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Serum protein binding displacement: theoretical analysis using a hypothetical radiopharmaceutical and experimental analysis with ^{123}I-*N*-isopropyl-*p*-iodoamphetamine

Keiichi Kawai[a,b], Ryuichi Nishii[c], Naoto Shikano[d,*,1], Nobuo Makino[e], Noriyuki Kuga[a,f], Mitsuyoshi Yoshimoto[a], Seishi Jinnouchi[g], Shigeki Nagamachi[g], Shozo Tamura[g], Norito Takamura[h]

[a]*Division of Health Science, Graduate School of Health Sciences, Kanazawa University, 5-11-80 Kodatsuno, Kanazawa, Ishikawa 9200-942, Japan*
[b]*Biomedical Imaging Research Center, University of Fukui, 23-3 Matsuokashimoaizuki, Eiheiji-cho, Yoshida-gun, Fukui 910-1193, Japan*
[c]*Research Institute, Shiga Medical Center, Moriyama City, Shiga 524-8524, Japan*
[d]*Department of Radiological Sciences, Ibaraki Prefectural University of Health Sciences, Inashiki-gun, Ibaraki 300-0394, Japan*
[e]*Center for Humanities and Sciences, Ibaraki Prefectural University of Health Sciences, Inashiki-gun, Ibaraki 300-0394, Japan*
[f]*Department of Radiology, University of Miyazaki Hospital, Kiyotake-cho, Miyazaki-gun, Miyazaki 889-1692, Japan*
[g]*Department of Radiology, Faculty of Medicine, University of Miyazaki, Miyazaki-gun, Miyazaki 889-1692, Japan*
[h]*School of Pharmaceutical Sciences, Kyusyu University of Health and Welfare, Nobeoka City, Miyazaki 882-8508, Japan*

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Abstract

Introduction: The binding of radiopharmaceutical to serum proteins is thought to be an important factor that restricts its excretion and accumulation in tissue. We calculated the effect of inhibitors of serum protein binding using a hypothetical radiopharmaceutical. In vitro experiments and protein binding inhibitor-loaded monkey scintigraphy were then conducted using ^{123}I-*N*-isopropyl-*p*-iodoamphetamine (IMP) as the radiopharmaceutical.

Methods: Free fraction ratios of radiopharmaceutical were calculated with one radiopharmaceutical, two serum proteins and two specific inhibitors in the steady state at various serum protein concentrations. In vitro protein binding inhibition studies using human, rat and monkey sera were performed with site-selective displacers of specific binding sites: 400 μM 6-methoxy-2-naphthylacetic acid (6MNA; a major nabumeton metabolite) as a serum albumin Site II inhibitor and 400 μM erythromycin (ETC) as an α_1-acid glycoprotein (AGP) site inhibitor. Scintigraphy with or without 6MNA loading of monkeys was performed.

Results: The theoretical findings roughly corresponded to the experimental results. Approximately 75% of IMP bound to serum albumin Site II and AGP in the species examined. The free fraction of IMP (25.0±0.6% for human, 22.8±0.4% for monkey, 23.7±0.3% for rat) increased with loading of specific protein binding inhibitors (6MNA: 28.0±0.3% for human, 24.5±0.7% for monkey, 24.3±0.2% for rat; ETC: 26.3±0.4% for human, 29.5±1.1% for monkey, 26.0±0.7% for rat) and was serum protein concentration dependant based on the results of calculations. Simultaneous administration of 6MNA and ETC produced a higher free fraction ratio of IMP (31.9±1.0% for human, 34.6±0.4% for monkey, 27.0±0.3% for rat) than summation of the single administrations of 6MNA and ETC (domino effect) in human, rat and monkey sera. Rapid cerebral accumulation was observed with 6MNA loading in monkey scintigraphy.

Conclusions: 6MNA appears to change the pharmacokinetics and brain accumulation of IMP in monkeys. Further studies in human are required.

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Keywords: *N*-Isopropyl-*p*-iodoamphetamine; Pharmacokinetics; Protein binding; Human serum albumin

* Corresponding author. Tel.: +81 298 40 2217; fax: +81 298 40 23171.
E-mail address: sikano@ipu.ac.jp (N. Shikano).
[1] Current address: Department of Radiological Sciences, Ibaraki Prefectural University of Health Sciences, 4669-2 Ami, Ami-machi, Inashiki-gun, Ibaraki 300-0394, Japan.

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1. Introduction

The binding of drugs to serum proteins is an important factor that restricts both drug excretion and accumulation in tissue [1]. The free drug hypothesis assumes that only the free fraction of the drug in plasma can penetrate the tissue and that drug transport in tissue is governed by the free drug concentration gradient between plasma and tissue, until equilibrium is reached [1,2].

