# Entrapping Efficiency of an Oil-in-Water Emulsion Containing Isocarbacyclin Methyl Ester (TEI-9090) in Dog and Human Sera

**Toshiya Minagawa,[1,2] Yoshiro Kohno,[1] Toshio Suwa,[1] and Akira Tsuji[3]**

*Received January 31, 1994; accepted May 16, 1994*
KEY WORDS: o/w emulsion; serum; entrapping efficiency; kinetics; SDS.

## INTRODUCTION

It has been reported that intravenous administration of isocarbacyclin methyl ester (TEI-9090) (1) as the o/w emulsion formulation dramatically increased its *in vivo* antithrombotic activity compared with that of the aqueous solution (2). This effect of the o/w emulsion formulation is believed to be due to a significant drug entrapment and efficient drug delivery. Accordingly, evaluating the entrapping efficiency of the emulsion containing TEI-9090 is important for interpreting its biological activity and its pharmaceutical characteristics.

In our previous study (3), the drug release profiles of the emulsion containing TEI-9090 were characterized by a new technique using glass beads coated with polydimethylsiloxane (PDMS-GB), which can adsorb the released drug without changing the characteristics of the emulsion. Furthermore, the protein binding of TEI-9090 was also successfully determined by applying PDMS-GB assay (4). However, the conventional procedure in PDMS-GB assay is unsuitable for the serum samples containing the emulsion, because not only TEI-9090 released from the emulsion particles but the drug bound to the serum protein, was not adsorbed to PDMS-GB and was apparently assayed as an entrapped drug. In this study, therefore, PDMS-GB assay was designed to use a suitable ligand which can displace TEI-9090 bound to the serum protein, resulting in the successful determination of the entrapping efficiency of the emulsion in dog and human sera by differentiating technically that from the protein binding of the drug.

## MATERIALS AND METHODS

*Chemicals.* 11-β-[$^3$H]TEI-9090 (sp act, 0.68 TBq/mmol; >99% pure), the unlabeled TEI-9090 and isocarbacyclin (TEI-7165) were kindly donated by Teijin Co., Ltd., Tokyo. The chemical structures and labeled position are shown in Fig. 1. [$^{35}$S]Sodium dodecyl sulfate (SDS, 1.3 GBq/mmol) was purchased from Amersham Int. (Buckinghamshire, UK). Essentially fatty acid-free human serum albumin (HSA) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

*Preparation of an o/w emulsion containing [$^3$H]TEI-9090.* A 10% (w/v) soybean oil o/w emulsion containing [$^3$H]TEI-9090 was prepared with a French press (Aminco Instrument Co., MD) (3). The particle size distribution was determined by photon correlation spectroscopy (Nicomp Model 370, Nicomp Instruments Division Pacific Scientific, CA).

*Serum samples and HSA solutions.* Dog serum was obtained from male beagle dogs of 12.0–14.5 kg body weight (Laboratory Research Enterprises, Inc., Tokyo). Human serum was obtained from three healthy volunteers, aged 25–32 years, who had taken no medication for a week before blood sampling. HSA solutions (4%, w/v) were prepared in a 0.1 M isotonic sodium phosphate buffer (PBS, pH 7.4). Dog and human serum samples were used immediately after separation from red blood cells. The protein concentration was measured using Biuret reagent (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan).

*PDMS-GB assay.* The preparation of PDMS-GB, and the assay procedure to determine protein binding of [$^3$H]TEI-9090 and the entrapping efficiency of the o/w emulsion, have been described in the previous papers (3,4). To study the effects of SDS on [$^3$H]TEI-9090 binding to serum protein, PDMS-GB assay was performed at 37°C for 1 ml of HSA solutions and the serum samples containing 10 ng/ml of [$^3$H]TEI-9090, 10 sec after addition of 10 μl SDS stock solution prepared with distilled water. To establish the standard curves for [$^3$H]TEI-9090, the incubation time of 1 min was used to avoid hydrolysis of the drug in the serum samples. The extent of hydrolysis of the drug was measured using a high performance liquid chromatography by the method described previously (3).

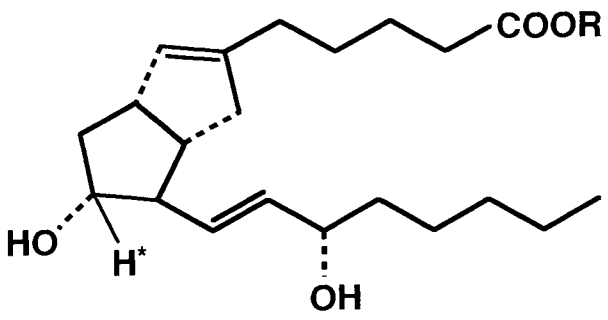
*Interaction between SDS and o/w emulsion.* By means of Amberlite XAD 2 (Rohm and Haas Co., PA) column chromatography (5) and a filtration method using a Mylex® membrane (VV13, 100 nm pore size, Nihon Millipore Ltd., Tokyo) (2), the binding of SDS to the o/w emulsion particles was examined.

The XAD 2 resins, which were washed with a sufficient volume of acetone and distilled water, were packed into a 10 × 150 mm column. The o/w emulsion containing unlabeled TEI-9090 was diluted 100 times with PBS. One milliliter of the diluted emulsion, which was mixed with 10 μl of [$^{35}$S]SDS solution to provide a final concentration of 10 mM, was passed through the column at 37°C. After the column was washed with 100 ml of distilled water, the labeled compound was eluted with 100 ml of MeOH containing 10% (v/v) of diethylamine (DEA/MeOH). Radioactivity in the wash and MeOH elute was counted in a liquid scintillation counter (LS6000TA, Beckman Instruments, Inc., CA) and total concentration of triacylglycerol which is the main contents of emulsion was determined by an enzymatic colorimetric kit, Anasolv TG-2 (Daiichi Pure Chemicals Co., Ltd., Tokyo).

