BIOPHARMACEUTICS & DRUG DISPOSITION
Biopharm. Drug Dispos. **34**: 125–136 (2013)
Published online 24 January 2013 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/bdd.1829

A diclofenac suppository–nabumetone combination therapy for arthritic pain relief and a monitoring method for the diclofenac binding capacity of HSA site II in rheumatoid arthritis

Nao Setoguchi[a], Norito Takamura[b,*], Ken-ichi Fujita[c], Kenji Ogata[b], Jin Tokunaga[b], Toyotaka Nishio[d], Etsuo Chosa[e], Kazuhiko Arimori[e], Keiichi Kawai[f], and Ryuichi Yamamoto[g]

[a]*Second Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino, Nobeoka, Miyazaki, 882-8508, Japan*

[b]*Second Department of Clinical Pharmacy, Graduate School of Clinical Pharmacy, Kyushu University of Health and Welfare, 1714-1 Yoshino, Nobeoka, Miyazaki, 882-8508, Japan*

[c]*Department of Medical Oncology, International Medical Center, Saitama Medical University, 1397-1 Yamane, Hidaka, Saitama, 350–1298, Japan*

[d]*Shinomiya Pharmacy, NEXUS, Inc., 1-3-57 Shinomiya, Hiratsuka, Kanagawa, 254-0014, Japan*

[e]*University of Miyazaki Hospital, 5200 Kihara, Kiyotake, Miyazaki, Miyazaki, 889-1692, Japan*

[f]*School of Health Sciences, Faculty of Medical, Kanazawa University, 5-11-80 Kadatsuno, Kanazawa, Ishikawa, 920-0942, Japan*

[g]*First Department of Pharmacology, Graduate School of Clinical Pharmacy, Kyushu University of Health and Welfare, 1714-1 Yoshino, Nobeoka, Miyazaki, 882-8508, Japan*

ABSTRACT: Diclofenac suppository, a non-steroidal anti-inflammatory drug (NSAID), is used widely in rheumatoid arthritis (RA) patients with severe arthritic pain. As the binding percentage of diclofenac to serum proteins is high, its free (unbound) concentration after rectal administration is low. To increase temporarily the free concentration of diclofenac and to enhance its analgesic effect by inhibiting the protein binding of diclofenac, the analgesic effect of diclofenac was examined before and after the start of an inhibitor administration to RA patients with insufficient control of arthritic pain, and the protein binding capacity of diclofenac was evaluated. Binding experiments were performed by ultrafiltration, and arthritic pain was recorded by the face scale. Free fractions of diazepam and diclofenac were augmented by increasing 6-methoxy-2-naphthylacetic acid (6-MNA; the active metabolite of the NSAID nabumetone) concentrations. The free fraction of diazepam increased after the start of nabumetone administration to RA patients, and arthritic pain relief was observed. These results suggest that 6-MNA has an inhibitory effect on the protein binding of diclofenac and the free fraction of diazepam can be used to evaluate the binding capacity of diclofenac. It is considered that diclofenac suppository–nabumetone combination therapy and the method for protein binding monitoring by diazepam can positively benefit RA patients with insufficient control of arthritic pain. Copyright © 2013 John Wiley & Sons, Ltd.

Key words: rheumatoid arthritis; diclofenac; nabumetone; human serum albumin; protein binding inhibition

Introduction

The first-choice drug for arthritic pain relief in patients with rheumatoid arthritis (RA) is a non-steroidal anti-inflammatory drug (NSAID), and a diclofenac suppository is used widely in RA patients with severe arthritic pain [1,2]. Diclofenac has the following characteristics: (1) in the case of rectal administration, it reaches its maximum plasma concentration quickly (fast-acting) [3]; (2) it is distributed well to synovial membranes and the synovial fluid [4]; and (3) it has a high

*Correspondence to: Second Department of Clinical Pharmacy, Graduate School of Clinical Pharmacy, Kyushu University of Health and Welfare, 1714–1 Yoshino, Nobeoka, 882–8508, Japan.
E-mail: noritotaka@phoenix.ac.jp

Received 21 September 2012
Revised 1 November 2012
Accepted 30 November 2012

inhibitory activity on cyclooxygenase-2 (COX-2) in human synovial cells [5,6]. Diclofenac suppository is important for reducing severe arthritic pain in RA patients: however, since the binding percentage of diclofenac to serum proteins is high [7], the free (unbound) concentration of diclofenac in plasma is low. As a result, the distribution of diclofenac to tissues is also low. In general, since the pharmacological effects of a drug depend on its free concentration [8], only the free drug that is distributed to tissues reflects the effect of the drug. Therefore, when the relief effect of arthritic pain is not fully acquired by administering a diclofenac suppository to RA patients, its free concentration needs to be increased.

Our previous study investigated enhancements in the diuretic effect of furosemide for the purpose of restoring an attenuated response to furosemide in patients with nephrotic syndrome [9]. The results showed that bucolome inhibited furosemide by binding to serum protein, thereby increasing its free concentration in plasma and urine, and as a result, restored the diuretic response to furosemide in rats.

If some drugs can temporarily inhibit the binding of diclofenac to serum protein using the previous inhibition method (furosemide–bucolome–serum protein interaction), the free concentration of diclofenac in plasma and the distribution of diclofenac to tissues could be increased, and as a result, its analgesic effect may be enhanced. Oral NSAIDs are used together with a diclofenac suppository in RA patients with severe arthritic pain [2]. If an oral NSAID drug can be found that inhibits the binding of diclofenac to serum protein, more effective drug treatment than the present treatment for arthritic pain may be possible.