Several drugs bind with high affinity to one of the serum albumin sites and to α_1-acid glycoprotein (AGP), and there are individual variations in free/bound drug ratios [3]. The specific binding sites in human serum albumin have been well characterized. In fact, three sites for drug binding have been reported: Site I (also known as the warfarin binding site), Site II (the benzodiazepine binding site) and Site III (the digitoxin binding site). Of these, Sites I and II are known to be the main binding sites of various therapeutic drugs [4]. However, few studies have examined competitive displacement by radiopharmaceuticals of specific serum protein binding sites.

In this study, free fraction ratios of hypothetical radiopharmaceutical or radiolabeled ligand (L) in model sera were calculated in an attempt to clarify the influence of the drug–radiopharmaceutical interaction with protein binding on molecular imaging. Free fraction ratios of radioactive ligand were calculated with one radiopharmaceutical (L), two serum proteins (P_1 and P_2) and two specific inhibitors (I_1 and I_2) (referred to as the LP_2I_2 model) at the steady state. Because the concentration of serum protein changes depending on the disease condition, free fraction ratios with various P_1 and P_2 concentrations were also calculated.

Although ^{123}I-*N*-isopropyl-*p*-iodoamphetamine (IMP) is widely used for cerebral blood flow assessment [5,6], the effects of serum protein binding displacement by coadministered drugs are unknown. It is noteworthy that the obtained values determined with SPECT may or may not be influenced by serum protein binding as well as by metabolism. Furthermore, it is important to establish the magnitude of the influence of plasma protein binding of radiopharmaceuticals. We compared the theoretical results with experimental results of serum protein binding displacement by IMP and the specific inhibitor for Site II of serum albumin, 6-methoxy-2-naphthylacetic acid (6MNA; a nabumeton metabolite), and the specific inhibitor for the AGP

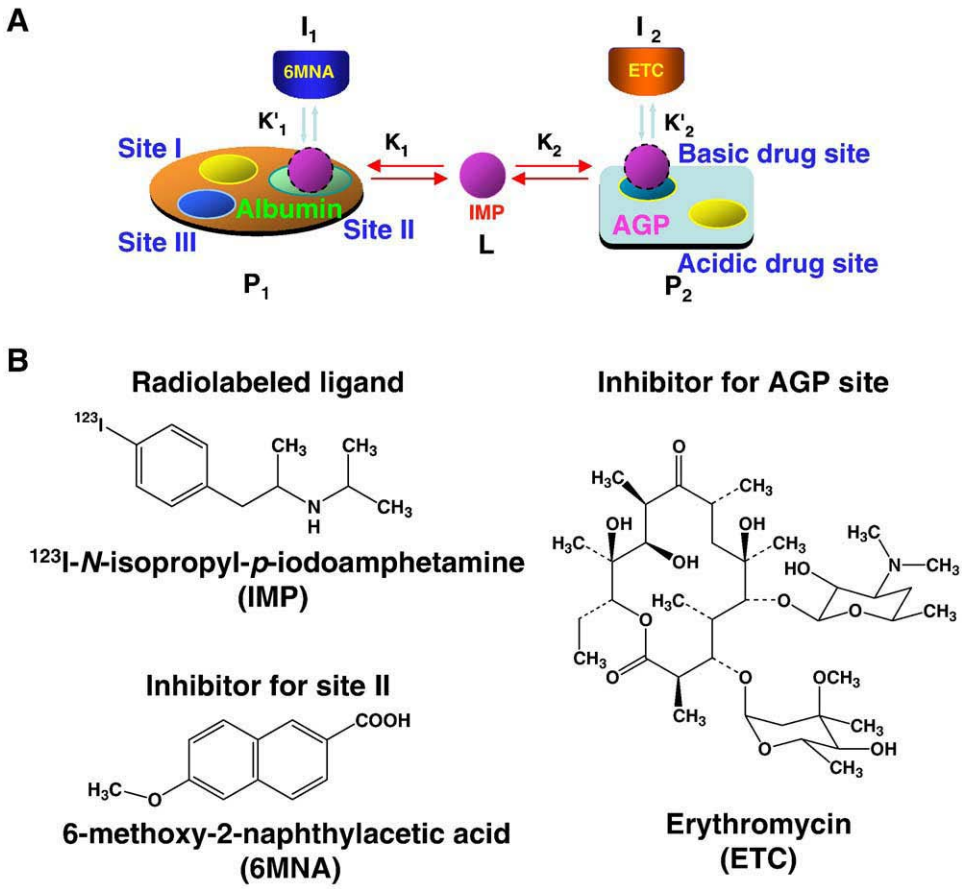

Fig. 1. LP_2I_2 model serum with one hypothetical radioactive ligand (L), two serum proteins (P_1 and P_2) and two specific inhibitors (I_1 and I_2) at steady state. L can bind to free P_1 and P_2. I_1 or I_2 competitively inhibits binding of L to P_1 or P_2, respectively. K_1, K_2, K'_1 and K'_2 are binding constants (A). Chemical structures of radioactive ligand and specific serum protein binding inhibitor for the in vitro study (B).

binding site, erythromycin (ETC), in human, monkey and rat sera (Fig. 1). As an in vivo study, we used scintigraphy to evaluate the effects of 6MNA on the biodistribution of IMP in monkeys intravenously injected with 6MNA.

