Albumin-binding of diclofenac and the effect of a site II inhibitor in the aqueous humor of cataract patients with the instillation of diclofenac

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Abstract

Diclofenac instillation has been widely used in cataract surgery to prevent postoperative inflammation. Since diclofenac strongly binds to albumin in the circulation, it does not have a sufficient effect on patients in whom diclofenac strongly binds to albumin in the aqueous humor. To decrease in diclofenac binding and increase free diclofenac levels are necessary in these patients. We investigated the binding of diclofenac to albumin in the aqueous humor. In a diclofenac binding assay with albumin in the aqueous humor of individual patients, diclofenac was extracted from aliquots of the aqueous humor, and its total levels were measured using ultra high performance liquid chromatography (UHPLC). Free diclofenac levels were measured using ultrafiltration and UHPLC. The albumin-binding fraction of diclofenac was 0.8 or higher in the aqueous humor of some patients. Ibuprofen significantly inhibited diclofenac binding to site II of albumin in mimic aqueous humor, but not in pooled aqueous humor. This difference may have been due to the weak binding of diclofenac to site II in pooled aqueous humor. We used flurbiprofen instead of diclofenac. Flurbiprofen has been shown to bind more strongly than diclofenac to the same site of albumin. Thus, we investigated the inhibitory effect of ibuprofen on the binding of flurbiprofen to albumin in pooled aqueous humor. The results indicated that ibuprofen significantly inhibited the flurbiprofen binding. An effective diclofenac administration method may be established for clinical application by the instillation of an appropriate inhibitor of binding to albumin site II.

Key words: diclofenac ophthalmic solution, cataract patient, albumin, aqueous humor, protein binding

1. Introduction

Diclofenac, the non-steroidal anti-inflammatory drug (NSAID), has been administered to many patients for its analgesic and anti-inflammatory effects. Although absorbed drugs generally bind to albumin after their distribution to the circulation, their binding capacities are various [1]. Two drug-binding sites have been identified in albumin: sites I and II [2-4], and diclofenac has been shown to strongly bind to site II [5]. Thus, the effect of this drug may be insufficient due to low levels of free diclofenac. To increase its effect, an elevation in the free concentration of diclofenac is needed by inhibiting its binding to albumin site II.

We previously succeeded in strengthening the analgesic effect of diclofenac in patients with rheumatoid arthritis with the combination use of nabumetone (albumin-binding of diclofenac to site II was inhibited by the main active metabolite of nabumetone, 6-methoxy-2-naphthylacetic acid), which inhibits albumin-binding of diclofenac when administered as a suppository and absorbed into the circulation (distribution of diclofenac to the target extravascular inflammatory tissue was promoted by inhibiting intravascular albumin-binding of diclofenac) [5].

Diclofenac instillation has been widely used in cataract surgery to prevent intraoperative miosis [6], macular edema [7, 8] and postoperative inflammation [8]. The aqueous humor contains albumin, which diclofenac binds to [9]. If diclofenac strongly binds to albumin in the aqueous humor, the drug is likely to be excreted in the venous system through the canal of Schlemm without having an effect on the eye. However, albumin-binding of diclofenac has not yet been examined in the aqueous humor.

Unlike previous studies, in which diclofenac distributed to the extravascular target tissue was increased by elevating free diclofenac levels through the inhibition of

intravascular albumin-binding of diclofenac, we used a unique method that directly increased the effect of diclofenac on the target tissue in the eye by inhibiting albumin-binding of diclofenac in the aqueous humor with an inhibitor, as shown in Figure 1 (elevation in free diclofenac levels in a special environment, target ocular tissue).

In this study, we investigated albumin-binding capacities of diclofenac in the aqueous humor by measuring the total and free levels of diclofenac administered by instillation and albumin levels in the aqueous humor. A binding inhibitory experiment should be conducted for patients with diclofenac strongly bound to albumin in the aqueous humor, but not for patients with diclofenac weakly bound to albumin in the aqueous humor. We also examined the inhibitory effect of ibuprofen, which was used as a representative drug that binds to albumin site II, on the albumin-binding of diclofenac in the aqueous humor as basic research.

