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Determination of Protein Binding of a Highly Lipophilic Drug, Isocarbacyclin Methyl Ester (TEI-9090), Using a Polydimethylsiloxane-coated Glass Beads Assay

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Abstract—An adsorption technique with polydimethylsiloxane-coated glass beads (PDMS-GB) was developed to determine the protein binding of a highly lipophilic and hydrophobic drug. The present assay method is based on the quantitative adsorption of unbound drug to the PDMS-GB. This method of batch separation in a glass assay tube has an advantage of simplicity and rapidity. To evaluate the reliability of PDMS-GB assay, we compared the protein binding of diazepam in serum in-vitro measured by ultrafiltration and PDMS-GB assay. There was no significant difference between the extent of binding measured by each method. Using PDMS-GB assay, we determined the protein binding of the prostaglandin I_2 (PGI_2) analogue isocarbacyclin methyl ester (TEI-9090), whose binding cannot be measured by commonly employed techniques (equilibrium dialysis, ultrafiltration, gel filtration or ultracentrifugation) because of a high degree of adsorption to membranes, resins or tubes. The percentage of TEI-9090 bound in human serum, 4% human serum albumin (HSA, fatty acid-free) and dog serum were ~98, ~87 and ~95%, respectively, and these values were independent of TEI-9090 concentration up to 10 ng mL^{-1}. The binding of isocarbacyclin (TEI-7165) to serum protein in man, dogs, rabbits and rats, determined by ultrafiltration, was also high (>90%). While the displacement of TEI-9090 and TEI-7165 binding to HSA by aspirin, salicylic acid and indomethacin was not observed, clofibric acid and free fatty acids significantly inhibited the protein binding of both compounds. These results indicate that the binding site of TEI-9090 and TEI-7165 on HSA could be identical with the possible binding site of PGI_2.

The protein binding of drugs is closely related to the pharmacokinetics, pharmacological effects and adverse effects of drugs (Jusko & Gretch 1976; Levy & Moreland 1984). To determine the protein binding of drugs, equilibrium dialysis, ultrafiltration, ultracentrifugation and gel filtration are employed (Kurz et al 1977). These methods are designed to determine the concentration of the drug in a protein-free solution separated from a protein-drug solution. Accordingly, adsorption of the drug to the system materials may cause errors. For example, diazepam adsorbs to containers (Cloyd et al 1980), and also to the ultrafiltration apparatus MPS-3. To calculate the tme concentration of free diazepam, ultrafiltration of the protein-free diazepam solution must be performed and a recovery factor of free diazepam applied (Graben & Parsons 1988). In the case of some highly lipophilic and hydrophobic drugs, which have been thought to bind extensively to serum protein (Carillet et al 1990), it is difficult to apply these techniques because of the extensive adsorption of the drugs to membranes, tubes or resins. A technique to determine accurately percent binding of these drugs is required (Maulard et al 1990; Urien et al 1992).

Previously, we demonstrated a polydimethylsiloxane-coated glass beads (PDMS-GB) assay (Minagawa et al 1994) to evaluate the entrapping efficiency of an oil-in-water (o/w) emulsion containing a prostaglandin I_2 (PGI_2) analogue, isocarbacyclin methyl ester (TEI-9090, Fig. 1, Shibasaki et al (1983)). This newly developed method has the advantage of simple and rapid measurement of the free drug released from the oil droplets in the o/w emulsion. In the present study, we have established a new application of the PDMS-GB assay to determine the protein binding of a highly lipophilic and hydrophobic drug. To evaluate the reliability of the PDMS-GB assay the binding of diazepam was measured and compared with measurements made by ultrafiltration. The PDMS-GB assay was used to determine the protein binding of TEI-9090 in human and animal serum samples in-vitro. In addition, by displacement experiments with several ligands, the possible binding site of TEI-9090 and its metabolite isocarbacyclin (TEI-7165, Fig. 1) on human serum albumin (HSA) was also characterized.