In the present study, in order to identify the binding protein and binding site of diclofenac, binding studies of diclofenac to proteins were performed in human serum. Moreover, an oral NSAID drug was found that inhibits the binding of diclofenac to serum protein, and the analgesic effect of diclofenac was verified before and after the start of inhibitor administration to RA patients with insufficient control of arthritic pain. The study examined simultaneously how to evaluate the protein binding capacity of diclofenac.

Materials and Methods

Materials

Each chemical was obtained from the following sources: pool plasma samples from healthy adults from Cosmo Bio (Tokyo, Japan); diclofenac from Novartis Pharma (Tokyo, Japan); diazepam from Wako Pure Chemicals (Osaka, Japan); warfarin from Eisai (Tokyo, Japan); flurbiprofen from Kaken Pharmaceutical (Tokyo, Japan); ibuprofen from Tokyo Chemical Industry (Tokyo, Japan); 6-methoxy-2-naphthylacetic acid (6-MNA; the active metabolite of nabumetone) from SmithKline Beecham (Tokyo, Japan); and etodolac, human serum albumin (HSA; essentially fatty acid free), human α_1-acid glycoprotein (AGP) and human γ-globulins from Sigma-Aldrich (St Louis, MO). All other chemicals were of analytical grade.

Plasma samples

Blood samples were obtained from three rheumatoid arthritis patients. This study was approved by the Ethical Committee in the Kyushu University of Health and Welfare, and informed consent was obtained before starting the study.

Blood samples were centrifuged immediately at 1670 $\times$ *g* for 10 min, and plasma was stored at -80 °C until analysis. The concentrations of albumin were measured by the bromcresol green method [10], using Cobas Integra 400 plus (Roche Diagnostics, Basel, Switzerland). The assay kit for albumin was ALB II (Roche Diagnostics, Basel, Switzerland).

Identification of the binding protein of diclofenac in human plasma

Identification of the binding protein of diclofenac in human serum was determined with human plasma and each isolated human serum protein (HSA, AGP, or γ-globulins) solution at 25 °C by the following procedures. Human plasma obtained from each healthy adult was mixed and diluted with 0.067 M phosphate buffer (pH 7.4) to adjust the HSA concentration to 600 μM. Concentrations of isolated HSA (600 μM), AGP (14 μM) and γ-globulins (152 μM) were adjusted by 0.067 M phosphate buffer (pH 7.4). Diclofenac was added to the diluted plasma and each

DOI: 10.1002/bdd

isolated serum protein solution to give a final concentration of 3 μM, corresponding to the therapeutic plasma concentrations after a single rectal administration at a dose of 50 mg [11]. Solutions of unbound diclofenac were prepared by ultrafiltration at 2970 × *g* for 15 min at 25 °C, using a Tosoh plastic ultrafiltration apparatus, Ultracent-10 (Tosoh Co., Tokyo, Japan). Adsorption of diclofenac onto the membrane or apparatus was negligible.

The free concentrations of diclofenac were determined by the Shimadzu high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) consisting of an SPD-20A UV/VIS detector, LC-10ADvp pump, SIL-10ADvp auto injector, SCL-10Avp system controller and CTO-10Avp column oven, equipped with a LiChrosorb RP-18 column (7 μm) (Kanto Reagents, Tokyo, Japan). The HPLC was performed at a flow rate of 1.0 ml/min at 40 °C. The eluent was monitored at 275 nm. The mobile phase was a mixture of acetonitrile, distilled water, methanol and acetic acid at a ratio of 120: 79: 20: 1 (v/v).

The bound fraction of diclofenac was calculated as follows:

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

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

Interaction mode between two ligands at binding sites of HSA

Binding parameters were determined by fitting experimental data to the Scatchard equation with a non-linear least squares program (MULTI program) [12]:

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{m} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \quad (2)$$

where r is the number of moles of bound drug per HSA molecule, $[P_t]$ is the total HSA 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 analysed using previously reported equations [13], as follows:

$$r_A = \frac{[A_b]}{[P_t]} = \frac{K_A[A_f] + \chi K_{BA} K_B [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \quad (3)$$

$$r_B = \frac{[B_b]}{[P_t]} = \frac{K_B[B_f] + \chi K_{AB} K_A [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \quad (4)$$

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 are independently bound to HSA, χ is equal to 1. The results $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anti-cooperative interactions between ligands. Competitive displacement between ligands is indicated by $\chi = 0$. In this analysis, $r < 0.31$ was used to suppress the contribution of a low-affinity binding site.

Solutions of unbound diclofenac, diazepam, warfarin and 6-MNA were prepared by ultrafiltration at 2970 × *g* for 10 min at 25 °C with Ultracent-10. As well as diclofenac, the adsorption of diazepam, warfarin and 6-MNA onto the membrane or apparatus was negligible.

Free concentrations of diclofenac, diazepam, warfarin and 6-MNA were determined by the HPLC system described above. The mobile phase was a mixture of acetonitrile, distilled water, methanol and acetic acid. The ratios were 100:99:20:1 (v/v) for diclofenac, 110:89:20:1 (v/v) for diazepam and 80:119:20:1 (v/v) for warfarin and 6-MNA.