2. Materials and Methods

2.1. Chemicals

Each chemical was obtained from the following sources: diclofenac from Novartis Pharma (Tokyo, Japan); 0.1% diclofenac ophthalmic solution from Wakamoto (Tokyo, Japan); flurbiprofen from Kaken Pharmaceutical (Tokyo, Japan); ibuprofen from Tokyo Chemical Industry (Tokyo, Japan); and human serum albumin (essentially fatty acid free) from Sigma-Aldrich (St Louis, MO). All other chemicals were of analytical grade.

2.2. Aqueous humor samples from cataract patients

This study was performed after approval by the Miyazaki University Ethical Committee and obtaining informed consent from patients at the Ozaki Eye Hospital. One drop of diclofenac ophthalmic solution was administered to patients 3, 2, 1 and 0.5 hours before cataract surgery. The aqueous humor was collected from the anterior chamber after the initiation of cataract surgery, and was stored at -80°C until later use.

2.3. Preparation of pooled aqueous humor

Pooled aqueous humor was prepared by combining 150 μL or smaller volumes of aqueous humor samples from 34 patients. The albumin level in the pooled aqueous humor was 1.55 μM.

2.4. Preparation of mimic aqueous humor

Mimic aqueous humor was prepared by dissolving and adjusting albumin to 1.55 μM with 0.067 M phosphate buffer (pH 7.4). The albumin level in mimic aqueous

humor was adjusted to that in the above pooled aqueous humor.

2.5. Measurement of total diclofenac levels in the aqueous humor of each patient

Calibration curve samples were prepared by adding diclofenac to 30 μL of distilled water in order to adjust the concentration to 0.3, 0.6 and 0.9 μM. A total of 200 μL of 3 M hydrochloric acid and 2.5 ml of cyclohexane were added to 30 μL of each calibration curve sample and aqueous humor of the patient, followed by the addition of the internal standard, hexyl 4-hydroxybenzoate, to a concentration of 0.5 μM. These mixtures were shaken for 10 minutes for extraction and centrifuged at 2,970 *g* for 10 minutes. The supernatant (2 mL) was collected from the organic layer and dried by suction under reduced pressure. Dried samples were dissolved with the mobile phase to prepare samples for ultra high performance liquid chromatography (UHPLC).

2.6. Measurement of free diclofenac levels in the aqueous humor of each patient

Calibration curve samples for UHPLC were prepared by adding diclofenac to aliquots of 0.067 M phosphate buffer (pH 7.4) in order to adjust the final diclofenac concentration to 0.15, 0.3 and 0.45 μM. Aqueous humor samples were prepared by applying 55 μL to a Tosoh plastic ultrafiltration apparatus (Minicent-10; Tosoh Co., Tokyo, Japan) followed by centrifugation (at 2,270 *g* for 10 minutes), and these filtrates were then used as samples for UHPLC.

2.7. Conditions of drug measurements by UHPLC and calculation of the binding fraction

The concentrations of diclofenac and flurbiprofen were determined by the

UHPLC system (Shimadzu, Kyoto, Japan) consisting of a SPD-20A UV/VIS detector, LC-30AD pump, SIL-30AC auto injector, CBM-20A system controller, and CTO-10ACvp column oven, equipped with an Inert Sustain® C18 column (2 μm) (GL Sciences Inc., Tokyo, Japan). UHPLC was performed at a flow rate of 1.0 mL/min at 40 °C. The eluent was monitored at 270 nm. The mobile phase was a mixture of acetonitrile, distilled water, methanol, and acetic acid at a ratio of 108: 91: 20: 1 (v/v).

The bound fraction of diclofenac and flurbiprofen was calculated as follows:

$$\text{bound fraction (fb)} = \frac{[D_b]}{[D_f]+[D_b]} \quad (1)$$

where $[D_f]$ and $[D_b]$ are unbound and bound drug concentrations, respectively.

2.8. Measurement of the albumin concentration in the aqueous humor

The concentrations of albumin in aqueous humor samples were measured by immunonephelometry using Cobas Integra 400 plus (Roche Diagnostics, Basel, Switzerland). The assay kit for albumin was U-ALB II (Roche Diagnostics, Basel, Switzerland).