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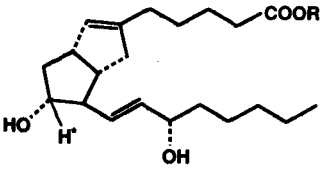
Materials and Methods

Materials

[11β-^{3}H]TEI-9090 (sp. act. 0·67 TBq mmol^{-1}) and unlabelled compound were kindly donated by Teijin Co. Ltd



R = CH_3 : TEI-9090
R = H : TEI-7165

FIG. 1. Chemical structures of [^{3}H]TEI-9090 and [^{3}H]TEI-7165. *Labelled position of ^{3}H.

(Tokyo, Japan). [11β-^{3}H]TEI-7165 was obtained by hydrolysis of [^{3}H]TEI-9090 in 1 mL of a 1 : 1 mixture of methanol and 0·05 M NaOH at 100°C for 1 h. The solution was diluted with distilled water to 10 mL, acidified to pH 3 with 1 M HCl and passed through a C18 Bond-Elut column (500 mg, Analytichem Int., Harbor City, CA, USA) activated by elution with 5 mL methanol and 10 mL 0·01 M HCl. After the column was washed with 10 mL 0·01 M HCl, the labelled compounds were eluted with 20 mL diethyl ether. The extract was evaporated to dryness, reconstituted in 1 mL of 1% EtOH in *n*-hexane, and added to an Si Bond-Elut column. The labelled compounds were extracted from the column by stepwise elution with 3, 5 and 10% ethanol in *n*-hexane and 10 mL 99% ethanol. This eluate, containing [^{3}H]TEI-7165, was used in the tests. [^{3}H]Diazepam (3·09 TBq mmol^{-1}) was purchased from Amersham Int. (Buckinghamshire, UK). The radiochemical purities of [^{3}H]TEI-9090, [^{3}H]TEI-7165 (Minagawa et al 1994) and [^{3}H]diazepam (Okiyama et al 1987) were more than 98·7% as determined by high-performance liquid chromatography (HPLC). Fraction V (A 1653) and essentially fatty acid-free human serum albumin (A 1887) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aspirin, salicylic acid and indomethacin were obtained from Wako Pure Chemicals Co. Ltd, Osaka, Japan, and clofibric acid was purchased from Tokyo Kasei Kogyo Co. Ltd, Tokyo, Japan. All other chemicals were of analytical grade.

Serum samples and HSA solutions

Human serum was obtained from three healthy volunteers, aged 25–32 years, who had taken no medication for a week before blood sampling. Fraction V and essentially fatty acid-free HSA solutions were prepared in a 0·1 M isotonic sodium phosphate buffer (PBS, pH 7·4). Animal serum samples were obtained from male beagle dogs (Laboratory Research Enterprises, Inc., Tokyo, Japan; 12·0–14·5 kg), male Japanese albino rabbits (Shin-Nihon Animals Ltd, Saitama, Japan; 3–4 kg) and male Wistar rats (SLC Co., Shizuoka, Japan; 170–180 g). Human and animal serum samples were used immediately after separation from red blood cells. The protein concentration was measured using Biuret reagent (Wako Pure Chemicals Co. Ltd, Osaka, Japan).

Measurement of serum protein binding by ultrafiltration

Ultrafiltration was performed using a commercially available apparatus MPS-3 (Amicon Corporation, Danvers, MA, USA). One millilitre of serum and 1 mL HSA were mixed with 10 μL of ethanol stock solutions of [^{3}H]diazepam or [^{3}H]TEI-7165, and incubated for 10 min at 37°C. The MPS-3 devices loaded with the samples were centrifuged at 1500 *g* for 10 min at 37°C. Filtrate (0·1 mL) and the initial solutions were placed in counting vials containing 10 mL Instagel (Packard Instruments Co., IL, USA), and the radioactivity was counted in a liquid scintillation counter (LS 6000TA, Beckman Instruments, Inc., CA, USA). A non-specific adsorption of unbound [^{3}H]diazepam to the ultrafiltration membrane was corrected by the method of Graben & Parsons (1988), after confirming that no adsorption of the drug to the reservoir of the MPS-3 apparatus, which disturbed the equilibrium between the protein-bound and unbound drugs, was observed in the following simple recovery test. The recovery tests for [^{3}H]diazepam in PBS, HSA solutions and the serum samples were performed by measuring radioactivities in the reservoirs.