Influence on the HSA binding of diclofenac by NSAIDs

Non-steroidal anti-inflammatory drugs including flurbiprofen, etodolac, ibuprofen and 6-MNA (active metabolite of nabumetone) were used as inhibitors of diclofenac binding to HSA, since these

DOI: 10.1002/bdd

NSAIDs have been reported to bind to site II of HSA [14,15] and their therapeutic plasma concentrations are high [16–19]. The following procedures were performed at 25 °C. Plasma obtained from each healthy adult was mixed and diluted with 0.067 M phosphate buffer (pH 7.4) to adjust the HSA concentration to 600 μM. Diclofenac was added to diluted plasma to give a final concentration of 3 μM. Flurbiprofen, etodolac, ibuprofen or 6-MNA was further added to give a final concentration of 30, 50, 100 or 200 μM after the addition of diclofenac. These concentrations corresponded to therapeutic plasma levels after single or continuous oral administrations (40 mg for flurbiprofen, 200 mg for etodolac, 200 mg for ibuprofen and 800 mg for nabumetone) [16–19]. Solutions of unbound diclofenac were prepared by ultrafiltration at 2970 × *g* for 15 min at 25 °C with Ultracent-10.

Free concentrations of diclofenac were determined by the HPLC system described above. The mobile phase was a mixture of acetonitrile, distilled water, methanol and acetic acid at a ratio of 110:89:20:1 (v/v).The free fraction of diclofenac was calculated as follows:

$$\text{free fraction (fu)} = \frac{[D_f]}{[D_f] + [D_b]} \qquad (5)$$

Establishment of a monitoring method for the in vivo HSA binding of diclofenac

An *ex vivo* monitoring method to evaluate the *in vivo* inhibitory effect of 6-MNA on the HSA binding of diclofenac was established by *in vitro* experiments as follows. The occupancy of the HSA site II with or without the addition of 6-MNA was monitored with diazepam. All procedures were performed at 25 °C. Plasma obtained from each healthy adult was mixed and diluted with 0.067 M phosphate buffer (pH 7.4) to adjust the HSA concentration to 400 μM. Plasma containing 500 or 600 μM HSA was prepared by adding HSA powder to diluted plasma. Diclofenac and diazepam were added to each plasma to give a final concentration of 20 μM. Moreover, the final concentration of 6-MNA added to each plasma ranged from 0 to 600 μM (6-MNA/HSA = 0, 0.25, 0.5, 0.75, 1.0). Solutions of unbound diclofenac and diazepam were prepared by ultrafiltration at 2970 × *g* for 15 min at 25 °C with Ultracent-10.

The free concentrations of diclofenac and diazepam were determined by the HPLC system described above. The mobile phase consisted of acetonitrile, distilled water, methanol and acetic acid at a ratio of 110:89:20:1 (v/v) for diclofenac and 100:99:20:1 (v/v) for diazepam.

Free fractions of diclofenac and diazepam were calculated using Equation (5).

Evaluation of the HSA binding capacity of diclofenac and arthritic pain by 6-MNA in RA patients

The binding capacity of diclofenac to site II of HSA in the absence and presence of 6-MNA was monitored by diazepam as established above. Diazepam was added to the plasma of each patient before and after the start of nabumetone administration to give a concentration of 20 μM in a final volume of 0.5 ml, and two free fractions were compared. Blood collection after the start of nabumetone administration was performed from day 4 after the start of nabumetone administration where the plasma concentration of 6-MNA had already reached a steady-state [19].

Solutions of unbound diazepam were prepared by ultrafiltration at 2970 × *g* for 15 min at 25 °C with Ultracent-10.

Free concentrations of diazepam were determined by the HPLC system described above. The mobile phase was a mixture of acetonitrile, distilled water, methanol and acetic acid at a ratio of 100:99:20:1 (v/v).

Free fractions of diazepam were calculated using Equation (5).

Arthritic pain caused by RA was recorded by the face scale. The face scale shows various faces from a laughing face, corresponding to a state of no pain, to a very unhappy face, corresponding to a state of worst pain. Faces are arranged in enhancing order of pain and are numbered from 1 to 10, with 1 representing 'no pain' and 10 representing 'worst pain'. Arthritic pain was evaluated just before diclofenac suppository administration for 5 days before and after the start of nabumetone administration. Arthritic pain in patient 2 was evaluated just before diclofenac suppository administration at 17:00. Assessment of arthritic pain for 5 days after the start of nabumetone administration was performed from day 4 after the start of nabumetone administration, as well as at the time of blood collection timing.

DOI: 10.1002/bdd

Statistical analysis

Statistical differences were assessed using Dunnett's test for multiple comparisons after a one-way analysis of variance (ANOVA), with a probability level of $p < 0.01$ regarded as significant.

Results

Identification of the binding protein of diclofenac in human plasma

To evaluate the binding capacity of diclofenac to HSA, AGP and γ-globulins, the free concentration of diclofenac was measured and its binding fraction calculated. Diclofenac strongly bound to HSA and the binding fraction was 99.99%. On the other hand, binding fractions to AGP and γ-globulins were low at 17.75% and 18.71%, respectively (Figure 1). This *in vitro* study indicated that diclofenac strongly binds to HSA in human plasma.

