Pharmaceutical Research, Vol. 5, No. 4, 1988

Report

The Effect of Azone on Ocular Levobunolol Absorption: Calculating the Area Under the Curve and Its Standard Error Using Tissue Sampling Compartments

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Received June 12, 1987; accepted November 10, 1987

Methods of calculating the area under the concentration–time curve and the associated standard error are proposed for studies in which each animal contributes one independent data point to a pool of data. This approach can be used for data analysis in bioequivalence studies employing tissue sampling compartments. Application of this method indicated that an azone-containing ophthalmic formulation of levobunolol did not produce better ocular bioavailability than a formulation containing no penetration enhancer.

KEY WORDS: area under the curve; standard error; variance; bioequivalence; tissue concentrations; azone; levobunolol.

INTRODUCTION

Bioavailability is the term indicating the measurements of both the relative amount of an administered drug that reaches the general circulation and the rate at which it occurs (1). General circulation usually refers to the circulating blood that can be sampled to determine systemic drug concentrations. In some circumstances, such as ophthalmic drug instillation, a specific biologic fluid (e.g., the aqueous humor) carries the drug to the site of action (2–4). Bioavailability is then the fraction of a dose that reaches the site of action. It can be directly evaluated by either examining the drug concentration–time profile in a specific tissue or determining its pharmacologic effects at the site of action (5,6).

In bioequivalence studies, the bioavailability of generic dosage forms is compared. Efforts are usually made to detect any difference in bioavailability between a test and a reference preparation and to estimate the probability that a difference does not arise through chance alone. To address these issues, estimation of the means of the area under the curve (AUC) values and their associated variances is required. This variance is proportional to the sum of squares of the deviations from the mean of the AUC values. It is generally used in the statistical analysis of the difference between the mean AUC values of different treatments. Calculation of the variance of the mean AUC value is not usually done in ocular pharmacokinetic studies or in tissue distribution studies because, in these studies, the animals need to be euthanized to collect tissue samples. Therefore, the collection of multiple samples over time from each animal to generate an individual temporal profile is impossible. Alternatively, tissues may be obtained from a group of animals euthanized at each time point. The differences between the mean tissue concentrations of different treatments at each time point are then compared independently. The results of this method can be difficult to interpret when the statistical tests do not provide consistent results for all time points. That is, statistically significant differences may be detected at some time points but not at others.

A composite approach is therefore desired in which data at all time points can be considered together. A population kinetic approach (7) is a solution. However, more than one parameter (e.g., bioavailability, volume of distribution, and clearance) will have to be estimated simultaneously by the use of fitting techniques. We have employed the AUC, a parameter that can be directly quantitated in tissue distribution studies, for comparison of the extent of absorption. A study to determine whether azone in ophthalmic formulations has any enhancement effect on ocular bioavailability of levobunolol was initiated. The aqueous humoral fluid was collected from individual animals so that each animal contributed only one data point to a pool of data. Methods of calculating AUC and standard error values were proposed. A two-sample test was then conducted using mean AUC and standard error values associated with different treatments.

MATERIALS AND METHODS

Female New Zealand albino rabbits weighing 2.08 ± 0.19 kg (mean ± SD) were randomly divided into two groups and received one of the following treatments: group I, 0.5% levobunolol (LBUN); and group II, 0.5% LBUN with 0.025% azone.

Fifty microliters of the drug solution was instilled di-

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0724-8741/88-0400-0238$06.00/0

rectly into the lower conjunctival cul-de-sac of each eye using a Hamilton microliter syringe. After instillation, the eyelids were held closed for 1 min.

One eye was used for each animal. The aqueous humor (in 100-μl aliquots) was collected over a 4-hr period postdose: 0, 5, 10, 15, 30, 45, 60, 120, 180, and 240 min. Four rabbit eyes were used at each sampling time point for each treatment. Experimental details were similar to those reported previously (8). Aqueous humor concentrations of LBUN were quantitated by a high-performance liquid chromatographic (HPLC) method (9).

The two-sample t test was used to test for statistically significant differences between the mean aqueous humor concentrations of these two treatments at individual time points. In addition, the mean AUC as calculated using the linear trapezoidal rule was compared between treatments using the two-sample t test with a Satterthwaite (10) approximation for degrees of freedom. The 0.05 level of significance was the criterion of statistical significance in all tests.