2. Materials and methods

2.1. Materials

Reagent-grade chemicals were used in this experiment. ETC and 6MNA were purchased from Abbott Japan (Tokyo, Japan) and SmithKline Beecham Co., Ltd. (West Sussex, UK), respectively. IMP (Nihon Medi-Physics Co., Ltd., Tokyo, Japan) was used as the radiopharmaceutical. The radiochemical purity of the IMP was greater than 98.0%.

2.2. Model serum protein binding calculations

In this study, free fraction ratios of hypothetical radiopharmaceutical or radiolabeled ligand (L) that assume IMP in the model sera were calculated in an attempt to clarify the influence of drug–radiopharmaceutical interactions with protein binding on imaging. Radiolabeled ligand binds serum protein-1 (P_1), which assumes serum albumin, and also serum protein-2 (P_2), which assumes AGP (Fig. 1). I_1 and I_2, which assume 6MNA and ETC, respectively, were the specific inhibitors of P_1 and P_2, respectively. Therefore, this is a one-ligand, two-serum-protein and two-specific-inhibitor model (denoted the LP_2I_2 model). Free fraction ratios of L were calculated repeatedly using step-by-step approximation until values reached the steady state. The normal range of human serum albumin and AGP is 3.8–5.0 g/dl and 70.0–80.0 mg/dl, respectively [7]. Because the concentration of serum protein changes depending on the disease condition, free fraction ratios with various P_1 and P_2 concentrations were also calculated. The scheme of calculation of the concentration of L, [*L*], in the steady state is described in the Appendix A. Parameters determined from the normal range of clinical laboratory tests and therapeutic concentrations of inhibitors were used in this calculation.

2.3. Measurement of IMP free fraction ratios of protein binding

We determined the IMP free fraction ratios of protein binding using the method published by Nishio et al. [8]. The human and monkey blood samples were obtained from the cephalic vein, while the rat blood samples were obtained by heart puncture. In brief, the serum protein binding of IMP was evaluated by ultrafiltration (Ultracent-10; Tosoh, Tokyo,