Identification of the HSA binding site of diclofenac

To identify the HSA binding site of diclofenac, a typical HSA site I binding drug, warfarin, and a HSA site II binding drug, diazepam, were used. The interaction mode between diclofenac and the site-specific probe was analysed according to the method of Kragh-Hansen [20]. Figure 2 shows the results of a quantitative analysis of mutual displacement between diclofenac and warfarin or diazepam. The kinetic binding constant ($n_1 \cdot K_1$) of diclofenac, diazepam and warfarin on binding to HSA were determined to be 3.8×10^6 M^{-1}, 1.4×10^6 M^{-1} and 2.1×10^5 M^{-1}, respectively (data not shown). Taking their parameters into consideration, a 30 μM concentration of these ligands will inhibit the binding to HSA. In this condition, the binding isotherm of diclofenac in the presence of diazepam, and vice versa, was fairly close to the theoretical curve that assumed competition between these two ligands at a common site (Figure 2, A and B). On the other hand, the binding isotherm of diclofenac in the presence of warfarin, and vice versa, was fairly close to the theoretical curve that assumed independence between these two ligands at a common site (Figure 2, C and D).

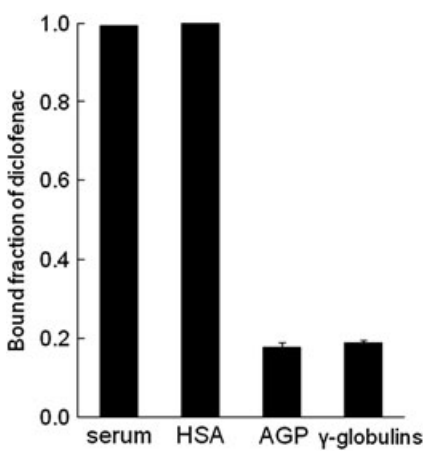

Figure 1. Binding of diclofenac to human serum, HSA, AGP and γ-globulins by ultrafiltration at pH 7.4 and 25 °C. The following concentrations were used: [serum] (as HSA), 600 μM; [HSA], 600 μM; [AGP], 14 μM; [γ-globulins], 152 μM; and [diclofenac], 3 μM. Each column is the mean of three experiments ± SD

Influence of NSAIDs on the HSA binding of diclofenac

To identify which oral NSAIDs inhibit the binding of diclofenac to site II of HSA, the inhibitory effect of flurbiprofen, etodolac, ibuprofen and 6-MNA were examined at therapeutic plasma concentrations. The results indicated that 6-MNA significantly inhibited the HSA binding of diclofenac, and there were no significant differences in flurbiprofen, etodolac and ibuprofen (Figure 3).

Interaction mode between diclofenac and 6-MNA at binding sites of HSA

The type of binding inhibition was analysed according to the method of Kragh-Hansen [20] because 6-MNA at therapeutic plasma concentrations inhibited diclofenac binding to site II of HSA. Figure 4 shows the results of a quantitative analysis of mutual displacement between diclofenac and 6-MNA. The kinetic binding constant ($n_1 \cdot K_1$) of diclofenac and 6-MNA on binding to HSA were determined to be 4.2×10^6 M^{-1} and 1.2×10^7 M^{-1}, respectively (data not shown). Taking these parameters into consideration, a 30 μM concentration of these ligands will inhibit the binding to HSA. In this condition, the binding isotherm of diclofenac in the presence of 6-MNA, and vice versa, was fairly close to the theoretical curve that assumed competition between these two ligands at a common site.

