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Dosage plan of a flurbiprofen injection product using inhibition of protein binding by lipid emulsion in rats

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Abstract

Flurbiprofen-axetil (FP-ax), a bolus injection product of a non-steroidal anti-inflammatory drug (NSAID), is a prodrug of flurbiprofen, an NSAID. As flurbiprofen strongly binds to site II of human serum albumin (HSA), the free (unbound) concentration of flurbiprofen after injection of FP-ax is low. We have examined the inhibitory effect of free fatty acid (FFA), a binding inhibitor for site II of HSA, on the binding of flurbiprofen in-vitro and in-vivo by ultrafiltration, to establish an effective dosage of FP-ax. In-vitro, fatty acid mixtures (FAs) inhibited the binding of flurbiprofen to rat serum albumin. The free fraction of flurbiprofen was remarkably increased by FAs in rat serum. In-vivo, FP-ax was injected into a control group (low FFA concentration in serum) and a lipid emulsion group (high FFA concentration in serum). The area under the curve of the free concentration of flurbiprofen during the alpha phase and the distribution volume of the central compartment of flurbiprofen were significantly higher in the lipid emulsion group than the control group (5.0- and 1.2-times, respectively). When FP-ax was administered at high FFA concentration, the free concentration of flurbiprofen and distribution of flurbiprofen to tissues increased transiently. This administration method may be useful for patients with cancer pain, having a potent analgesic effect.

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Introduction

Currently, the guidelines of the World Health Organization for relieving cancer pain with analgesics advocate a so-called ‘analgesic ladder’: patients with mild pain (step 1) should be treated with non-steroidal anti-inflammatory drugs (NSAIDs), patients with moderate pain (step 2) and severe pain (step 3) should receive an opioid in combination with NSAIDs (World Health Organization 1986). Thus, NSAIDs are important for reducing cancer pain (Pace 1995; Cherny 2000).

The intravenous administration of NSAIDs is expected to have a rapid and potent analgesic effect. Flurbiprofen-axetil (FP-ax), a bolus injection product of NSAID, is a prodrug of flurbiprofen. FP-ax is rapidly hydrolysed in flurbiprofen by esterase in blood. As the analgesic effect of FP-ax is rapid and continuous, the drug is indispensable for relieving cancer pain. Flurbiprofen binds to human serum albumin (HSA) very strongly, its association constant is approximately 1.0×10^{-7} M^{-1} (Takla et al 1985), and so the free (unbound) concentration of flurbiprofen in serum is low. In general, since the pharmacological effect of a drug depends on its free concentration (Dasgupta 2002), it seems that the analgesic effect of flurbiprofen is not sufficiently exerted. We reported that bucolome, an NSAID, inhibited furosemide, which is a diuretic binding to site I of HSA, increasing the free concentration of furosemide in serum and urine, and as a result, restoring the diuretic response to furosemide (Takamura et al 2005). Flurbiprofen binds to site II of HSA (Sudlow et al 1976; Wanwimolruk et al 1983). If some drugs can temporarily inhibit the binding of flurbiprofen to site II using the previous inhibition method (furosemide–bucolome–HSA interactions), the free concentration of flurbiprofen in serum will be increased and the analgesic effect may be enhanced.

A lipid emulsion is often administered to patients with cancer (Dionigi et al 1985). Free fatty acid (FFA) is released from the emulsion by lipoprotein lipases in blood. The FFA mainly consists of long chain fatty acids such as oleic acid and linoleic acid. FFA binds to site II of HSA (Menke et al 1989); however, a study of flurbiprofen–FFA–HSA interactions

has not been reported. To establish an effective dosage of FP-ax, we have examined the inhibitory effect of FFA on the binding of flurbiprofen in rats.

Materials and Methods

Materials

Flurbiprofen powder and flurbiprofen-axetil (10 mg mL^{-1}) were donated by Kaken Pharmaceutical Co. (Tokyo, Japan). Oleic acid and linoleic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Intralipos (refined soybean oil 20%, concentrated glycerin 2.2% and refined egg yolk lecithin 1.2%), a lipid emulsion, was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan). HSA (essentially fatty acid free) was purchased from Sigma-Aldrich (St Louis, MO, USA). All other reagents were of analytical grade. A 0.067 M phosphate buffer (pH 7.4) was prepared with dibasic sodium phosphate and monobasic sodium phosphate.