Measurement of serum protein binding by PDMS-GB assay

Preparation of PDMS-GB and the PDMS-GB assay procedure has been described previously (Minagawa et al 1994). One millilitre of sample was mixed with 10 μL ethanol [^{3}H]diazepam or [^{3}H]TEI-9090 in a 3-mL glass assay tube with a polypropylene cup, and was incubated at 37°C. Assay was initiated by adding 3 g PDMS-GB, which can adsorb a proportion of the unbound drug. After shaking the assay tube vigorously for 15 s, the assay was terminated by withdrawing an 0·1-mL aliquot to determine the radioactivity. While the serum samples containing [^{3}H]diazepam were incubated for 10 min, the incubation interval for [^{3}H]TEI-9090 was 1 min to avoid a high degree of hydrolysis in serum.

Hydrolysis of [^{3}H]TEI-9090 in serum

The hydrolytic rate of [^{3}H]TEI-9090 to [^{3}H]TEI-7165 in the serum samples was measured to determine the concentration of unchanged drug at the protein binding determination. One millilitre of human serum, HSA solutions and animal serum containing [^{3}H]TEI-9090 were incubated at 37°C. At different time intervals, 4 mL ice-cold ethanol was added to the samples to precipitate the serum protein, and the mixtures were centrifuged at 1500 *g* for 10 min. After the supernatant was evaporated to dryness, the residue was dissolved in 200 μL ethanol, and a 50-μL aliquot was assayed by HPLC, as described previously (Minagawa et al 1994). The HPLC system consisted of an Auto-Analytical HPLC system (Gilson Medical Electronics Inc., France S.A.) and a Ramona LS-90 radiodetector (Raytest Isotopenemessgeräte GmbH, Germany) operated at 2 mL min^{-1} of the liquid scintillator, Instagel. A Lichrosorb RP-18 column (4 × 250 mm, 7 μm, E. Merck, Darmstadt, Germany) was used, and gradient elutions of acetonitrile/water/acetic acid from 50 : 50 : 0·1 to 100 : 0 : 0·1 in 20 min at a flow rate of 1 mL min^{-1} were performed.

Displacement studies on [^{3}H]TEI-9090 and [^{3}H]TEI-7165 binding to HSA

To study displacement effects of some ligands on [^{3}H]TEI-9090 and [^{3}H]TEI-7165 binding to HSA, PDMS-GB assay or ultrafiltration was performed on 1 mL fatty acid-free HSA solution mixed with each drug (1 ng mL^{-1}) and 10 μL ethanol solution of aspirin, salicylic acid, indomethacin or clofibric acid.

Results

Evaluation of PDMS-GB assay in comparison with ultrafiltration

The protein binding of [^{3}H]diazepam in-vitro in human serum, HSA solution and dog serum was measured by ultrafiltration and PDMS-GB assay. To determine the non-specific adsorption of [^{3}H]diazepam to the ultrafiltration device, [^{3}H]diazepam dissolved in PBS was filtrated at

Table 1. Comparison of the protein binding of [^{3}H]diazepam in serum in-vitro determined by ultrafiltration and PDMS-GB assay.