Estimation of the AUC and Its Standard Error

For a bioavailability study composed of n predetermined time points, let i vary from 2 to n. When the animals euthanized at time t_i are randomly selected from a homogeneous population, the tissue concentrations at any t_i are assumed to be independently and normally distributed with population mean μ_i and variance σ_i^2. The tissue concentrations at t_i and t_i' ($i \neq i'$), C_i and C_i', are also assumed to be independently and normally distributed with mean μ_i and μ_i', respectively, and with variances σ_i^2 and σ_i^2. At each sampling time t_i, the number of animals euthanized is m_i. The mean AUC from t_{i-1} to t_i is the mean of all possible trapezoids that are defined by t_i, t_{i-1}, $C_{j,i}$, and $C_{k,i-1}$, where $1 \leq j \leq m_i$ and $1 \leq k \leq m_{i-1}$. There are $m_i \cdot m_{i-1}$ possible combinations for this time interval. The mean AUC from t_{i-1} to t_i is then expressed as

$$\overline{\mathrm{AUC}}_{t_{i-1}}^{t_i} = \frac{1}{m_i \cdot m_{i-1}} \sum_{j=1}^{m_i} \sum_{k=1}^{m_{i-1}} \left(\frac{t_i - t_{i-1}}{2}\right)(C_{j,i} + C_{k,i-1}) \quad (1)$$

The mean AUC from t_1 to t_n is the sum of the mean AUC of each time interval:

$$\overline{\mathrm{AUC}}_{t_1}^{t_n} = \sum_{i=2}^{n} \overline{\mathrm{AUC}}_{t_{i-1}}^{t_i} \quad (2)$$

Combining Eqs. (1) and (2),

$$\overline{\mathrm{AUC}}_{t_1}^{t_n} = \sum_{i=2}^{n} \frac{1}{m_i \cdot m_{i-1}} \sum_{j=1}^{m_i} \sum_{k=1}^{m_{i-1}} \left(\frac{t_i - t_{i-1}}{2}\right)(C_{j,i} + C_{k,i-1}) \quad (3)$$

Equation (3) can be rearranged as follows:

$$\overline{\mathrm{AUC}}_{t_1}^{t_n} = \sum_{i=2}^{n} \left(\frac{t_i - t_{i-1}}{2}\right) \cdot \left(\frac{1}{m_i} \sum_{j=1}^{m_i} C_{j,i} + \frac{1}{m_{i-1}} \sum_{k=1}^{m_{i-1}} C_{k,i-1}\right) \quad (4)$$

Therefore

$$\overline{\mathrm{AUC}}_{t_1}^{t_n} = \sum_{i=2}^{n} \left(\frac{t_i - t_{i-1}}{2}\right)(\overline{C}_i + \overline{C}_{i-1}) \quad (5)$$

Equation (5) can then be expanded and rewritten as follows:

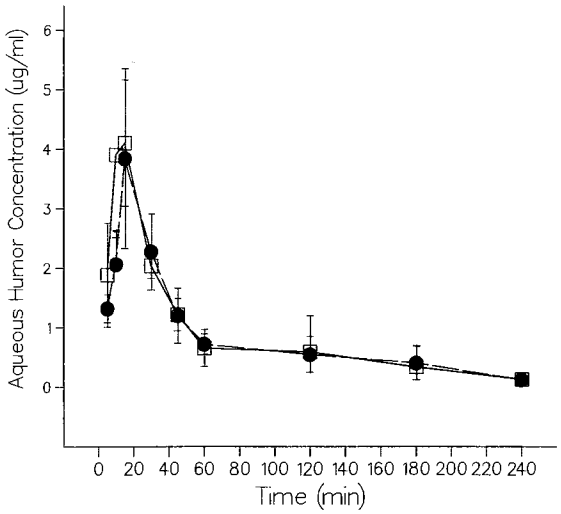

Fig. 1. The aqueous humor concentration–time profiles of levobunolol for group I (□) and group II (●).

$$\overline{\mathrm{AUC}}_{t_1}^{t_n} = \sum_{i=1}^{n} a_i \cdot \overline{C}_i \quad (6)$$

where

$$a_1 = \frac{t_2 - t_1}{2}$$

$$a_n = \frac{t_n - t_{n-1}}{2}$$

and

$$a_i = \frac{t_{i+1} - t_{i-1}}{2} \qquad \text{for } i = 2, \ldots, n-1.$$

In Equation (6), the mean AUC is expressed as a linear combination of the mean concentration at each sampling time. Since the $\overline{C}_i$ values are independent, the variance of the mean AUC is

$$\mathrm{Var}\,(\overline{\mathrm{AUC}}) = \sum_{i=1}^{n} a_i^2 \cdot \sigma_i^2/m_i \quad (7)$$

This variance is estimated by replacing σ_i^2 with the calculated sample variance s_i^2 for $i = 1, \ldots, n$. If we use S_i^2 to denote the term s_i^2/m_i, the standard error of the mean AUC is estimated by

$$\mathrm{SE}\,(\overline{\mathrm{AUC}}) = \sqrt{\sum_{i=1}^{n} a_i^2 \cdot S_i^2} \quad (8)$$

Since it cannot be assumed that σ_i^2 is constant (that is, the population variances are not necessarily homogeneous across the various sampling times), the degrees of freedom for the standard error of the mean AUC are estimated using a Satterthwaite procedure[4] as follows:

$$\mathrm{df} = \left(\sum_{i=1}^{n} a_i^2 \cdot S_i^2\right)^2 \Big/ \left[\sum_{i=1}^{n} (a_i^2 \cdot S_i^2)^2/(m_i - 1)\right] \quad (9)$$

[4] With this procedure, the distribution of a linear combination of independent mean squares is approximated using the chi-square distribution and sample estimators of the mean squares. It is assumed here that the underlying data follow a normal distribution.