2.9. Influences of ibuprofen on the binding of diclofenac and flurbiprofen to site II of albumin in mimic aqueous humor

The following experiment was performed to investigate the influence of ibuprofen on the binding of diclofenac and flurbiprofen to site II of albumin in mimic aqueous humor. Diclofenac was added to 55 μL of mimic aqueous humor in order to adjust the final concentration to 0.3 μM. Samples with and without the addition of

ibuprofen at 10 μM were prepared and centrifuged at 2,270 *g* for 10 minutes using the Tosoh plastic ultrafiltration apparatus. These filtrates were used as samples for UHPLC. Flurbiprofen samples for UHPLC were prepared in a similar manner.

Free diclofenac and flurbiprofen levels were measured using UHPLC, and the free fractions of diclofenac and flurbiprofen were determined using the equation below:

$$\text{free fraction (fu)} = \frac{[D_f]}{[D_f] + [D_b]} \quad (2)$$

2.10. Influence of ibuprofen on the binding of diclofenac and flurbiprofen to site II of albumin in pooled aqueous humor

The following experiment was performed to investigate the influence of ibuprofen on the binding of diclofenac and flurbiprofen to site II of albumin in pooled aqueous humor. Pooled aqueous humor samples containing 0.3 μM diclofenac (55 μL) with and without the addition of ibuprofen at 10 μM were prepared and centrifuged at 2,270 *g* for 10 minutes using the Tosoh plastic ultrafiltration apparatus, and these filtrates were used as samples for UHPLC. Flurbiprofen was added in order to adjust the final concentration to 0.3 μM, and samples for UHPLC were prepared as described above. Free diclofenac and flurbiprofen levels were measured using UHPLC, and the free fractions were determined using Equation (2).

2.11. Inhibition levels by the albumin-binding capacity of diclofenac in mimic aqueous humor

The following experiment was performed to investigate differences in the level of inhibition by ibuprofen in mimic aqueous humor due to differences in albumin site

II-binding of diclofenac. To prepare differences in the albumin-binding capacity of diclofenac in mimic aqueous humor, the albumin concentration was stepwise increased while the diclofenac concentration was maintained at 0.3 μM. Mimic aqueous humor containing albumin at stepwise increasing concentrations: 0.3, 0.5, 0.75, 1.0, 2.0 and 3.0 μM was prepared. Diclofenac was added to 55 μL of mimic aqueous humor, and was adjusted to a final concentration of 0.3 μM. Ibuprofen was then added in order to adjust the ibuprofen concentration to 6 times that of the albumin concentration ([ibuprofen]/ [albumin]=6). These samples were applied to the Tosoh plastic ultrafiltration apparatus, centrifuged at 2,270 *g* for 10 minutes, and these filtrates were used as samples for UHPLC. The free diclofenac level was measured using UHPLC, and the free fraction of diclofenac was determined using Equation (2).

2.12. Interaction mode between two ligands at the binding sites of albumin

Binding parameters were determined by fitting experimental data to the Scatchard equation with a non-linear least squares program (MULTI program) [10]:

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{m} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \tag{3}$$

where r is the number of moles of bound drug per albumin molecule, $[P_t]$ is the total albumin concentration, and K_i and n_i are the binding constant and number of binding sites, respectively, for the class of binding sites. The simultaneous binding of two ligands was analyzed using previously reported equations [11], as follows:

$$r_A = \frac{[A_b]}{[P_t]} = \frac{K_A[A_f] + \chi K_{BA} K_B [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \tag{4}$$

$$r_B = \frac{[B_b]}{[P_t]} = \frac{K_B[B_f] + \chi K_{AB} K_A [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \tag{5}$$

where K_A and K_B are the binding constants of ligands A and B, respectively, [A_f] and [B_f] are the respective free concentrations of ligands A and B, and [A_b] and [B_b] are the bound concentrations of ligands A and B, respectively. χ is a coupling constant, K_{BA} is the binding constant of ligand A in the presence of ligand B, and K_{AB} is the binding constant of ligand B in the presence of ligand A. Theoretical values of χ were calculated with these equations. The interaction mode of ligands on a macromolecule can be evaluated from the sign and magnitude of the value of χ. For example, if ligands A and B independently bind to albumin, χ is equal to 1. The results $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anti-cooperative interactions between ligands respectively. Competitive displacement between ligands is indicated by $\chi = 0$. In this analysis, $r < 0.7$ was used to suppress the contribution of a low-affinity binding site.