Method	[^{3}H]Diazepam (μg mL^{-1})	Binding (%)		
		Human[a]	HSA[b]	Dog[c]
Ultrafiltration	0·5	98·7 ± 0·07	98·2 ± 0·03	94·0 ± 0·09
	1·0	98·7 ± 0·12	98·4 ± 0·07	94·0 ± 0·06
	5·0	98·6 ± 0·07	98·3 ± 0·12	93·8 ± 0·09
PDMS-GB assay	0·5	98·5 ± 1·0	97·9 ± 1·1	95·7 ± 0·8
	1·0	98·6 ± 0·5	98·4 ± 1·2	95·7 ± 0·5
	5·0	97·7 ± 0·9	98·4 ± 1·2	95·8 ± 0·7

[a] Total protein in serum, 87·6 ± 3·6 mg mL^{-1}. [b] Essentially fatty acid-free human serum albumin, 39·2 ± 0·4 mg mL^{-1}. [c] Total protein in serum, 65·1 ± 1·9 mg mL^{-1}. Each value represents the mean ± s.e. of three samples.

37°C. Ultrafiltration with various concentrations of [^{3}H]diazepam up to 5 μg mL^{-1} indicated that free drug recovery was independent of the drug concentration. The mean recovery for all solutions was 83·7%. The extent of protein binding (R) was calculated from the following equation (Graben & Parsons 1988):

$$R = \frac{C_{tot} - C_{fapp}/0{\cdot}837}{C_{tot}} \times 100\ (\%) \qquad (1)$$

where C_{tot} and C_{fapp} were total and observed unbound [^{3}H]diazepam concentrations, respectively. The binding of [^{3}H]diazepam (0·5–5·0 μg mL^{-1}) in human serum, HSA solution and dog serum were 98·6–98·7, 98·2–98·4 and 93·8–94·0%, respectively (Table 1).

For PDMS-GB assay to determine the protein binding of a drug, a standard curve for the free drug is necessary. As shown in Fig. 2, the standard curve for [^{3}H]diazepam dissolved in PBS was linear up to 500 ng mL^{-1}. The linear regression equation was:

$$Y = 0{\cdot}236X + 0{\cdot}554\ (ng) \qquad (2)$$

with a correlation coefficient of 0·997.

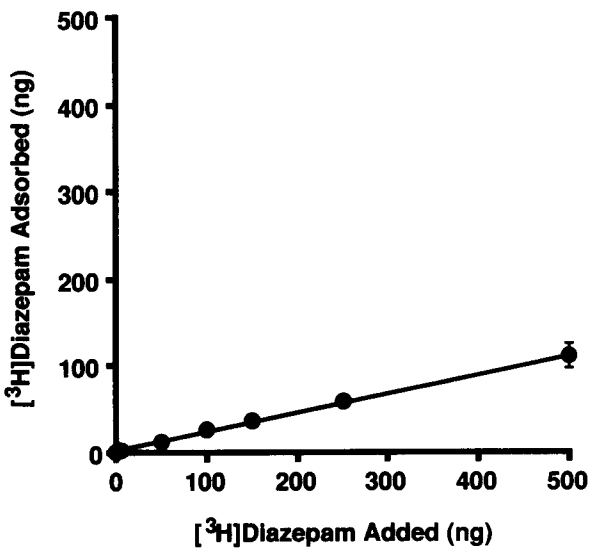


FIG. 2. Standard curve for [^{3}H]diazepam. PDMS-GB assay was performed under standard conditions as described in the text. Each value represents the mean ± s.e. of three samples.

Therefore, up to 500 ng mL^{-1} of [^{3}H]diazepam assayed, about 24% of the free drug was constantly adsorbed to PDMS-GB. The following equation to calculate the unbound drug concentration in serum or the protein solution (C_f) was obtained:

$$C_f = \frac{(C_{tot} - C_{sup}) - a}{b}\ (ng\,mL^{-1}) \qquad (3)$$

where C_{sup} is the drug concentration of the assayed solution, and a and b are the intercept (0·554) and slope (0·236) of the standard curve, respectively. Accordingly, the protein binding is determined from the difference between C_{tot} and C_f (eqn 4):

$$R = \frac{C_{tot} - C_f}{C_{tot}} \times 100\ (\%) \qquad (4)$$

The protein binding of [^{3}H]diazepam in human serum, HSA solution and dog serum, determined by PDMS-GB assay, was not significantly different from the value measured by ultrafiltration (Table 1).