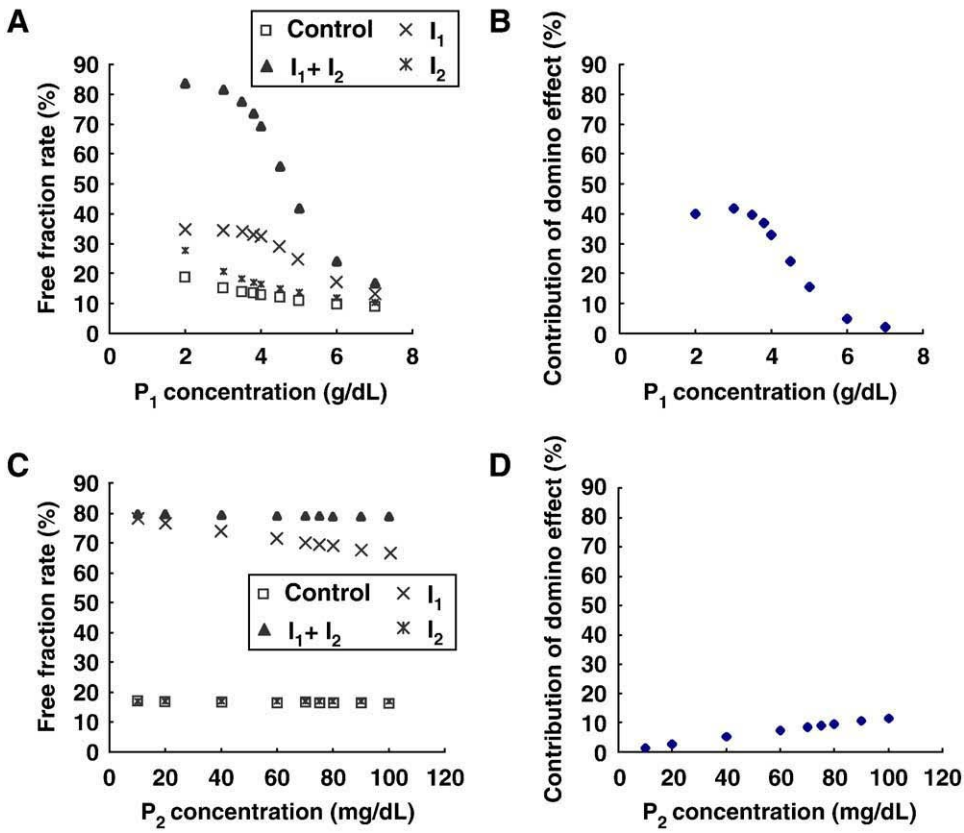

Fig. 2. Results of calculations. The following parameters were used: K_1=8000 M^{-1}, K_2=11,000 M^{-1}, K_1'=5.0×10^6 M^{-1}, K_2'=3.5×10^4 M^{-1}. For Panels A and B, P_1: 2–7 g/dl, P_2: 75 mg/dl, L: 1.25×10^{-9} M, I_1: 400 μM, I_2: 400 μM. For Panels C and D, P_1: 4 g/dl, P_2: 10–100 mg/dl, L: 1.25×10^{-9} M, I_1: 400 μM, I_2: 400 μM.

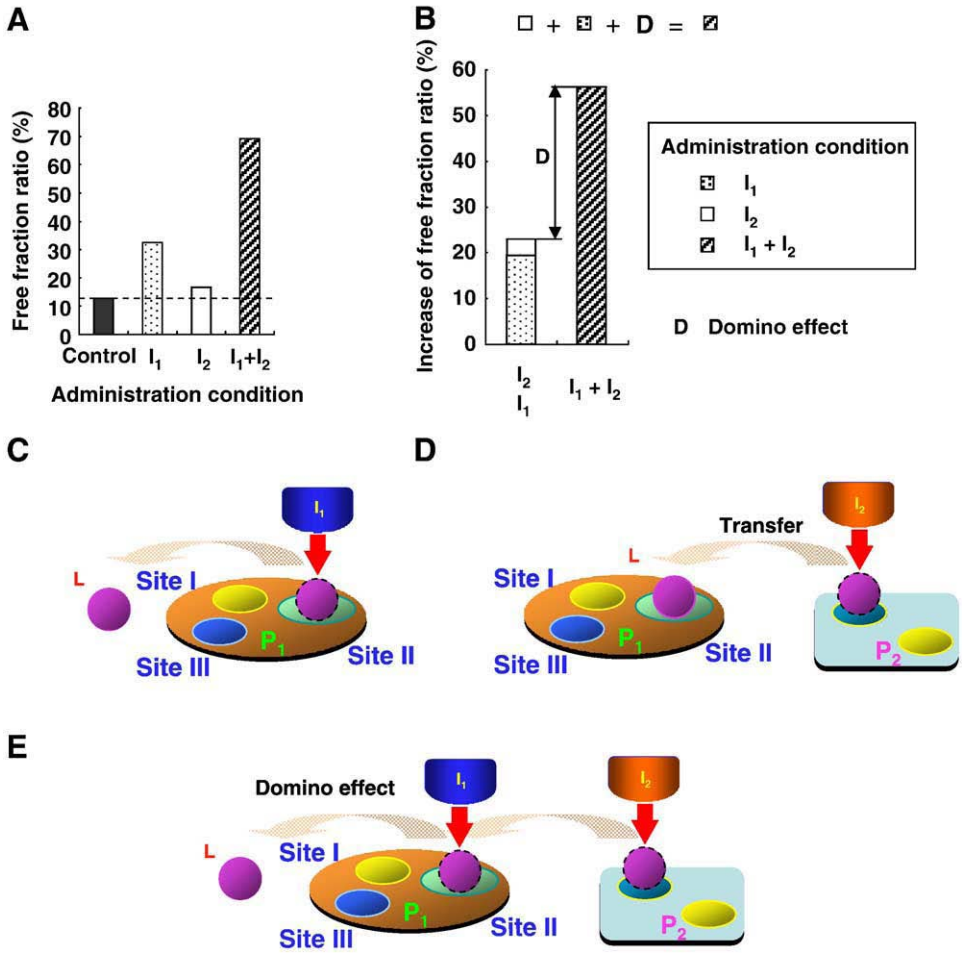

Fig. 3. Calculated in vitro displacement of IMP protein binding in rat serum (A and B). Proposed mechanisms by which the free fractions of L are determined with administration conditions I_1 (C), I_2 (D) and I_1+I_2 (E) are illustrated. The free fraction of L is increased by competitive inhibition with I_1 (C). The free fraction of L does not increase as much by competitive inhibition with I_2 because of P_2 to P_1 transfer of L (D). The combination of both binding inhibitors appears to exert a synergistic effect on the displacement of L serum binding, the domino effect (E).