DOI: 10.1002/bdd

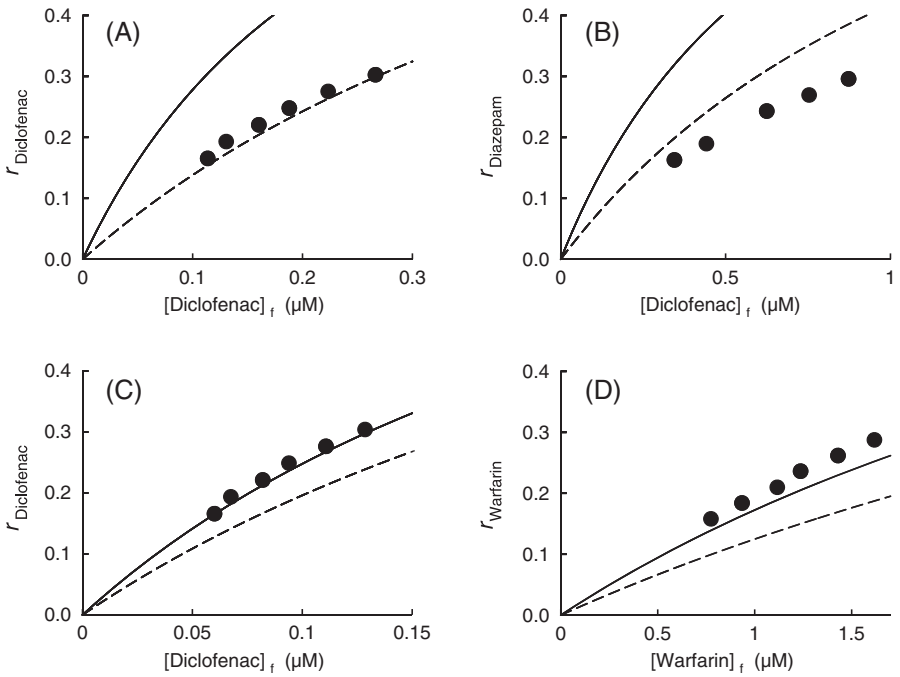

Figure 2. Binding of diclofenac in the presence of diazepam (A) and vice versa (B), warfarin (C) and vice versa (D) to HSA at pH 7.4 and 25 °C. (A) Binding of diclofenac (15–27.5 μM) to HSA (90 μM) in the presence of diazepam (30 μM); (B) Binding of diazepam (15–27.5 μM) to HSA (90 μM) in the presence of diclofenac (30 μM); (C) Binding of diclofenac (15–27.5 μM) to HSA (90 μM) in the presence of warfarin (30 μM); (D) Binding of warfarin (15–27.5 μM) to HSA (90 μM) in the presence of diclofenac (30 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, $n_1 \cdot K_1 = 3.8 \times 10^6$ M^{-1}; diazepam, $n_1 \cdot K_1 = 1.4 \times 10^6$ M^{-1}; warfarin, $n_1 \cdot K_1 = 2.1 \times 10^5$ M^{-1}]

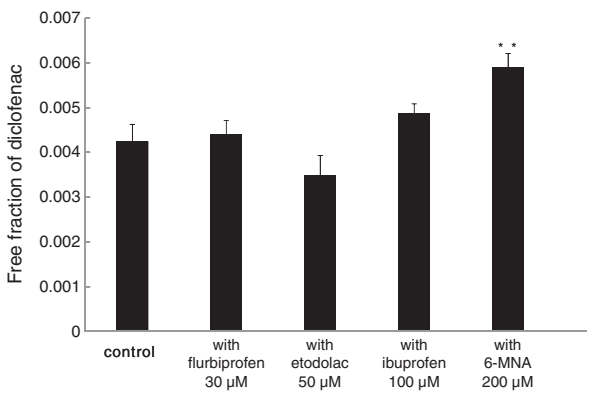

Figure 3. Influence on diclofenac HSA site II binding at the clinically highest blood levels by various NSAIDs binding to site II of HSA. The following concentrations were used: [serum] (as HSA), 600 μM; [diclofenac], 3 μM; [flurbiprofen], 30 μM; [etodolac], 50 μM; [ibuprofen], 100 μM; and [6-MNA], 200 μM. Each column is the mean of three experiments ± SD. $^{**}p < 0.01$ significantly different from the control

Establishment of a monitoring method for the in vivo HSA binding of diclofenac

As a monitoring method to evaluate the binding capacity of diclofenac to site II of HSA in the absence and presence of 6-MNA in RA patients, free fractions were compared using free and total concentrations of diclofenac before and after the start of nabumetone administration. However, these total concentrations before and after the start of nabumetone administration have to be equal [21]. It is very difficult to obtain blood that contains the same total concentrations of diclofenac because time-dependent changes in plasma concentrations vary widely according to changes in condition (including physical condition and endogenous substances) on the day. An attempt was made to establish a monitoring method that evaluates the binding capacity of diclofenac to site II of HSA using diazepam, which binds to the same HSA site II as diclofenac. It is considered

DOI: 10.1002/bdd

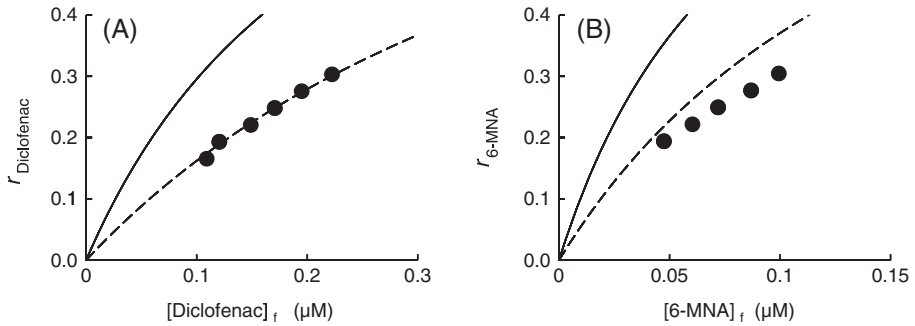

Figure 4. Binding of diclofenac in the presence of 6-MNA (A) and vice versa (B) to HSA at pH 7.4 and 25 °C. (A) Binding of diclofenac (15–27.5 μM) to HSA (90 μM) in the presence of 6-MNA (30 μM); (B) Binding of 6-MNA (15–27.5 μM) to HSA (90 μM) in the presence of diclofenac (30 μM). ● Experimental values; --------- Theoretical curve assuming competitive binding; ————, Theoretical curve assuming independent binding. All theoretical curves were constructed using n_1 and K_1 values [diclofenac, $n_1 \cdot K_1 = 4.2 \times 10^6$ M^{-1}; 6-MNA, $n_1 \cdot K_1 = 1.2 \times 10^7$ M^{-1}]

that it will be possible correctly to evaluate the binding capacity of diclofenac to site II of HSA by comparing these free fractions after adding diazepam at a fixed concentration to the plasma of each patient obtained by collecting blood before and after the start of nabumetone administration. Moreover, it will be possible to simply and speedily perform this evaluation because, in this monitoring method, it is not necessary to measure total diazepam concentrations in plasma by adding the same concentration of diazepam to the plasma of patients before and after the start of nabumetone administration. Diclofenac contained in the plasma of patients does not affect the binding of diazepam to site II of HSA because the therapeutic plasma concentration of diclofenac (approximately 3 μM) is markedly lower (1/200–1/133) than that of HSA (400–600 μM) (data not shown). To confirm whether the binding of diazepam as well as diclofenac to site II of HSA was inhibited by 6-MNA, the free fraction of diazepam was compared with that of diclofenac at [6-MNA/HSA] = 0, 0.25, 0.5, 0.75 and 1.0. As a result, free fractions of diazepam and diclofenac were augmented by increasing 6-MNA concentrations (Figure 5).