Rat serum was pooled from three adult male Wistar rats (380–400 g). Rats were anaesthetized with ether. Blood samples were drawn from the abdominal aorta with polyethylene disposable syringes and transferred into vacutainers. The blood samples were immediately centrifuged and the serum was separated. The concentration of rat serum albumin (RSA) in this serum was 525 μM. HSA solution (600 μM) was prepared with 0.067 M phosphate buffer (pH 7.4). Fatty acid mixtures (FAs) were prepared by mixing oleic acid and linoleic acid ([linoleic acid]/[oleic acid] = 2).

Binding experiments in-vitro

In-vitro binding of flurbiprofen to albumin (RSA or HSA) was determined in rat serum and in isolated HSA solution at 25°C by the following procedures. The concentration of flurbiprofen was 10 μg mL^{-1} (40 μM), corresponding to the maximum therapeutic concentration. To assess the inhibitory effect of FAs on the binding of flurbiprofen to RSA, FAs were added to rat serum to give final concentrations of 2625–7875 μM – multiples of the molar concentration of RSA. To study the inhibitory effect of oleic acid on the binding of flurbiprofen to HSA, oleic acid was added to isolated HSA solution to give final concentrations of 300–2400 μM – multiples of the molar concentration of HSA. Free species of flurbiprofen were prepared by ultrafiltration (1670 *g*, 8 min, 25 °C) using a Tosoh plastic ultrafiltration apparatus, Ultracent-10 (Tosoh Co., Tokyo, Japan). Adsorption of flurbiprofen onto the membrane or apparatus was negligible.

Free concentrations of flurbiprofen were determined by HPLC using a system consisting of a RF-10A$_{XL}$ fluorescence detector, a LC-10ADvp pump, a SIL-10ADvp auto injector, a SCL-10Avp system controller, and a CTO-10Avp column oven, all from Shimadzu (Kyoto, Japan). A LiChrospher RP-Select B column (5 μm) (Kanto Reagents, Tokyo, Japan) was used for the stationary phase. Flurbiprofen was assayed by fluorescence monitoring (ex. 248 nm, em. 320 nm). HPLC was performed at a flow rate of 1.0 mL min^{-1} at 40°C, using 0.1 M citric acid–0.1 M sodium acetate (pH 4.5)/acetonitrile (1:1, v/v) as the mobile phase.

The free fraction of flurbiprofen was calculated by dividing the unbound flurbiprofen concentration by the total (bound + unbound) flurbiprofen concentration:

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

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

Pharmacokinetics of flurbiprofen in rats

The protocol of the animal study was approved by the Animal Care and Use Committee of the Kyushu University of Health and Welfare. Adult male Wistar rats (327–408 g) were housed in an air-conditioned room with free access to commercial feed and water. Rats were anaesthetized with a 2 mL kg^{-1} urethane solution (60%, w/v). A cannula was inserted into the femoral vein and artery using polyethylene tubing (i.d. 0.5 mm; o.d. 0.8 mm; Natsume Seisakusyo, Tokyo, Japan). The body temperature of the rats was maintained by heating with a lamp. To adjust the solution for intravenous injection to a dose of 1.0 mg kg^{-1}, the FP-ax solution was diluted with saline. FP-ax was injected into two groups of rats intravenously. Saline (3 mL kg^{-1}) was administered to one group (control group), 30 min before the injection of FP-ax. A lipid emulsion (Intralipos, 3 mL kg^{-1}) was administered to the other group (lipid emulsion group) to increase the serum concentration of FFA, 30 min before the injection of FP-ax. After FP-ax injection, the cannula was flushed with a small volume of heparinized saline to ensure that the complete dose had been administered and to prevent the formation of clots.