Japan). Inhibitors of albumin binding were examined in human, Japanese monkey and rat sera. Mixtures (0.90 ml) of a radioactive tracer (IMP at 220 kBq in 20.0 μl saline; 1.25×10^{-9} M) and serum were centrifuged at 3000 rpm for 10 min at room temperature (Model 5900; Kubota, Tokyo, Japan). The ^{123}I free iodide in the radiotracer and "sticking" of radioactivity to the Ultracent-10 were not observed. The radioactivity (count/20.0 μl) of the initial sample [*I*] and filtrate [*F*] was measured using a well-type scintillation counter (ARC-360; Aloka, Japan), and the protein binding rate and free fraction rate were then determined using the following equations: Free fraction (%)=[*F*]/[*I*]×100, and Protein binding rate (%)=100−free fraction. The sera were adjusted to albumin concentrations of 375 μM with phosphate buffer (0.067 M, pH 7.4) as described elsewhere [9–11]. 6MNA was tested as a displacer of serum albumin binding site (Site II), while ETC was tested as a displacer of the AGP binding site. Test compounds were added at the therapeutic concentration of 400 μM before addition of the radioactive sample [7].

2.4. In vivo monkey study

Female Japanese monkeys (4 kg) were anesthetized with pentobarbital (50 mg/kg body weight, ip) after ketalar (5 mg/kg body weight, im) and were then administered 1.0 ml saline containing IMP (111 MBq, iv via the left cephalic vein, bolus). Saline was infused via the right cephalic vein to avoid a circulation failure. A warm patch was used to maintain body temperature. The depth of anesthesia was monitored based on the condition of the pupils and using pulse oximetry. Dynamic planar data for the whole body was collected (1 frame/min for 30 min) using a two-headed scintillation camera (Prism 2000; Picker International, Cleveland, OH, USA). In the displacement study, 6MNA (20 mg/kg body weight, iv) was administered to the same monkey immediately prior to IMP injection. Time–activity curves were obtained for the main tissue regions using a supercomputer (Odyssey; Picker International). The monkey studies were conducted three times in two different monkeys to confirm reproducibility. All

animal experiments were approved by the Ethics Committee of the University of Miyazaki.

2.5. Statistical analysis

The values obtained in each experiment are expressed as the mean±S.D. Statistical comparisons between groups were performed using Student's *t* test.

3. Results

3.1. Calculations

Fig. 2 shows the calculated free fraction ratio of hypothetical radiopharmaceutical L versus P_1 or P_2 concentration. As shown in Fig. 2A and C, the free fraction ratios of L increased upon addition of the inhibitors, compared to the control. The changes in the free fraction ratio of L depended on the P_1 and P_2 concentration.

Interestingly, as shown in Fig. 2B and D, simultaneous administration of I_1 and I_2 produced a higher free fraction ratio of L than the single administration of I_1 or I_2 (domino effect). This effect increased as the P_1 concentration decreased. In contrast, the effect increased as the P_2 concentration increased.

Fig. 3A shows the free fraction ratio of L when the P_1 concentration was 4 g/dl under various I_1 and I_2 administration conditions. Coadministration of I_1 and I_2 produced a higher free fraction ratio of L than single administration of I_1 or I_2 (Fig. 3B).

3.2. Measurement of IMP free fraction ratios in serum

The percentage of IMP bound to human serum protein was found to be 69.1±1.9%, and thus, we next evaluated the effects of the site-specific inhibitors (Fig. 4) on serum protein binding of IMP. IMP bound to human serum albumin and AGP almost equally. In human serum, the free fraction of IMP with 6MNA loading increased significantly, by 1.1-fold, when compared to the controls (free fraction of IMP without loading) in the presence of the human serum albumin Site II inhibitor, 6MNA. The free fraction of IMP increased significantly, by 1.1-fold, when compared to the free fraction of IMP without loading (controls), in the presence of the AGP inhibitor, ETC. Simultaneous administration of 6MNA and ETC produced a 1.3-fold-higher free fraction ratio of IMP than summation of the data from the single administrations of 6MNA and ETC. Similar effects were observed in the Japanese monkey and rat sera studies (Fig. 4A and B).