Evaluation of the HSA binding capacity of diclofenac and arthritic pain by 6-MNA in RA patients

Table 1 shows the information of three RA patients (two inpatients and an outpatient) who tried to increase free concentrations of diclofenac. Diclofenac suppository was administered to each

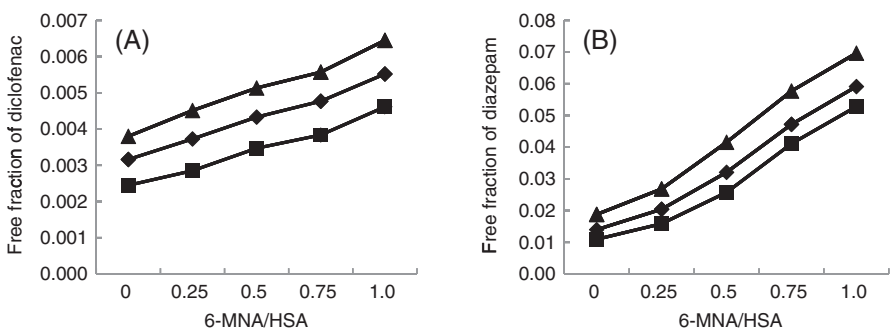

Figure 5. Effect of 6-MNA on the free fraction of diclofenac and diazepam to site II of HSA. The following concentrations were used: [serum] (as HSA), ▲ 400 μM; ♦ 500 μM; ■ 600 μM; [diclofenac], 20 μM; and [diazepam], 20 μM

DOI: 10.1002/bdd

Table 1. Rheumatoid arthritis patient information

	Gender, age	Rheumatoid arthritis	Medication for pain or fever control	
			Without nabumetone	With nabumetone
Patient 1 (inpatient)	Male, 59	Atlantoaxial subluxation (excision of vertebrae cervicales arcus vertebrae, occipital pexis), decrease in myodynamia, numbness in the upper limbs, lumbago	Diclofenac suppository 25 mg daily (17:00)	Diclofenac suppository 12.5 mg daily (17:00) Nabumetone tablet 800 mg daily (13:00)
Patient 2 (inpatient)	Female, 73	Occipitoaxial luxation (occipitoaxial pexis), numbness in the hands, pain in the inferior limb	Diclofenac suppository 50 mg daily (05:00) Diclofenac suppository 25 mg daily (17:00)	Diclofenac suppository 25 mg daily (05:00) Diclofenac suppository 12.5 mg daily (17:00) Nabumetone tablet 800 mg daily (13:00)
Patient 3 (outpatient)	Female, 47	Arthralgia	Diclofenac suppository 25 mg daily (06:00) Etodolac tablet 200 mg twice daily (07:30 and 19:30)	Diclofenac suppository 25 mg daily (06:00) Nabumetone tablet 800 mg daily (19:30)

DOI: 10.1002/bdd

RA patient in consideration of the degree of arthritic pain and the time at which pain was experienced. It has been reported that the time to maximum plasma concentration (T_{max}) of 6-MNA after oral administration of nabumetone is 4.0 h [19] in humans. To obtain the maximum inhibitory effect of 6-MNA on the binding of diclofenac to site II of HSA, nabumetone (800 mg) was given 4.0 h before diclofenac suppository administration. However, the administration time of nabumetone according to patient's life style was considered, as it was difficult to administer nabumetone 4.0 h before diclofenac suppository administration. Nabumetone was administered only once a day because 6-MNA has a long half-life ($t_{1/2}$) of approximately 20.5 h [19]. In patient 1, since the diclofenac suppository needed to be administered at 17:00, nabumetone was given after lunch (13:00), 4.0 h before diclofenac suppository administration. Patient 2 needed an administration of diclofenac suppository twice a day, and her arthritic pain was especially severe in the evening. Therefore, nabumetone was administered after lunch (13:00), 4.0 h before diclofenac suppository administration (17:00), to obtain the maximum inhibitory effect in the evening. In patient 3, since the diclofenac suppository needed to be administered at 06:00, nabumetone should have been given at 02:00, 4.0 h before the diclofenac suppository administration. However, nabumetone was administered after dinner (19:30), the day before the diclofenac suppository administration because it was difficult to administer nabumetone at 02:00. The increase in the free fraction of diazepam after the start of nabumetone administration in patients 1, 2, and 3 was 3.65 times (0.062/0.017), 2.15 times (0.043/0.020) and 1.78 times (0.032/0.018), respectively (Table 2). Arthritic pain relief was simultaneously observed in all patients. The HSA concentrations remained unchanged in patients 1 and 2, and increased in patient 3 who had nutritional recovery by increasing appetite during arthritic pain relief.