Blood samples (600 μL) for the measurement of flurbiprofen and FFA concentrations were taken from the femoral artery at 1, 5, 10, 30, 60 and 180 min after the administration of FP-ax. To obtain serum, the blood sample was placed in Capiject (Terumo Medical Co., USA) and centrifuged (1200 *g* for 10 min).

Serum concentrations of flurbiprofen (bound + unbound) were estimated by the following procedure. Serum (30 μL) was added to 50 μL 0.01 M NaOH (containing 3 μg mL^{-1} of 4-biphenylacetic acid, i.s.), 100 μL 3 M HCl, and 1.5 mL cyclohexane, shaken for 10 min, and centrifuged. A 20-μL organic layer sample was injected into the HPLC system. Free concentrations of flurbiprofen were estimated as follows. Serum (250 μL) was placed in an ultrafiltration apparatus, Minicent-10 (Tosoh Co., Tokyo, Japan). The filtrated solution (20 μL) was added to 50 μL 0.01 M NaOH (containing 3 μg mL^{-1} 4-biphenylacetic acid, i.s.), 100 μL 3 M HCl, and 1.5 mL cyclohexane, shaken for 10 min, and centrifuged. A 1.0-mL organic layer sample was evaporated and resolved by HPLC mobile phase (70 μL) and the sample was injected into the HPLC system (50 μL). HPLC conditions were as described above in-vitro.

Pharmacokinetic analysis

Total (bound + unbound) and free areas under the concentration of the flurbiprofen–time curve from 1 to 10 min ($AUC_{1\text{-}10,total}$ and $AUC_{1\text{-}10,free}$) after the administration of FP-ax were

calculated using the log trapezoidal rule. The distribution volume of the central compartment (Vd_c) of flurbiprofen was calculated with the following equation:

$$Vd_c = Dose_{FP}/C_{1\,min}$$

where $Dose_{FP}$ is the dosage of flurbiprofen calculated based on molecular weight (flurbiprofen, 244.26; FP-ax, 330.36) from the dosage of FP-ax. $C_{1\,min}$ was the maximum concentration of flurbiprofen hydrolysed from FP-ax in serum.

Measurement of albumin and FFA concentrations

Albumin and FFA were measured by the bromcresol green method (Doumas et al 1971) and enzyme method (Shimizu et al 1979), respectively, using Cobas Integra 400 plus (Roche Diagnostics, Basel, Switzerland). The assay kits for albumin and FFA were ALB II (Roche Diagnostics, Basel, Switzerland) and Nescauto NEFA-V2 (Alfresa Pharma Co., Osaka, Japan), respectively.

Statistical analysis

Student's *t*-test was used to analyse differences between two groups. Analysis of variance was used to analyse differences among more than two groups, and the significance of difference between two means in these groups was evaluated using Fisher's protected least significant difference.

Results

Effect of oleic acid and linoleic acid on the binding of flurbiprofen in rat serum

To examine FFA as a potential inhibitor of the binding of flurbiprofen to RSA, we measured the free concentration of flurbiprofen and calculated the free fraction of flurbiprofen. In-vivo, we examined the inhibitory effect of FFA using a lipid emulsion, but in-vitro, the inhibitory effect of FFA released from a lipid emulsion could not be evaluated because the activity of lipoprotein lipase was weak in the rat serum added to the lipid emulsion. Therefore, FAs that mimic soybean oil, which is the main component of lipid emulsion, were used in-vitro. To adjust to 5-, 10- and 15-times the RSA concentration ([FAs]/[RSA] were 5, 10 and 15), FAs were added to rat serum containing flurbiprofen ($10\,\mu g\,mL^{-1}$). Figure 1 shows that the free fraction of flurbiprofen increased significantly with increasing FAs. When [FAs]/[RSA] was 5, the free fraction of flurbiprofen was approximately 20.9-times that of the control. Further addition of FAs to serum increased the free fraction of flurbiprofen remarkably, the value being approximately 83.7-times that of the control at [FAs]/[RSA] = 15. This in-vitro study indicated that the binding of flurbiprofen to RSA was inhibited by FAs.

