Factor level	Data points	Average % ratio	95% confidence interval for average % ratio	
Main effects: ($p < 0.1$)				
pH				
5.6	14	115.50	74.39	156.60
7.0	16	168.81	130.36	207.26
Two factor interactions: ($p < 0.05$)				
pH by PVP				
5.6×0%	8	126.12	71.74	180.50
5.6×4%	6	101.33	38.54	164.12
7.0×0%	8	121.50	67.12	175.87
7.0×4%	8	216.12	161.74	270.50

TABLE 4

Aqueous humour levels and percent ratio (optimized/control) for optimized formulation of Enalkiren

Rabbit no.	Aqueous humour level (ng/ml)		Ratio (%)
	Control formula	Optimized formula	
1	134	130	97
2	280	357	128
3	126	313	248
4	301	532	177
5	162	223	138
6	154	156	101
7	268	430	160
8	194	357	184
Mean (SD)	202 (70)	312 (137)	154 (50)

The addition of EDTA, however, did not influence the penetration of Enalkiren into the aqueous humor. To confirm the validity of this statistical inference, an optimized formulation at pH 7.0 with 4% PVP K90 but without EDTA, i.e. a solution with the composition shown for trial 6 in Table 1 and the control formulation were evaluated in eight rabbits. For each rabbit the experimental formulation was instilled into one eye and the opposite eye received the control formula. The aqueous humor levels and the calculated percent ratio values are presented in Table 4.

The difference between the 'optimized' and control formulations was determined to be significant at the 5% level when the aqueous humor levels for each rabbit were compared by a paired-sample *t*-test (Bolton, 1984b). The relative absorption for the two formulations in each rabbit is expressed by the percent ratio (Table 4). A paired-sample *t*-test analysis of the ratio values with or without a log transformation (Bolton, 1984b) further indicated that the difference between the average percent ratio value of 154% for the optimized formulation and a hypothetical value of 100% was significant at the 5% level. Therefore, it has been demonstrated that using a factorial experimental design approach aqueous humor levels of Enalkiren can be significantly increased by formulating the ophthalmic solution at pH 7 with the addition of 4% PVP K90. Furthermore, this study showed that the factorial experimental

approach may be used to optimize the in vivo performance of an ophthalmic formulation.

Conclusions

Factorial designs could be a useful screening tool for the selection of additives for ophthalmic formulations. A 2^3 factorial formulation study demonstrated that the transport of Enalkiren into the rabbit eye was influenced by pH, and statistically significant interaction effects were observed between the variables pH and PVP K90. The aqueous humor levels of Enalkiren in rabbit were increased on an average by 50% when compared to a control solution by using an ophthalmic formulation at pH 7 containing 4% PVP K90. This investigation has demonstrated the applicability and advantages of factorial experiments in ophthalmic formulation development. Effects of various formulation factors and the interactions between them can be studied simultaneously with less experimentation in order to optimize the performance of the ophthalmic formulation.

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