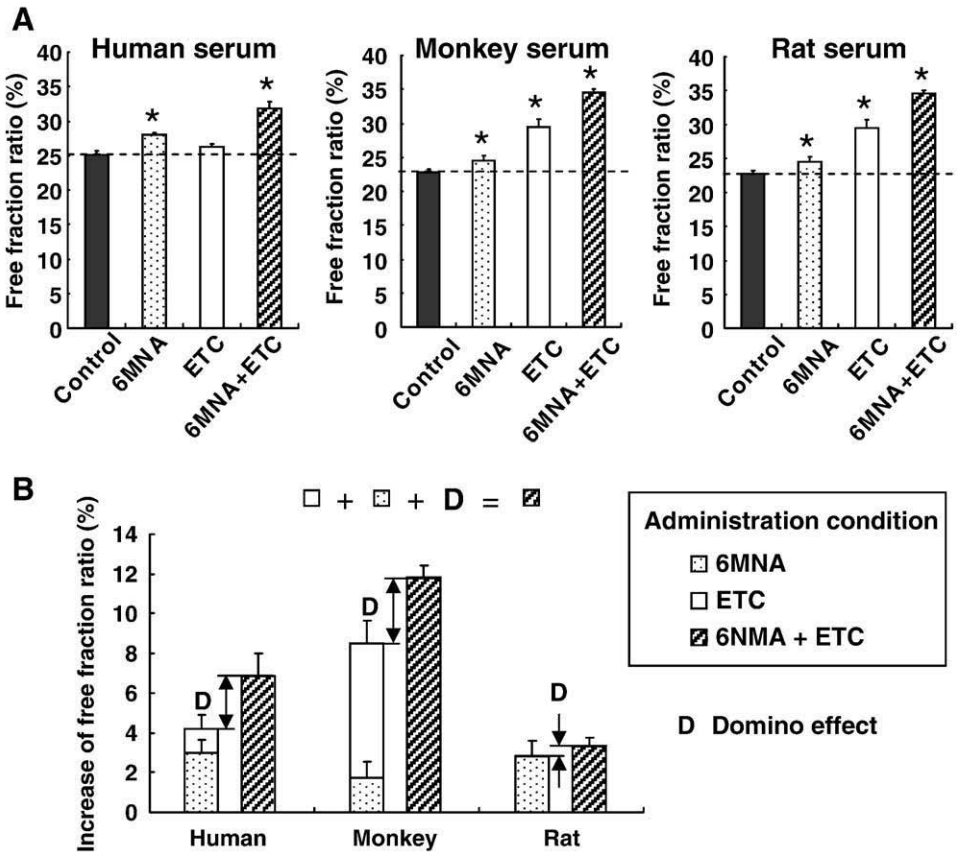

Fig. 4. In vitro displacement of IMP protein binding in rat serum. Inhibitor was loaded at a final concentration of 400 μM. *P<01 versus control, n=6 (A). Observed domino effect in human, monkey and rat sera (B).