Solutions of free diclofenac and flurbiprofen were prepared by ultrafiltration at 2,270 *g* for 10 minutes at 25°C with Minicent-10. The adsorption of flurbiprofen onto the membrane or apparatus was negligible, similar to that observed with diclofenac. Free concentrations of diclofenac and flurbiprofen were determined by the UHPLC system described above.

3. Result

3.1. Albumin-binding of diclofenac in the aqueous humor of each patient

Albumin-binding of diclofenac has not yet been reported in the aqueous humor. To evaluate this, we measured total and free diclofenac levels in the aqueous humor samples of 31 patients, and calculated the albumin-binding fraction of diclofenac (Fig. 2). The highest and lowest-albumin binding fraction of diclofenac in the aqueous humor were 0.995 and 0.085, respectively, which indicated marked variations in the binding fraction among individuals. The highest and lowest albumin levels in the aqueous humor were 12.8 and 0.71 μM, respectively, while those of diclofenac were 0.86 and 0.08 μM, respectively, again showing marked individual variations in albumin and diclofenac levels (Table 1); however, the albumin level was higher than that of diclofenac in the aqueous humor of each patient.

3.2. Influence of ibuprofen on albumin-binding of diclofenac in mimic aqueous humor

We selected the widely used representative drug binding to albumin site II, ibuprofen, as an inhibitor of albumin-binding of diclofenac [12]. To investigate the binding inhibitory effect of ibuprofen in mimic aqueous humor, we measured free concentration of diclofenac and calculated the free fraction of diclofenac in the presence of ibuprofen. Diclofenac and albumin concentrations were adjusted to 0.3 and 1.55 μM, respectively in mimic aqueous humor, which was similar to those in pooled aqueous humor. Binding inhibition by ibuprofen significantly increased the free fraction of diclofenac (Fig. 3).

3.3. Influence of ibuprofen on albumin-binding of diclofenac in pooled aqueous humor

Since ibuprofen was suggested to inhibit albumin-binding of diclofenac, an experiment was performed using the pooled aqueous humor. To investigate the influence of the site II-binding inhibitor, ibuprofen, on albumin-binding of diclofenac in pooled aqueous humor, we measured free concentration of diclofenac and calculated the free fraction of diclofenac in the presence of ibuprofen. Binding inhibition by ibuprofen slightly increased the free fraction of diclofenac but not significantly (Fig. 4).

3.4. Differences in the inhibition level due to differences in the albumin-binding capacity of diclofenac in mimic aqueous humor

To identify the reason for no significant inhibition due to ibuprofen of albumin-binding of diclofenac in pooled aqueous humor, we investigated the inhibitory effect of ibuprofen in samples with various albumin-binding fractions of diclofenac in mimic aqueous humor. The level of inhibition by ibuprofen decreased with decreasing in the albumin-binding fraction of diclofenac. No inhibitory effect was noted when the binding fraction of diclofenac was 0.2 (Fig. 5; free fraction of diclofenac = 0.8).

3.5. Mode of inhibition of binding to albumin site II between diclofenac and flurbiprofen

Ibuprofen did not inhibit albumin-binding of diclofenac in pooled aqueous humor, and this may have been due to weak albumin-binding of diclofenac in the pooled aqueous humor. If diclofenac strongly binds to albumin in pooled aqueous humor, ibuprofen may significantly inhibit the albumin-binding of diclofenac. Flurbiprofen

binds more strongly to albumin site II than diclofenac [13, 14]. Therefore, we used flurbiprofen instead of diclofenac to examine the albumin-binding inhibitory effect of ibuprofen. However, although the binding site of albumin for flurbiprofen was classified as site II, whether this binding site is identical to that for diclofenac has not yet been confirmed. Thus, we investigated the mode of binding inhibition between diclofenac and flurbiprofen based on the theoretical formula of Kragh-Hansen. The interaction between diclofenac and flurbiprofen was consistent with a theoretical curve prepared on the assumption of a competitive reaction (Fig. 6), which suggested that flurbiprofen and diclofenac bind to the same site II of albumin.