Effect of FFA on the pharmacokinetics of flurbiprofen in rats

To evaluate the effect of FFA on the pharmacokinetics of flurbiprofen, FP-ax was injected in the control group and lipid emulsion group. Figure 2 shows the time course of the change in serum concentration of FFA after the injection of FP-ax in the two groups. Serum concentrations of FFA were significantly higher in the lipid emulsion group than the control group until 30 min after the administration of FP-ax, and there were no significant differences in the FFA concentrations of the two groups at 60 min after the injection.

Figure 3 indicates the time course of the change in serum concentration of flurbiprofen after the injection of FP-ax in the two groups. During the alpha phase (until 10 min after FP-ax administration), serum concentrations of flurbiprofen were lower in the lipid emulsion group than the control group.

Figure 4 shows the time course of the change in free concentration of flurbiprofen after the injection of FP-ax in the two groups. As in the serum FFA profile, free concentrations of flurbiprofen were significantly higher in the lipid emulsion

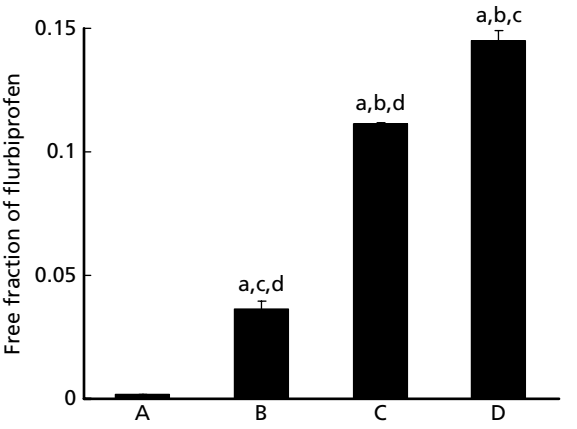

Figure 1 Effect of oleic acid and linoleic acid mixtures (FAs) on the free fraction of flurbiprofen in rat serum: A, control; B, [FAs]/[RSA] = 5; C, [FAs]/[RSA] = 10; D, [FAs]/[RSA] = 15. The following concentrations were used: rat serum (as RSA), 525 μM; flurbiprofen, $10\,\mu g\,mL^{-1}$. Each column is the mean of three experiments ± s.d. $^{a}P < 0.001$ vs A; $^{b}P < 0.001$ vs B; $^{c}P < 0.001$ vs C; $^{d}P < 0.001$ vs D.

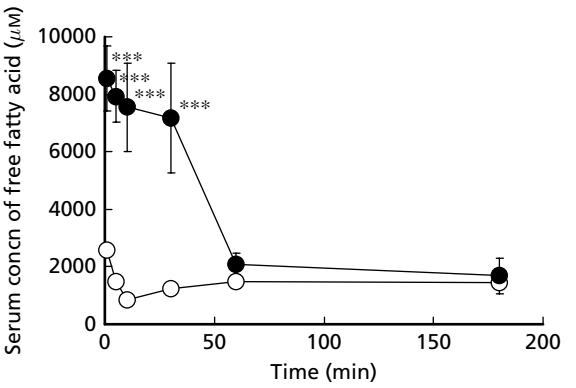

Figure 2 Serum concentration of free fatty acid–time profiles after FP-ax injection to the control group (○) and lipid emulsion group (●). Each point represents the mean ± s.d. (n = 6). ***$P < 0.001$ compared with the control group.

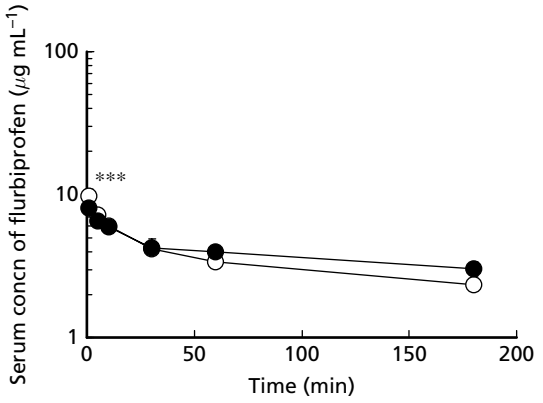

Figure 3 Serum concentration of flurbiprofen–time profiles after FP-ax injection to the control group (○) and lipid emulsion group (●). Each point represents the mean ± s.d. (n = 6). ***$P < 0.001$ compared with the control group.