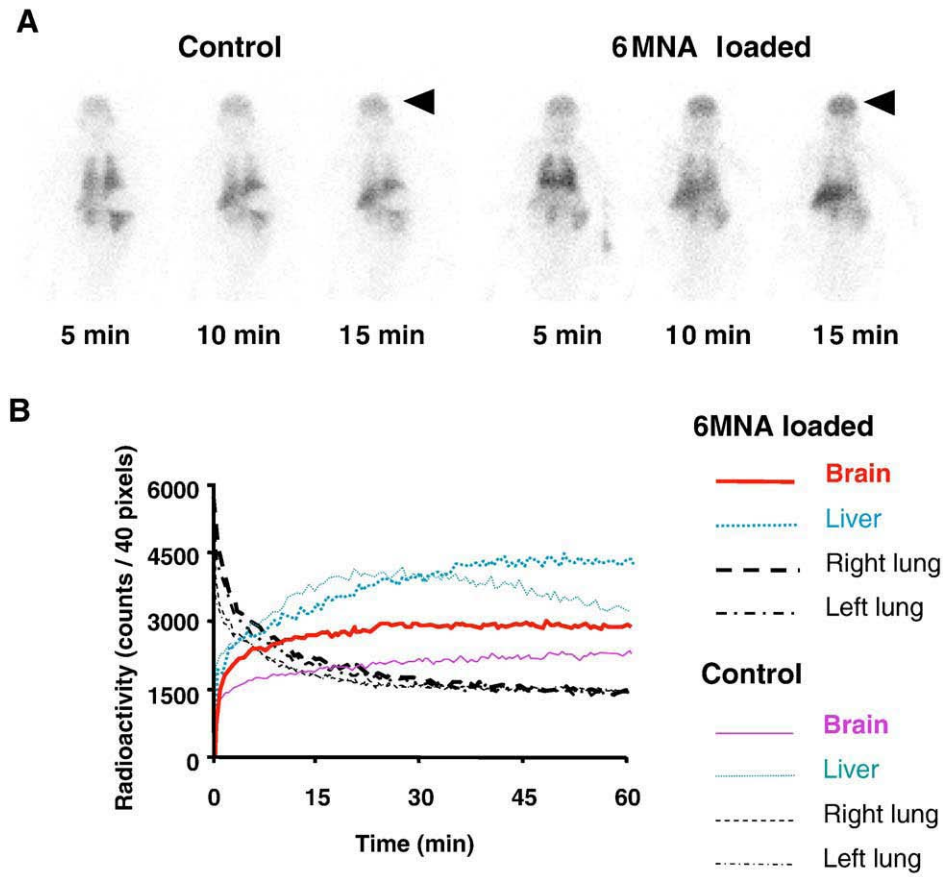

Fig. 5. Representative IMP whole-body scintigraphy of monkey. The arrowheads indicate the brain. 6MNA (20 mg/kg body weight) was simultaneously injected with IMP. Dynamic planar data for the whole body were collected (1 frame/min for 60 min) by scintillation camera (A). The time–activity curve of Panel A (B).

3.3. In vivo monkey study

Representative IMP whole-body planar scintigraphy of a Japanese monkey and the resultant time–activity curves are shown in Fig. 5A and B, respectively. Rapid cerebral accumulation was observed with 6MNA loading. The plateau level of brain radioactivity was 1.3-fold higher than in the controls. In vitro monkey serum analysis revealed that the free fraction of IMP with 6MNA loading increased significantly (1.3-fold), from 27.9% without loading to 36.9% with 6MNA loading.

4. Discussion

As predicted from the calculation using the LP_2I_2 model in Fig. 1, the free fraction of IMP was affected by the change in human serum albumin and AGP. The change in serum albumin was more effective than that in AGP, as shown in Fig. 2. The free fraction of IMP would increase in a patient with low serum protein concentration and decrease in a patient with high serum protein concentration. Possible causes of a decrease in human serum albumin include renal damage, hepatic damage, diabetes, inflammatory disorders (rheumatism, cancer and infection), nephrosis and hemorrhage (due to surgery, blood in urine and hematochezia). Human serum albumin increases in hemodialysis due to the concentrated blood. On the other hand, AGP increases in renal damage, hemodialysis and inflammatory disorders (rheumatism, cancer and infection), while AGP decreases in hepatic damage, nephrosis and hemorrhage (due to surgery, blood in urine and hematochezia) [12].

Figs. 3 and 4 show that the theoretical and experimental results roughly corresponded, although species differences were observed in the experimental data. Furthermore, the free fraction data differed between the theoretical and experimental data, possibly caused by naturally occurring compounds in the sera. Although the principle was provisionally proven by the calculation, the appropriate binding constants K_1, K_2, K_1' and K_2' should be determined experimentally in future research.

We predicted the domino effect using the LP_2I_2 model. Significant inhibition of 6MNA and ECT showed that IMP was bound to Site II on human serum albumin and to the basic binding site on AGP (Fig. 4A). The combination of both binding inhibitors appears to exert a synergistic effect

on the displacement of IMP serum binding (domino effect, Fig. 3E). The domino effect was observed in all sera studied in this experiment (Fig. 4B).

We used 6MNA for loading in the monkey study based on the results of Fig. 2. As shown in Fig. 5, 6MNA loading resulted in high cerebral accumulation (1.30 times control level) and rapid clearance of IMP. The free fraction of IMP in in vitro monkey serum increased with 6MNA loading (1.32 times control level). Acceleration of cerebral accumulation with 6MNA loading was considered the result of IMP protein binding displacement on only the serum albumin binding site. This condition corresponds to Fig. 3C. The use of binding site inhibitors may result in more rapid production of better diagnostic images using lower radiation doses.

There are several clinical implications of these findings, and the following points should be noted when a protein binding inhibitor is used. The distribution of drugs depends not only on their interaction with proteins but also on a number of other factors, such as metabolism and excretion [1]. For example, an increase in IMP brain uptake by loading imipramine has been demonstrated in an animal study, and the imipramine bound plasma proteins and erythrocytes [2]. Moretti et al. [13] found that post-loading of imipramine resulted in an increase in the brain activity of IMP in rabbits, which was thought to occur because IMP competed with imipramine in a metabolic reaction related to the mixed function oxidase system in the lung. On the other hand, Riant et al. [2] reported that in vivo studies demonstrated that the protein-bound drug as well as imipramine or hormone, as measured in vitro, is partly available for transport into tissue, including brain, depending on the nature of the drug and of the binding protein.