3.6. Influence of ibuprofen on albumin-binding of flurbiprofen in mimic aqueous humor

To investigate the influence of the site II-binding inhibitor, ibuprofen, on albumin-binding of flurbiprofen in mimic aqueous humor, we measured free concentration of flurbiprofen and calculated the free fraction of flurbiprofen in the presence of ibuprofen. Ibuprofen inhibited albumin-binding of flurbiprofen and significantly increased the free fraction of flurbiprofen (Fig. 7).

3.7. Influence of ibuprofen on albumin-binding of flurbiprofen in pooled aqueous humor

Since the inhibition of albumin-binding of flurbiprofen using ibuprofen was suggested, we performed an experiment using pooled aqueous humor.

To investigate the influence of the site II-binding inhibitor, ibuprofen, on albumin-binding of flurbiprofen in pooled aqueous humor, we measured free

concentration of flurbiprofen and calculated the free fraction of flurbiprofen in the presence of ibuprofen. Ibuprofen inhibited albumin-binding of flurbiprofen and significantly increased the free fraction of flurbiprofen (Fig. 8).

4. Discussion

The aqueous humor is known to contain albumin [9], which binds to various drugs; however the binding of drugs to albumin in the aqueous humor has not yet been examined.

If externally applied diclofenac strongly binds to albumin in the aqueous humor, its pharmacological effect may not be sufficient. We detected a 0.8 or higher albumin-binding fraction of diclofenac in the aqueous humor samples of some patients (Fig. 2). Therefore, we recognized to be important to inhibit diclofenac binding to albumin with an inhibitor. Although albumin-binding of diclofenac was significantly inhibited with ibuprofen in mimic aqueous humor (Fig. 3), no significant inhibition was noted in pooled aqueous humor (Fig. 4), despite a slight increase in the free fraction of diclofenac, and this difference may have been due to weak albumin-binding of diclofenac in the pooled aqueous humor. Thus, we demonstrated that flurbiprofen, which exhibits markedly higher albumin-binding capacity than that of diclofenac, bound to a binding site identical to that for diclofenac (Fig. 6). Flurbiprofen was subsequently used, instead of diclofenac, to examine the inhibitory effect of ibuprofen on albumin-binding in mimic and pooled aqueous humor, and significant binding inhibition of flurbiprofen was noted (Figs.7 and 8).

Albumin-binding of externally applied diclofenac in the aqueous humor was found to markedly vary among patients, and very strong binding was observed in some patients. It is important to inhibit diclofenac binding to albumin with an inhibitor in aqueous humor of the local intraocular region of patients in whom diclofenac strongly bound to albumin in the aqueous humor. The aim of this study was to determine whether

diclofenac binding to albumin in the aqueous humor could be displaced with an inhibitor. However, the albumin-binding of diclofenac could not be significantly inhibited by ibuprofen in pooled aqueous humor. Furthermore, preliminary experiments (these experiments were performed only once) revealed that flurbiprofen, 6MNA, and long-chain fatty acids hardly inhibited the albumin-binding of diclofenac in pooled aqueous humor (data not shown). We investigated the reason for this unexpected result. The point of interest here is that the albumin-binding fraction of diclofenac in the pooled aqueous humor was generally low (Fig. 4; free fraction of diclofenac = 0.62). Only a few aqueous humor samples had a high bound fraction of diclofenac, as shown in Figure 2. Accordingly, the binding capacity of diclofenac was inevitably low when the experiment was performed using pooled aqueous humor.