Discussion

In the present study, it was shown that diclofenac strongly bound to site II of HSA (Figures 1 and 2). On the basis of these results, drugs that inhibit the binding of diclofenac to site II of HSA were investigated, and, as a result, 6-MNA, the active metabolite of the NSAID nabumetone, inhibited the HSA binding of diclofenac at a therapeutic plasma concentration (Figures 3 and 4). The degree of inhibition of diclofenac was enhanced with increasing concentrations of 6-MNA, HSA (site II) binding of diazepam to the same HSA site II as diclofenac was also inhibited by 6-MNA, and the degree of inhibition was enhanced with increasing concentrations of 6-MNA (Figure 5). These results suggest that diazepam can evaluate the binding capacity of diclofenac to site II of HSA (Figure 6). On the basis of the preliminary examination results, the binding capacity of diclofenac to site II of HSA and the degree of arthritic pain in diclofenac suppository–nabumetone combination therapy were evaluated in three RA patients. As a result, marked increases in the free fraction of diazepam and arthritic pain relief were observed after the start of nabumetone administration in all patients (Table 2).

The diclofenac suppository demonstrated an analgesic effect with a single dose and was

Table 2. Diagnosis of HSA site II binding property in rheumatoid arthritis patients

		Blood collection time	Free fraction of diazepam	HSA (μM)	Face scale
Patient 1 (inpatient)	Without nabumetone	17:30	0.017	433	7.6 ± 0.5
	With nabumetone	17:50	0.062	468	4.7 ± 0.2^{a}
Patient 2 (inpatient)	Without nabumetone	05:45	0.020	519	7.0 ± 0.9
	With nabumetone	17:30	0.043	513	4.6 ± 0.7^{b}
Patient 3 (outpatient)	Without nabumetone	11:00	0.018	414	8.0 ± 0.0
	With nabumetone	10:36	0.032	511	4.0 ± 0.0^{c}

[a] $p < 0.01$ vs before inhibition for patient 1.
[b] $p < 0.01$ vs before inhibition for patient 2.
[c] $p < 0.01$ vs before inhibition for patient 3.

DOI: 10.1002/bdd

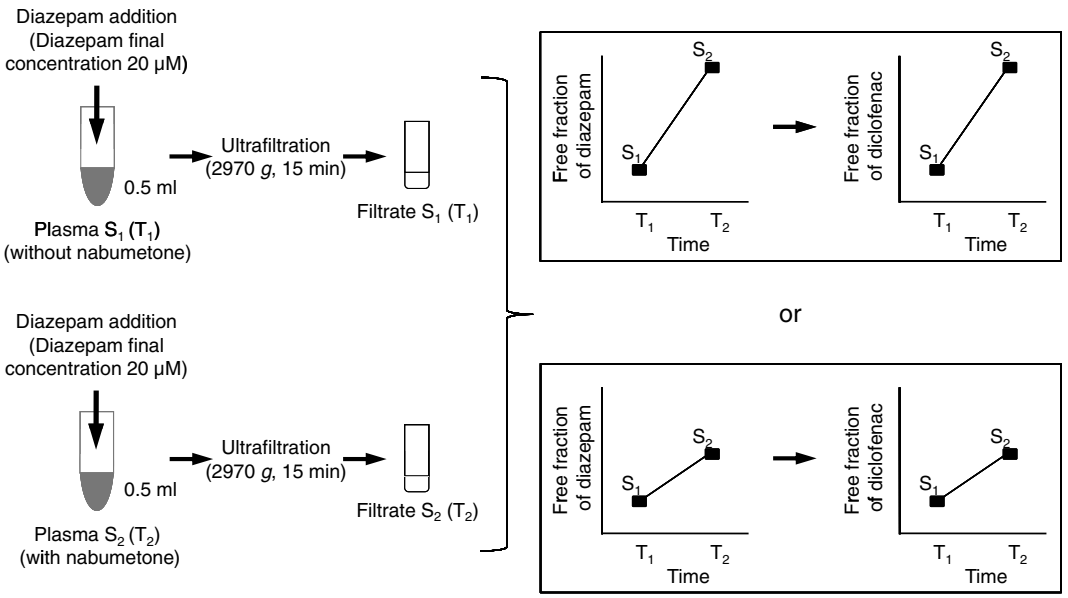

Figure 6. Monitoring method for the binding capacity of diclofenac to site II of HSA by diazepam in RA patients. Diazepam was added to the patient's plasma sample 1 (S_1) and sample 2 (S_2) of 0.5 ml before (T_1) and after (T_2) the start of nabumetone administration to give a final concentration of 20 μM. Each filtrate was obtained by ultrafiltration. Free fractions of diazepam of S_1 and S_2 were compared, and the degree of increase in the free fraction of diazepam from T_1 to T_2 can be calculated. The degree of increase in the free fraction of diclofenac from T_1 to T_2, i.e. the degree of binding capacity of diclofenac to HSA site II before (T_1) and after (T_2) the start of nabumetone administration is predicted by the free fraction of diazepam

distributed well to synovial membranes and the synovial fluid [4]. Therefore, there is a high probability that arthritic pain relief is observed even with transient increases in the free concentration of diclofenac in plasma. For this reason, because diclofenac has very high inhibitory activity on COX-2 compared with nabumetone [22], the analgesic effect of the diclofenac suppository was much higher than that of nabumetone. Therefore, it is considered that nabumetone gives little arthritic pain relief to RA patients with insufficient control of arthritic pain from a diclofenac suppository. In fact, arthritic pain was significantly reduced when changing from etodolac to nabumetone in patient 3, although the inhibitory effect of etodolac (400 mg/day) on COX-2 was greater than that of nabumetone (800 mg/day) (Tables 1 and 2). Therefore, it is considered that the pain relief was due to transient increases in the free concentration of diclofenac by 6-MNA in plasma.