Table I. Comparison of Mean Concentrations (SD) Between Treatment I and Treatment II

Time (min)	I	II
5	1.88 (0.94)	1.32 (0.25)
10	3.91 (1.37)*	2.06 (0.59)*
15	4.11 (1.14)	3.84 (1.63)
30	2.04 (0.23)	2.28 (0.69)
45	1.22 (0.29)	1.20 (0.50)
60	0.66 (0.34)	0.72 (0.19)
120	0.29 (0.09)	0.50 (0.19)
180	0.19 (0.13)	0.22 (0.07)
240	0.12 (0.02)	0.09 (0.06)

* Significantly different ($P = 0.049$).

Comparison of Two Mean Areas Under the Curve

As stated earlier, a two-sample test can be used to compare AUC values calculated as described above when determining whether two treatments differ in terms of bioavailability. One method used for normally distributed data with unknown and possibly unequal variances is the two-sample t test employing a Satterthwaite approximation for degrees of freedom. This approach (also known as the Welch procedure) has been recommended as the standard approach when comparing two means from normally distributed populations with unknown variances (11).

The t statistic for comparing the AUC values of treatments I and II is calculated as follows:

$$t = \frac{\overline{AUC}_I - \overline{AUC}_{II}}{\sqrt{SE\,(\overline{AUC}_I)^2 + SE\,(\overline{AUC}_{II})^2}} \quad (10)$$

The corresponding degrees of freedom, calculated using the Satterthwaite procedure to account for possible heterogeneity between treatments, are as follows:

$$df_{diff} = \frac{[SE(\overline{AUC}_I)^2 + SE(\overline{AUC}_{II})^2]^2}{[SE(\overline{AUC}_I)^2]^2/df(\overline{AUC}_{II}) + [SE(\overline{AUC}_I)^2]^2/df(\overline{AUC}_{II})} \quad (11)$$

It should be noted here that if the degrees of freedom are large enough (say, greater than or equal to 30), the t statistic given in Eq. (10) can be tested for significance using the standard normal distribution.

RESULTS AND DISCUSSION

After an eyedrop instillation, levobunolol penetrated the cornea and appeared in the aqueous humor readily. The absorption phase was over in 45 min postdose. Subsequently, the drug concentration in the aqueous humor declined in a first-order fashion (Fig. 1).

The mean concentrations and standard deviations at each time point for treatments I and II are shown in Table I. It is obvious from this table that the sample standard deviations are not uniform over time within either treatment group. Application of Levene's test (12) to these data indicated statistically significant variance heterogeneity within both treatment groups ($P \leq 0.05$).

Comparisons of mean concentrations at each time point between treatment I and treatment II showed a statistically significant difference between treatments only at the 10-min time point. Mean concentrations at all other sampling time points were not statistically different between treatments. To determine whether these two treatments were bioequivalent, the rates (t_{max} and C_{max}) and extents (AUC) of absorption were then compared.

Table II. Estimated Area Under the Concentration–Time Curve of Levobunolol (Mean ± SE)

Treatment	Mean ± SE	df[a]
I	176 ± 11	13.8
II	180 ± 13	11.7
Difference	4 ± 17	24.2

[a] Degrees of freedom.

The time to peak seemed to occur in the 10-min to 30-min interval, within which three time samples were taken. Further analysis showed no significant difference between the mean concentrations of the 10-min and the 15-min samples of each treatment.

The estimated mean AUC values and associated degrees of freedom are listed in Table II. The AUC values were not statistically significantly different from each other ($P = 0.826$).

Although statistically significant differences in mean concentrations were detected in one of nine sampling time points, the contribution of the 10-min time point to the overall bioavailability profile did not appear to be significant. This supports the contention that both treatments produced the same ocular bioavailability. Considering both the rate and the extent of ocular absorption, it follows that both treatments were bioequivalent to each other.

Levobunolol penetrates the cornea well (13). In this study, an azone-containing formulation failed to enhance the ocular bioavailability of levobunolol *in vivo*. The role of azone as an ocular penetration enhancer will be reported elsewhere.

In this report, we have described a method for calculating the standard error of the mean AUC value of a drug concentration in ocular tissues. This calculation is simple and, therefore, is proposed as one approach which may be used in analyzing data from comparative bioavailability studies employing tissue samples.

ACKNOWLEDGMENT

The author thanks Ms. S. Liu for technical assistance.

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