We analyzed differences in the influence of ibuprofen on albumin-binding between diclofenac and flurbiprofen in mimic and pooled aqueous humor. Firstly, when controls in assays using mimic aqueous humor were compared (Figs. 3 and 7), the free fractions of diclofenac and flurbiprofen were 0.37 and 0.058, respectively, which indicated that the binding fraction of flurbiprofen was markedly higher than that of diclofenac. The binding-inhibitory effects of ibuprofen on albumin-binding of diclofenac and flurbiprofen were 1.6 and 8.3, respectively, showing that binding of flurbiprofen was more strongly inhibited than that of diclofenac. Controls in assays using pooled aqueous humor were then compared (Figs. 4 and 8). The free fractions of diclofenac and flurbiprofen were 0.62 and 0.25, respectively, which indicated that the binding capacity of flurbiprofen was markedly higher than that of diclofenac, and the binding-inhibitory effects of ibuprofen on albumin-binding of diclofenac and flurbiprofen were 1.02 and 1.62, respectively. These differences in binding inhibition

should have been due to differences in albumin-binding capacities between diclofenac and flurbiprofen in the pooled aqueous humor. Therefore, it is likely that ibuprofen inhibits binding in patients in whom diclofenac strongly binds to albumin, such as those shown in Figure 2.

We wanted to demonstrate an inhibitory effect using aqueous humor in which diclofenac strongly binds to albumin; however, as shown in Figure 2, only a few patients had a 0.8 or higher bound fraction of diclofenac, and only a very small volume of aqueous humor could be collected from a patient. Since measuring total and free concentration of diclofenac is necessary to identify the capacity of diclofenac-binding in each aqueous humor sample, only a very small volume was left after this measurement. Therefore, it is impossible to perform a similar binding inhibition assay using the left aqueous humor of a single patient having strong diclofenac binding capacity with our current measurement technique.

We initially considered that the binding inhibition assay using pooled aqueous humor only was sufficient, and using mimic aqueous humor was unnecessary. However, using mimic aqueous humor was needed for the following reasons: Firstly, investigating the albumin binding capacities (diclofenac << flurbiprofen) of diclofenac ($K_1 = 3.3 \times 10^6$ M^{-1}) and flurbiprofen ($K_1 = 2.53 \times 10^7$ M^{-1}), which compete for the same albumin site II in mimic and pooled aqueous humor, was necessary to identify the qualitative problem of albumin (e.g. slight change of albumin binding site structure) in pooled aqueous humor. Since the albumin-binding fraction of flurbiprofen, with a greater binding constant, was markedly higher than that of diclofenac in both mimic and pooled aqueous humor, we concluded that there was no qualitative problem of albumin in pooled aqueous humor. Secondly, an increase in endogenous substances has been

reported in cataracts [15]. To assume the influence of endogenous substances on binding in pooled aqueous humor, it is necessary to compare the albumin-binding capacities of diclofenac and flurbiprofen between mimic and pooled aqueous humor. These binding capacities were lower in pooled aqueous humor than in mimic aqueous humor, which suggested the presence of endogenous substances in the pooled aqueous humor. If these endogenous substances inhibited albumin-binding of diclofenac in pooled aqueous humor before the addition of ibuprofen, it may have decreaced the inhibitory effect of ibuprofen on diclofenac binding.

Regarding the advantages of inhibiting albumin-binding of drugs in the local intraocular region, to distribute an intravascular drug to the local extravascular target tissue, the following physical properties of drugs are generally problematic: 1) a high targeting ability to a specific tissue and 2) a small distribution volume (high hydrophilicity), in addition to strong binding to albumin; however, considering these physical properties is unnecessary if albumin-binding of the drug is inhibited in the local target of ocular tissue. This local intraocular binding inhibition method may increase the effects of eye drops, including those of NSAIDs, adrenocortical steroids, and prostaglandins having strong albumin-binding capacity.

Regarding the dosing plan conforming to medical practice, we used ibuprofen as an inhibitor to ensure the inhibition of site II-binding based on our experience because the aim of the present study was to investigate whether diclofenac binding to albumin in the aqueous humor could be displaced with an inhibitor. However, eye drops concomitantly applied with diclofenac should be used for clinical application. Fatty

acids that strongly bind to albumin site II are administrable for dry eyes, so they are candidates for concomitantly applicable eye drops, although this is still in the experimental step (In preliminary experiments, fatty acids inhibited the albumin-binding of flurbiprofen in pooled aqueous humor, we did not show the data). The candidate long-chain fatty acids, alpha-linolenic acid and linoleic acid [16] may be promising binding inhibitors, and these may facilitate the preparation of a clinically optimal dosing plan in which these fatty acids are applied before diclofenac instillation to inhibit albumin-binding of diclofenac in the aqueous humor, thereby increasing the pharmacological effect of diclofenac.