Protein binding of [^{3}H]TEI-9090 and [^{3}H]TEI-7165

Before measurement of the binding of [^{3}H]TEI-9090, the stability of the drug in serum and HSA solution was examined. Hydrolysis of [^{3}H]TEI-9090 (10 ng mL^{-1}) in human serum, HSA solution and dog serum at 37°C is shown in Fig. 3. Although remaining [^{3}H]TEI-9090 in human serum samples was already 88·9 ± 1·7% at 1 min and 17·7 ± 4·5% at 60 min, no hydrolysis was observed in HSA solution. In dog serum, hydrolytic activity was lower than that in human serum. While remaining [^{3}H]TEI-9090 was 64·4 ± 0·58% at 60 min, hydrolysis of the drug was not observed within 1 min.

The standard curve for free [^{3}H]TEI-9090 in PBS, determined by PDMS-GB assay, was linear up to 10 ng mL^{-1}, and linear regression was $Y = 0{\cdot}819X - 0{\cdot}024$ (ng) ($r = 0{\cdot}999$). The protein binding of [^{3}H]TEI-9090 was calculated from equations 3 and 4, as in the case of diazepam. In the course of the incubation for the protein binding measurement, 0·5–10 ng mL^{-1} [^{3}H]TEI-9090 in human serum decreased to 0·45–8·9 ng mL^{-1} in 1 min at 37°C. The protein binding of [^{3}H]TEI-9090, determined by PDMS-GB assay, were as high as 97·0–98·1% up to 8·9 ng mL^{-1} (Table 2). Adsorption of [^{3}H]TEI-7165, which was generated from [^{3}H]TEI-9090 in the samples, to PDMS-GB was far less than [^{3}H]TEI-9090 (<10%). Accordingly, the contamination of [^{3}H]TEI-7165 must be negligible to

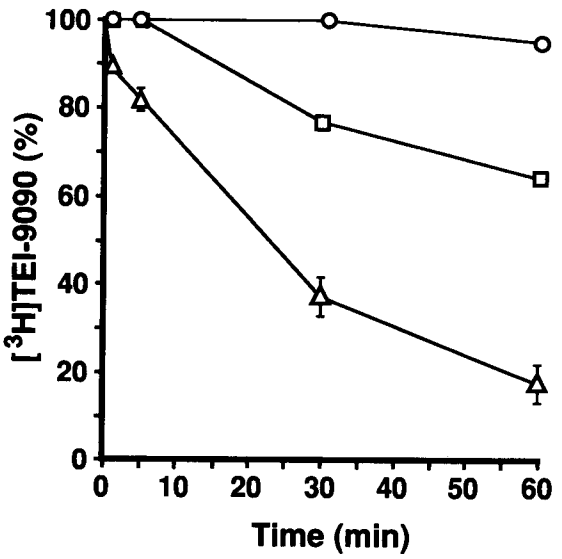


FIG. 3. Hydrolysis of [^{3}H]TEI-9090 in human serum (△), 4% HSA solution (○) and dog serum (□). Each point represents the mean ± s.e. of three samples.

calculate the binding of [^{3}H]TEI-9090. In HSA solution, the protein binding of [^{3}H]TEI-9090 was 84·3–86·3%, which was less than that in human serum. The binding of [^{3}H]TEI-9090 in dog serum were 93·9–95·1%. The protein binding of [^{3}H]TEI-9090 in rabbit and rat serum could not be determined, because the drug was completely hydrolysed in these serum samples within 1 min at 37°C.

The protein binding of [^{3}H]TEI-7165 in human, dog, rabbit, rat sera and HSA solution was measured by ultrafiltration. In all species, the binding was greater than 90% up to 10 ng mL^{-1} (Table 2).