The therapeutic plasma concentrations of 6-MNA (250 and 266 μM at 4 h after administration for 3 and 42 days, respectively) [19] corresponded to about one-half of the HSA concentrations 468–513 μM after the start of nabumetone administration (Table 2). As shown in Figure 3, since 6-MNA (200 μM), corresponding to one-third the concentration of HSA (600 μM) in plasma, significantly inhibited the binding of diclofenac to site II of HSA, it is considered that the therapeutic plasma concentration of 6-MNA is a level sufficient as a binding inhibitor of site II of HSA. Furthermore, nabumetone is available early after administration because the plasma concentration of 6-MNA after nabumetone administration reaches a sufficiently high concentration as the binding inhibitor of site II of HSA. In addition, since 6-MNA has a long $t_{1/2}$ [19], it is considered that 6-MNA sufficiently inhibits the binding of diclofenac to site II of HSA even if the timing of diclofenac suppository administration was shifted too far from the T_{max} of 6-MNA.

In general, if the free concentration of a drug is markedly increased by inhibiting protein binding, its elimination from blood is increased and its plasma concentration is decreased [23,24]. As a result, since accumulation of the drug does not take place, it is expected that the occurrence of

DOI: 10.1002/bdd

side effects should be low. When the metabolism of a drug is also inhibited, its free concentration can be expected to increase due to higher plasma concentrations. However, since the inhibition of drug metabolism suppresses elimination of the drug, plasma concentrations increase markedly. As a result, the occurrence of side effects may be high. The inhibition of diclofenac metabolism does not occur in diclofenac suppository–nabumetone combination therapy because nabumetone and 6-MNA do not inhibit CYP2C9 that is the rate-limiting enzyme in the metabolic clearance of diclofenac [19,25]. Therefore, since it has been suggested that increases in free plasma diclofenac concentrations occurred due to protein binding inhibition, it is considered that the occurrence of side effects should be low. In fact, no side effects were observed in the three patients.

The binding capacity of diclofenac to site II of HSA in diclofenac suppository–nabumetone combination therapy was evaluated. In this evaluation diazepam was added to the plasma of each patient before and after the start of nabumetone administration at the same final concentration, and these free fractions of diazepam were compared (Figure 6). Blood collection after the start of nabumetone administration is recommended at a time between immediately after administration of the diclofenac suppository and the T_{max}. It is considered that plasma concentrations of 6-MNA with a long $t_{1/2}$, that is, the inhibitory effect on the binding of diclofenac to HSA, remains mostly unchanged in that time range because diclofenac reaches C_{max} immediately after rectal administration. After the start of nabumetone administration, blood was collected from patients 1 and 2 and was evaluated at the optimal time for the maximum inhibitory effect on the binding of diclofenac to HSA. The increase in the free fraction of diazepam after the start of nabumetone administration was 3.65 and 2.15 times, respectively, proving that the inhibitory effect of 6-MNA on the binding of diclofenac to HSA was strong. Since there were large differences in the inhibitory effect between patients 1 and 2, it is possible to conjecture that there were large individual differences in the C_{max} of nabumetone between the two patients. On the other hand, in the case of the outpatient, it was difficult to collect blood at the optimal time. Patient 3 was an outpatient, and blood collection after the start of nabumetone administration was performed at 10:36 (consultation time), 4 h 36 min after the diclofenac suppository administration (06:00). It seems that the plasma concentration of 6-MNA was lower than that immediately after administration of the diclofenac suppository because the blood collection point may have passed approximately 15 h after nabumetone administration (19:30). Therefore, it seems that the maximum inhibitory effect on diclofenac binding to HSA was higher than that (1.78 times) in the blood that was collected and evaluated. Thus, even if blood collection cannot be performed at the optimal time, the inhibitory effect of 6-MNA on HSA binding immediately after administration of diclofenac suppository can be predicted using the administration time, T_{max} and $t_{1/2}$ of nabumetone. Moreover, the monitoring method can obtain useful information by comparing the free fraction of diazepam between patients before the start of nabumetone administration. For example, although the concentration of HSA (519 μM) in patient 2 before the start of nabumetone administration was higher than that (433 μM) in patient 1, the free fraction of diazepam (0.020) in patient 2 was higher than that (0.017) in patient 1. It can be predicted that there was a difference in the microenvironment around the HSA site II [26,27] or in the molecular structures [28,29] between the two patients.

In conclusion, it was shown that when the diclofenac suppository was administered at a therapeutic plasma concentration of 6-MNA, the binding to site II of HSA was inhibited and the analgesic effect of diclofenac increased because of this inhibitory effect. The diclofenac suppository–nabumetone combination therapy can be performed safely only by considering the combination of drugs for RA and the administration timing. Furthermore, it was found that the free fraction of diazepam can be used to monitor the inhibitory effect of 6-MNA on the HSA binding of diclofenac. The monitoring method can also predict the microenvironment around the HSA site II [26,27] or the molecular structure [28,29] by comparing the binding capacity to site II of HSA between patients. It is considered that this monitoring method is the optimal approach for evaluating binding capacities in clinical practice because there is very little patient burden and it is possible to perform the evaluation simply and speedily. It is considered that diclofenac

DOI: 10.1002/bdd

suppository–nabumetone combination therapy and the method for monitoring HSA binding by diazepam described in the present study can positively benefit rheumatoid arthritis patients with insufficient control of arthritic pain.

Conflicts of Interest

The authors have no conflicts of interest.

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DOI: 10.1002/bdd