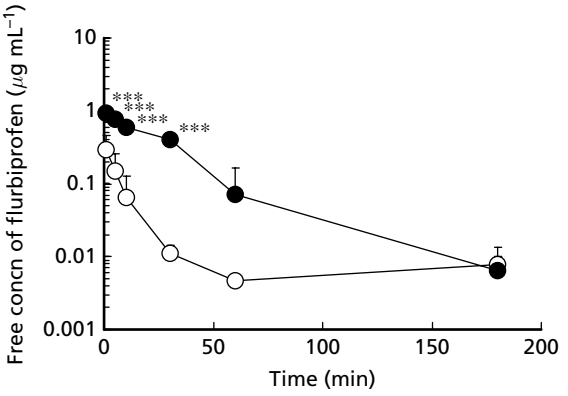

Figure 4 Free concentration of flurbiprofen–time profiles after FP-ax injection to the control group (○) and lipid emulsion group (●). Each point represents the mean ± s.d. (n = 6). ***$P < 0.001$ compared with the control group.

group than the control group until 30 min after the administration of FP-ax, and there were no significant differences in free flurbiprofen concentrations of the two groups at 60 min after the injection.

Table 1 shows the pharmacokinetic parameters of flurbiprofen obtained from the alpha phase. The AUC of serum concentrations of flurbiprofen during the alpha phase ($AUC_{1\text{-}10,total}$) in the lipid emulsion group was significantly lower than the value obtained from the control group. The AUC of free concentrations of flurbiprofen during the alpha phase ($AUC_{1\text{-}10,free}$) was remarkably higher in the lipid emulsion group than the control group. The ratio of $AUC_{1\text{-}10,free}$ in the lipid emulsion group to $AUC_{1\text{-}10,free}$ in the control group was approximately 5-times. The distribution volume of the central compartment (Vd_c) of flurbiprofen was significantly larger in the lipid emulsion group than the control group.

Table 1 Kinetic parameters obtained from flurbiprofen concentrations during the alpha phase after FP-ax injection in the control group and lipid emulsion group

Parameter	Control	Lipid emulsion
Vd_c (L kg^{-1})	0.077 ± 0.009	0.092 ± 0.007**
$AUC_{1-10,\ total}$ (μg min mL^{-1})	66.4 ± 5.34	60.0 ± 3.09*
$AUC_{1-10,\ free}$ (μg min mL^{-1})	1.35 ± 0.94	6.71± 1.00***

Each value represents the mean ± s.d. (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group.

Discussion

To administer FP-ax with an effective dosage plan, a way to increase the free concentration of flurbiprofen in serum is required. In this study, the inhibitory effect of FFA on the binding of flurbiprofen to RSA was investigated. When FFA inhibited flurbiprofen binding to RSA in-vivo, the serum concentrations of flurbiprofen during the alpha phase changed markedly; therefore, we mainly observed the pharmacokinetics of flurbiprofen during the alpha phase. The free concentrations and Vd_c values obtained from the pharmacokinetics of flurbiprofen during the alpha phase were higher in the lipid emulsion group than the control group (Figure 4 and Table 1). This indicated that the inhibition of flurbiprofen bound to RSA by FFA could allow flurbiprofen to distribute to the target tissues. It may be speculated that the concentration of FFA was increased by injecting a lipid emulsion in rats at the time when FP-ax was administered, FFA inhibited the binding of flurbiprofen to RSA, and as a result, flurbiprofen was distributed to the target tissues.

