Errors in Measurement of the Activity of Carbonic Anhydrase Inhibitors

To the Editor:

The paper by Putnam, Schoenwald, Duffel, Barknecht, Segarra and Campbell (Invest Ophthalmol Vis Sci 28:1373, 1987) has serious errors in the measurement of the potency of sulfonamide inhibitors of carbonic anhydrase. Additionally, there are errors and omissions in citing the literature, and all of this leads to questionable conclusions. I do not question their finding that aminozolamide lowers IOP in the normal rabbit or a chymotrypsin-treated rabbit. We have the same result in the normal (Drug Dev Res 10:225, 1987). Still, where are data for untreated eyes following application of drug suspension?

The chief experimental flaw is the use of the wrong isozyme (B or I instead of C or II), and the wrong substrate (nitrophenylacetate instead of CO$_2$ or HCO$_3^-$) in the in vitro assay of sulfonamide activity. This results in obtaining I$_{50}$ values for their compounds in the $\mu$M instead of the nM range, and is entirely predictable. For when such a system is used the turnover number is very low (10$^{-4}$ that for CO$_2$), so that high concentrations of enzyme must be used, 100 mg/L or 3 $\mu$M. (In the usual CO$_2$ assay with carbonic anhydrase C or II, I–2 nM is used.) Thus, the lowest I$_{50}$ possible is 1.5 $\mu$M, and that is just what Putnam et al found. However, it can readily be shown that under these conditions, the K$_i$ or potency for a strong inhibitor is not measureable. One would get within experimental error the same I$_{50}$ whether the K$_i$ was 10$^{-7}$ M or 10$^{-10}$ M, that is, an I$_{50}$ one-half of the enzyme concentration added.

Carbonic anhydrase B does not occur in the ciliary process epithelium (Wistrand and Garg, Invest Ophthalmol Vis Sci 18:802, 1979; Wistrand, Schenholm and Lonnerholm, Invest Ophthalmol Vis Sci 27:419, 1986). Its use by Putnam et al is incomprehensible since enzyme C is available. Equally hard to fathom is the use of p-nitro-phenylacetate instead of CO$_2$ or HCO$_3^-$. Putnam et al duly note that their I$_{50}$ values are 1000 times greater than literature values, but do not appear concerned. Didn’t this surprise the reviewers?

Not knowing the true K$_i$, it is impossible to calculate the fractional or percent inhibition in vivo from the drug concentration at the active site. Authors give a figure of 99+%, but no hint as to where it came from. Equally unsatisfactory is their use of drug concentration in the “ciliary body/iris” rather than ciliary process. Thus, there are no real pharmacological results from this work.

Curious misreading of the literature occurs on p. 1361 where authors say that retention of inhibitor in the eye is an important requirement for use in glaucoma “because of the rapid turnover of carbonic anhydrase.” This enzyme actually has so low a turnover that it is barely measureable, so I hastened to look for the source of the statement only to find that it was me, in a 1960 paper where this was not even measured or mentioned!

Authors conclude that accession to the anterior chamber does not predict potency of a drug to lower IOP. But if they included the K$_i$ of the drug, and measured the concentration in the ciliary process, they might be close to the mark.

Finally they mention a paper from their group saying that aminozolamide in a gel preparation lowers IOP in ocular hypertension in man, but fail to record that in that study, the lowering from gel alone was considerable. They do not cite a more recent paper from their group which shows that a suspension of...
aaminozolamide fails to lower IOP or aqueous flow in hypertensives (Kalina, Lewis, and Brubaker, Suppl. to Invest Ophthalmol Vis Sci 28:268, 1987). Why the suspension of aminozolamide works in rabbit and not in man is an important subject for study.

To the Editor:

In the following paragraphs we intend to answer the criticism directed towards our recent article (Invest Ophthalmol Vis Sci 28:1373, 1987) by Dr. Maren in his letter to the editor.

As Dr. Maren has discussed in his letter, we are aware of the limitations inherent in K_i determinations using the ester hydrolysis assay. We must emphasize again, however, that we did not attempt to calculate K_i values. Because of the nonlinear relationship of figure 5 (slope from Lineweaver-Burk plot vs. inhibitor concentration), it was not possible to calculate a K_i value with certainty.

We must also point out that the commonly used CO_2 hydration assay (Maren TH: J Pharmacol Exp Ther 130:26, 1960) in which the time of the reaction is measured over a pH change from 10 to 7.4 cannot be used to accurately determine K_i. The change in hydronium ion concentration can have a significant effect on enzyme activity. Indeed, Dr. Maren was aware of this when he reported that the turnover number of carbonic anhydrase (CA) was 20 \times 10^8 \text{ min}^{-1} when using a pH range of 10 to 7.4, but was one-half this when he used a pH range of 7.9 to 7.4 (our reference 20: J Pharmacol Exp Ther 130:389, 1960). The advantage of the ester hydrolysis assay is that it can be conducted at a single pH value. It is correct that this particular assay is not as physiologically relevant as a CO_2 hydration assay since p-nitrophenylacetate is used as a substrate; however, the esterase assay has been documented to produce similar inhibition characteristics (our reference 12: J Am Chem Soc 87:5497, 1965, and Pocker Y and Stone JT: Biochemistry 7:3021, 1968).

In reference to the use of isoenzyme B, a careful examination of the title of our paper reveals that the work was done in the rabbit eye, not the human eye. While Wistrand and coworkers (Invest Ophthalmol Vis Sci 18:802, 1979 and 27:419, 1986) demonstrated the absence of isoenzyme B in human ciliary process, the work of Kishida and Ochi, referenced in our paper (Experientia 36:42, 1980), establishes the fact that both isoenzymes are present in the rabbit ciliary process.

With regard to the alleged misquotation, we disagree with Dr. Maren. His 1960 paper gives experimental results clearly showing the high turnover number of CA. Moreover, I. H. Segal in "Enzyme Kinetics" (J. Wiley and Sons, 1975, p. 80) states that "Carbonic anhydrase has one of the highest turnover numbers (36 \times 10^6 \text{ min}^{-1})."

Finally, it is true that if the K_i values of CA inhibitors are very low (<10^{-7} M), the IC_{50}s would tend to be similar. However, the results of Figure 5 show the similarity in inhibition of aminozolamide, its acetamido metabolite, and ethoxzolamide at all inhibitor concentrations. The differences in IC_{50} values from Dr. Maren's publications were discussed in the paper.

Our statement regarding 99% inhibition was made in general reference to the often published fact that this % inhibition is necessary for IOP reduction and since we obtained a significant reduction in both rabbits, monkeys and man, we therefore must have achieved 99% inhibition in vivo. We were not referring to our in vitro results.

We have certainly been concerned over our choice of models; however, we would like to emphasize that the purpose for which a model is intended depends greatly on the objective of the work. Our overall goal is to identify which processes are responsible for a lack of topical activity of a CA inhibitor. To this end, we have hypothesized that distribution and subsequent retention at the active site is critical to topical activity of a CA inhibitor. Adequate bioavailability is an important factor, but only secondary to retention. It is not our intention to determine absolute numbers for physiological processes in man but rather to interpret our results for the purpose of developing new antiglaucoma agents. It is pertinent to observe that although there has been an explosion of new therapeutic agents in many medical disciplines over the last 30+ years, a similar phenomenon has not occurred in ophthalmology. No new drug entities have been developed exclusively for use in the eye, but instead have been discovered from screening programs of drugs designed solely for systemic use.

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