Effects of the ligands on binding of [^{3}H]TEI-9090 and [^{3}H]TEI-7165 to HSA

Displacement of [^{3}H]TEI-9090 and [^{3}H]TEI-7165 binding to HSA by the ligands was tested to examine the binding sites of these drugs (Table 3). Although the binding of [^{3}H]TEI-9090 and [^{3}H]TEI-7165 to fatty acid-free HSA was not inhibited by aspirin, salicylic acid and indomethacin, the binding was significantly decreased by the site II (Sudlow et al 1975)-ligand clofibric acid. Moreover, the binding of both [^{3}H]TEI-9090 and [^{3}H]TEI-7165 to Fraction V of HSA were significantly less than those to fatty acid-free HSA.

Table 3. Effects of several drugs on [^{3}H]TEI-9090 and [^{3}H]TEI-7165 binding to human serum albumin.

	Drug (mM)		Binding (%)	
			[^{3}H]TEI-9090[a]	[^{3}H]TEI-7165[b]
HSA (fatty acid-free)[c]	None		84·6 ± 1·2	98·1 ± 0·03
	Aspirin	(1·0)	83·4 ± 1·1	97·1 ± 0·2
		(10·0)	78·5 ± 1·7	96·2 ± 0·1
	Salicylic acid	(1·0)	83·4 ± 0·9	95·3 ± 1·1
		(10·0)	83·1 ± 4·2	95·3 ± 0·1
	Indomethacin	(0·5)	84·8 ± 1·1	98·0 ± 0·1
	Clofibric acid	(1·0)	76·7 ± 1·3**	96·2 ± 0·1
		(10·0)	69·2 ± 0·8**	69·8 ± 0·3**
HSA (Fraction V)[d]	None		73·7 ± 1·3*	95·1 ± 0·1**

Concentration of [^{3}H]TEI-9090 and [^{3}H]TEI-7165 was 1 ng mL^{-1}. [a]Protein binding was determined by PDMS-GB as described in the text. [b]Protein binding was determined by ultrafiltration as described in the text. [c]Total protein, 39·2 ± 0·4 mg mL^{-1}. [d]Total protein, 40·9 ± 1·1 mg mL^{-1}. Each value represents the mean ± s.e. of three samples. *$P < 0·05$, **$P < 0·01$ (Student's t-test) compared with essentially fatty acid-free human serum albumin values.

Discussion

In equilibrium dialysis, ultrafiltration and gel filtration to determine the protein binding of a highly lipophilic and hydrophobic drug TEI-9090, the free drug was not detected in the dialysis buffer, filtrate and eluate because the

Table 2. Protein binding of [^{3}H]TEI-9090 and [^{3}H]TEI-7165 in serum of several species in-vitro.

Species	Protein (mg mL^{-1})	[^{3}H]TEI-9090		[^{3}H]TEI-7165	
		Concn (ng mL^{-1})	Binding[a] (%)	Concn (ng mL^{-1})	Binding[b] (%)
Human	87·6 ± 3·6	0·5(0·45)[c]	97·0 ± 1·3	0·1	94·2 ± 1·1
		1·0(0·85)[c]	98·1 ± 0·5	1·0	96·6 ± 0·3
		10·0(8·9)[c]	97·5 ± 0·1	10·0	96·5 ± 0·2
HSA[d]	39·2 ± 0·4	0·5	84·3 ± 0·7	0·1	98·0 ± 0·7
		1·0	84·9 ± 2·2	1·0	98·5 ± 0·1
		10·0	86·3 ± 0·4	10·0	98·3 ± 0·1
Dog	65·1 ± 1·9	0·5	93·9 ± 0·8	0·1	90·4 ± 0·4
		1·0	94·3 ± 0·9	1·0	93·1 ± 0·4
		10·0	95·1 ± 0·7	10·0	92·7 ± 0·6
Rabbit	69·4 ± 1·8	–	–	0·1	96·5 ± 0·6
		–	–	1·0	95·3 ± 0·3
		–	–	10·0	95·6 ± 0·2
Rat	74·7 ± 1·5	–	–	0·1	95·2 ± 0·6
		–	–	1·0	94·5 ± 0·1
		–	–	10·0	94·1 ± 0·2