In general, if the free concentration of a drug is increased remarkably by inhibiting protein binding, the serum concentration is decreased (Arimori et al 1984). This study showed that serum concentrations of flurbiprofen during the alpha phase were significantly lower in the lipid emulsion group than the control group, whereas those during the beta phase were slightly higher without a significant difference in the lipid emulsion group (Figure 3). This result would be due to the higher concentration of RSA in the lipid emulsion group (lipid emulsion group, 445 ± 25 μM; control group, 371 ± 13 μM). It could be speculated that the retention of flurbiprofen in serum would be increased during the beta phase, in which the affinity of flurbiprofen was increased by reducing the concentration of FFA, a site II binding inhibitor; however, it is notable that the binding of flurbiprofen to RSA was strongly inhibited by FFA during the alpha phase, though the RSA concentration was higher in the lipid emulsion group than the control group.

We revealed that the free fraction of flurbiprofen increased significantly as the binding of flurbiprofen to site II of HSA was inhibited by oleic acid, which is abundant in human serum (control, free fraction = $4.9\times10^{-5}\pm0.1\times10^{-5}$; [oleic acid]/[HSA] = 2, free fraction = $6.6\times10^{-5}\pm0.2\times10^{-5}$, $P<0.05$ vs control; [oleic acid]/[HSA] = 3, free fraction = $13.1\times10^{-5}\pm0.1\times10^{-5}$, $P<0.01$ vs control; [oleic acid]/[HSA] = 4, free fraction = $23.9\times10^{-5}\pm0.7\times10^{-5}$, $P<0.01$ vs control, each value is the mean of three experiments±s.d.). It was reported that HSA has six to seven different binding sites of FFA and the second binding site of FFA corresponds to site II (Petitpas

et al 2001; Chuang & Otagiri 2002). Arg410 and Tyr411, which are amino acid residues in site II, interact electrostatically with the carboxy group of FFA (Bhattacharya et al 2000; Petitpas et al 2001). These amino acid residues play an important role in the binding of ketoprofen, which is an NSAID of the arylpropionic acid class involving flurbiprofen and has a carboxy group (Watanabe et al 2000). Here, the identity of the amino acid sequence, which is the primary structure, between HSA and RSA was high, and Arg410 and Tyr411 in site II of HSA exist in the RSA binding site (Carter & Ho 1994); therefore, the binding inhibitory mechanism of flurbiprofen by FFA was speculated to be similar in HSA and RSA.

When a lipid emulsion is administered to patients, the serum concentration of FFA can increase to approximately 3000 μM, comparable with 5-times the HSA concentration (Zimmerman et al 1981). The increment of FFA concentration can inhibit flurbiprofen binding to HSA. It was predicted that the injection of FP-ax would lead to an increase in the free concentration of flurbiprofen when FFA concentration was increasing by administering a lipid emulsion. Though a lipid emulsion was used in this study to increase the serum concentration of FFA, there are other ways to increase FFA concentrations. For example, the injection of heparin, an anticoagulant drug, to patients at risk for thrombus formation activates lipoprotein lipase, and can increase the FFA concentration transiently by approximately 3000 μM (Naranjo et al 1980; Brown et al 1981). Furthermore, the FFA concentration fluctuates in a normal physiological state and is generally high during fasting (Reaven et al 1988; Wolever et al 1995). FP-ax can be administered with the result of increasing FFA concentrations by heparin injection for 'anticoagulant' therapy and fasting. So far, we have elucidated that when a diclofenac suppository (NSAID: distribution volume is small; binding affinity of HSA is high) was administered to patients with rheumatoid arthritis at high FFA concentration during fasting, FFA inhibited the binding of diclofenac to HSA, and the analgesic effect of diclofenac increased (Takamura et al 2007).

The control of cancer pain has tended to involve opioids alone, and many patients are supported with high doses of opioids; however, administering a combination of NSAID and opioids has an opioid-sparing effect as well as better analgesia (Bjorkman et al 1993; Joishy & Walsh 1998; Zelcer et al 2005). Combination therapy prevents side effects such as severe constipation and somnolence which are induced by excess opioids; therefore, it is important to establish an effective dosage plan for FP-ax to enhance the analgesic effect of NSAIDs.

We elucidated that when FP-ax was administered at a high FFA concentration, the free concentration of flurbiprofen and distribution of flurbiprofen to tissues increased transiently. This administration method may give patients with cancer pain a potent analgesic effect.

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