The safety aspects of administering this kind of protein binding inhibitor to patients require analysis and discussion. Because the protein displacer affects not only the binding of IMP but also the binding of other drugs or small molecules, the use of a protein displacer needs to be investigated carefully not only in patients taking other medications but also in patients with low plasma proteins, dialysis patients, and so forth. Nabumeton, with a major metabolite of 6MNA, is easily administered perorally [14], and its pharmacokinetics with continuous and single oral administration have been well studied in humans [15]. Although the risks of NSAIDs including nabumeton are generally low, clinically safe inhibitors without pharmacological activity need to be identified.

In conclusion, we studied serum protein binding displacement by theoretically using a hypothetical radiopharmaceutical and experimentally with IMP. The theoretical results with the LP_2I_2 model roughly corresponded to the experimental findings in human, rat and monkey sera with administration of IMP, 6MNA and ECT. In the in vivo monkey scintigraphy study, rapid cerebral accumulation was observed with 6MNA loading. The search for strategies to improve imaging may facilitate further research into the combined use of radiopharmaceuticals with drugs.

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Appendix A. *Scheme*

P_1: serum protein 1
P_2: serum protein 2
L: radioactive ligand
I_1: specific inhibitor of P_1
I_2: specific inhibitor of P_2

$$P_1 + L \Leftrightarrow P_1 - L, \quad K_1 = [P_1 - L]/[P_1][L] \tag{1}$$

$$P_2 + L \Leftrightarrow P_2 - L, \quad K_2 = [P_2 - L]/[P_2][L] \tag{2}$$

$$P_1 + I_1 \Leftrightarrow P_1 - I_1, \quad K'_1 = [P_1 - I_1]/[P_1][I_1] \tag{3}$$

$$P_2 + I_2 \Leftrightarrow P_2 - I_2, \quad K'_2 = [P_2 - I_2]/[P_2][I_2] \tag{4}$$

L can bind to free P_1 and P_2. I_1 or I_2 competitively inhibits binding L with P_1 or P_2, respectively.

K_1, K_2, K'_1 and K'_2 are binding constants. The unit of these binding constants is M^{-1}.

From Eqs. (1–4),

$$[P_1 - L] = K_1[P_1][L] \tag{5}$$

$$[P_2 - L] = K_2[P_2][L] \tag{6}$$

$$[P_1 - I_1] = K'_1[P_1][I_1] \tag{7}$$

$$[P_2 - I_2] = K'_2[P_2][I_2] \tag{8}$$

Initial concentration of P_1, $[P_1]_0$, can be described as follows:

$$[P_1]_0 = [P_1] + [P_1 - L] + [P_1 - I_1] \tag{9}$$

From Eqs. (5), (7) and (9),

$$[P_1]_0 = [P_1](1 + K_1[L] + K'_1[I_1])$$

Concentration of free P_1, $[P_1]$, can be described as follows:

$$[P_1] = [P_1]_0/(1 + K_1[L] + K'_1[I_1]) \tag{10}$$

In a similar way, the next expression consists of:

$$[P_2] = [P_2]_0/(1 + K_2[L] + K'_2[I_2]) \tag{11}$$

Initial concentration of L, $[L]_0$, can be described as follows:

$$[L]_0 = [L] + [P_1 - L] + [P_2 - L] \tag{12}$$

From Eqs. (5), (6) and (12),

$$[L]_0 = [L](1 + K_1[P_1] + K_2[P_2])$$

The above equation is rewritten as follows:

$$[L] = [L]_0/(1 + K_1[P_1] + K_2[P_2]) \quad (13)$$

$[L]/[L]_0$ is experimentally measured in this study.
In a similar way, the next expression consists of:

$$[I_1] = [I_1]_0/(1 + K'_1[P_1]) \quad (14)$$

$$[I_2] = [I_2]_0/(1 + K'_2[P_2]) \quad (15)$$

Calculation of [L] in the steady state

Step 1. At first, free P_1 concentrations $[P_1]_1$ and $[P_2]_1$ are calculated from the initial concentrations of $[P_1]_0$, $[P_2]_0$, $[P_2]_0$, $[L]_0$, $[I_1]_0$ and $[I_2]_0$ using Eqs. (10) and (11). At Step 1, $[P_1]_1$ and $[P_2]_1$ are not true values but approximate values, because instead of initial concentrations, the free concentration should be used with this calculation.

Step 2. Then, from the free plasma protein concentrations $[P_1]_1$ and $[P_2]_1$ obtained above, the concentrations of free radioactive ligand and specific inhibitors $[L]_1$, $[I_1]_1$ and $[I_2]_1$ are calculated using Eqs. (13–15)). In addition, we tried to calculate $[P_1]_2$ and $[P_2]_2$ using the values of $[L]_1$, $[I_1]_1$ and $[I_2]_1$.

Step 3 and further steps. After Step 2, the accuracy of the approximation is improved by repeating Step 2.

The values are obtained with the accuracy of six digits within Step 10 or less.

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