[a] Protein binding was determined by PDMS-GB as described in the text. [b] Protein binding was determined by ultrafiltration as described in the text. [c] A part of [^{3}H]TEI-9090 was hydrolysed in serum. Concentration of the unchanged drug at 1 min is shown in the parentheses. [d] Essentially fatty acid-free human serum albumin. Each value represents the mean ± s.e. of the three samples.

drug adsorbed completely to the membranes and resins (Minagawa et al 1994). Moreover, in ultracentrifugation, about 50% TEI-9090 in PBS adsorbed to the centrifugation tubes (data not shown). To determine the protein binding of a lipophilic drug, such as TEI-9090, we developed an application of an adsorption technique using PDMS-GB. Binding of [^{3}H]diazepam in human serum, HSA solution and dog serum measured by ultrafiltration was similar to the values reported in the literature (Klotz et al 1975; Allen & Greenblatt 1981; Denson et al 1984), and were not significantly different from the values determined by PDMS-GB assay. These results indicate that the PDMS-GB assay is a reliable technique to measure simply and rapidly the protein binding of a lipophilic drug.

Binding of TEI-9090 in HSA solutions was found to be less than that of TEI-7165. This result indicates that esterification of α-chains of prostaglandins must affect the binding characteristics of their binding to albumin molecules, and was consistent with a previous report (Gueriguian 1976). However, in human serum the binding of TEI-9090 was elevated and any differences could not be elucidated for both drugs. Accordingly, it was deduced that a part of TEI-9090 could bind to a serum protein other than albumin. Papp et al (1985) reported that human serum Fraction VI glycoprotein as well as albumin may play an important role in the binding and stabilization of PGI_2. On the other hand, it has been suggested that PGI_2 could bind mainly to human high density lipoprotein and apolipoprotein A-I (Yui et al 1988), but there have been some objections to this statement (O'Brien et al 1989b; Tsai et al 1991). Although further studies are required to reveal the other binding protein for TEI-9090, as in the case of PGI_2, we conclude that albumin is the main component binding TEI-9090 in human serum.

It has been shown that PGA_1 and PGE_1 binding to HSA are displaced by salicylate and indomethacin (Attallah & Lee 1980), and that these prostaglandins, and prostaglandins with β-hydroxy ketone groups bind to Sudlow's site I on albumin molecules (Fitzpatrick & Wynalda 1981). In contrast, Kurono et al (1982) demonstrated that the PGI_2 binding site on HSA differs from site I, since PGI_2 binding to HSA was displaced by clofibric acid, which can bind to the R-site considered to be identical with site II (Ozeki et al 1980; Kurono et al 1987). Moreover, it has been reported that free fatty acids would compete with PGI_2 for binding to HSA (Goodnight et al 1985; O'Brien et al 1989a). These characteristics of PGI_2 closely resemble those of TEI-9090 and TEI-7165, observed in our displacement studies. However, Tsai et al (1989) have suggested that a PGI_2 analogue, iloprost, could bind to site I on HSA molecules, since iloprost binding to HSA was displaced by the site I ligand warfarin and only weakly competed with free fatty acids. The binding mechanism of PGI_2 analogues with albumin is not clearly understood. Recently, the three-dimensional structure of HSA has been determined and further investigation of the ligand binding domains is in progress (He & Carter 1992). These studies will enlighten the complex problems on the ligand binding mechanisms of serum albumin.

In summary, the protein binding of a highly lipophilic and hydrophobic drug, PGI_2 analogue TEI-9090, could be successfully determined by applying PDMS-GB assay. Results obtained in the present study demonstrate that the binding characteristics of TEI-9090 and its metabolite TEI-7165 were similar to those of endogenous PGI_2.

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