In conclusion, we demonstrated that diclofenac strongly bound to albumin in the aqueous humor of some patients, the pharmacological effect of which may be markedly reduced. Therefore, an effective administration method may be established for these patients by the instillation of an appropriate binding inhibitor prior to diclofenac instillation.

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Table 1 Concentration of albumin and total concentration of diclofenac in aqueous humor samples from 31 cataract patients.

	Maximum value	Minimum value	Mean
Concentration of albumin (μM)	12.8	0.71	2.58 ± 2.51
Total concentration of diclofenac (μM)	0.86	0.08	0.32 ± 0.19

Each value represents the mean ± S.D. (n = 31)

Figure legends

Figure 1. Intraocular distribution of free diclofenac by inhibitory drug.

Monotherapy: Binding of diclofenac to albumin in the aqueous humor in the absence of an inhibitory drug.

Combination: Binding inhibition of diclofenac to albumin an inhibitory drug in the aqueous humor.

Figure 2. Binding of diclofenac to albumin in aqueous humor samples from 31 cataract patients.

Figure 3. Influence on the binding of diclofenac to site II of albumin by ibuprofen in mimic aqueous humor.

The following concentrations were used: [albumin in mimic aqueous humor], 1.55 μM; [diclofenac], 0.3 μM; [ibuprofen], 10 μM. Each column shows the mean of three experiments ± S.D.. $^{**}P<0.01$ significantly different from the control.

Figure 4. Influence on the binding of diclofenac to site II of albumin by ibuprofen in pooled aqueous humor.

The following concentrations were used: [pooled aqueous humor] (as albumin), 1.55 μM; [diclofenac], 0.3 μM; [ibuprofen], 10 μM. Each column is the mean of three experiments ± S.D.

Figure 5. Degree of inhibition of the binding of diclofenac to site II of albumin by ibuprofen in mimic aqueous humor.

[albumin in mimic aqueous humor], 3.0, 2.0, 1.0, 0.75, 0.5, 0.3 μM; [diclofenac], 0.3 μM; [ibuprofen], 18, 12, 6.0, 4.5, 3.0, 1.8 μM. Each column shows the mean of three experiments ± S.D.

Figure 6. Binding of diclofenac to albumin in the presence of flurbiprofen (A) and vice versa (B) at pH 7.4 and 25°C.

(A), Binding of diclofenac (30, 32.5, 35, 37.5, 40 μM) to albumin (90 μM) in the presence of flurbiprofen (40 μM); (B), Binding of flurbiprofen (30, 32.5, 35, 37.5, 40 μM) to albumin (90 μM) in the presence of diclofenac (40 μM). ●, Experimental values; ---------, Theoretical curve assuming competitive binding; ————— , Theoretical curve assuming independent binding. All theoretical curves were constructed using n_1 and K_1 values [diclofenac, n_1= 1.0, $K_1 = 3.3\times10^6$ M^{-1}; flurbiprofen , $n_1 = 1.0$, $K_1 = 2.5\times10^7$ M^{-1}].

Figure 7. Influence on the binding of flurbiprofen to site II of albumin by ibuprofen in mimic aqueous humor.

The following concentrations were used: [albumin in mimic aqueous humor] (as albumin), 1.55 μM; [flurbiprofen], 0.3 μM; [ibuprofen], 10 μM. Each column shows the mean of three experiments ± S.D. $^{**}P<0.01$ significantly different from the control.

Figure 8. Influence on the binding of flurbiprofen to site II of albumin by ibuprofen in pooled aqueous humor.

The following concentrations were used: [pooled aqueous humor] (as albumin), 1.55 μM; [flurbiprofen], 0.3 μM; [ibuprofen], 10 μM. Each column shows the mean of three

experiments ± S.D. ** $P<0.01$ significantly different from the control.