The triacylglycerol-rich creamy layer was fractionated from the emulsion containing unlabeled TEI-9090 by the ul-

[1] Research Center, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshinocho, Ohmiya 330, Japan.
[2] To whom correspondence should be addressed.
[3] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan.


0724-8741/94/1100-1677$07.00/0 




R = $CH_3$ : TEI-9090

R = H : TEI-7165

Fig. 1 Chemical structures of TEI-9090 and TEI-7165. *: labeled position of $^3H$.

tracentrifugation method (3). Triacylglycerol-rich particles suspended in 2.2% (w/v) grycerol solution (TGRP), with a mean particle size of 320 nm and with 98% of the particles were above 100 nm, was diluted 100 times with PBS. The diluted suspension mixed with [$^{35}$S]SDS (10 mM) was filtered through the Mylex® membrane at 37°C.

*Data analysis.* The *in vitro* half-lives were determined by plotting the logarithm of the entrapping efficiency and the amount of [$^3$H]TEI-9090 in serum samples against time. Student's *t*-test was utilized to determine the significance of differences.

## RESULTS AND DISCUSSION

*Effects of SDS on the protein binding of TEI-9090.* The binding of [$^3$H]TEI-9090 to HSA was evaluated. The standard curve for [$^3$H]TEI-9090 in PBS determined by PDMS-GB assay, was linear up to 10 ng/ml. The linear regression was

$$Y = 0.819X - 0.024 \quad [ng] \qquad (1)$$

with a correlation coefficient of 0.999, where X and Y are the amount of [$^3$H]TEI-9090 added and adsorbed, respectively. The following equation for calculating the unbound drug concentration ($C_f$) in HSA solution or serum samples was obtained:

$$C_f = \frac{(C_{tot} - C_{sup}) - a}{b} \quad [ng/ml] \qquad (2)$$

where $C_{tot}$ is total drug concentration, $C_{sup}$ is a drug concentration of the assayed solution, and a and b are the intercept (−0.024) and slope (0.819) of the standard curve, respectively. Accordingly, the percent of protein binding is determined from the difference between $C_{tot}$ and $C_f$.

As shown in Table I, the extent of protein binding of [$^3$H]TEI-9090 at 10 ng/ml in HSA solution was 85%, and was diminished to 67% by the addition of 10 mM of SDS. Since the critical micelle concentration (c.m.c.) of SDS in the isotonic PBS was reported to be about 1 mM (6), SDS micelles would exist in HSA solutions in our study. It was reported that a considerable conformational change of the albumin molecules occurs above the c.m.c. of SDS (7). Accordingly, the inhibition of the binding of TEI-9090 to HSA by SDS should be due to the conformational change of HSA.

Table I. Inhibition on *in Vitro* Protein Binding of [$^3$H]TEI-9090 by SDS[a]

| SDS (mM) | Binding (%)[b] |
|---|---|
| −[c] | 85 ± 1 |
| 1 | 77 ± 1 |
| 10 | 67 ± 1* |

[a] PDMS-GB assay was performed for HSA solutions containing 10 ng/ml of [$^3$H]TEI-9090 and 1 or 10 mM of SDS at 37°C.
[b] Values represent the mean ± S.E. of triplicate assays.
[c] Control study was carried out without SDS.
* $p < 0.01$ (Student's t-test).

*Interaction between SDS and o/w emulsion.* [$^{35}$S]SDS in PBS at 10 mM was almost completely adsorbed by the XAD 2 resins and 96% of radioactivity was eluted by DEA/MeOH. In the case of the emulsion diluted 100 times with PBS containing [$^{35}$S]SDS, although triacylglycerols passed completely through the column into the wash, 94% of [$^{35}$S]SDS was eluted from the resins by DEA/MeOH.

Since the particle size of the main component of the emulsion, TGRP, was mostly above 100 nm, Mylex® membrane with a pore size of 100 nm was adequate to separate the particles from the aqueous phase. Prior to the use of the membrane to study the interaction between SDS and TGRP, the membrane binding of SDS was examined, by filtering a PBS solution of [$^{35}$S]SDS at a concentration of 10 mM. The recovery of [$^{35}$S]SDS from PBS almost reached saturation, above 96%, after more than 0.3 ml of the solution had been filtered. After filtration of 0.5 ml of 10 mM SDS in PBS, 99% of [$^{35}$S]SDS in the 1/100 TGRP suspension was filtered through the membrane, while triacylglycerols were negligible in the filtrate.

The mean particle diameters of the o/w emulsion determined by the photon correlation spectroscopy were not changed after addition of 10 mM of SDS (data not shown).

From these observations, there should be little influences of SDS on the emulsion particles.

*Entrapping efficiency of o/w emulsion containing [$^3$H]TEI-9090 in serum.* Because neither the [$^3$H]TEI-9090 entrapped in the emulsion particles nor the drug bound to the serum protein are adsorbed to PDMS-GB, the assay needs to be modified to evaluate the entrapping efficiency of the emulsion containing [$^3$H]TEI-9090 in serum. SDS can displace partly the drug bound to HSA, and scarcely binds to the emulsion particles. Consequently, we had the idea that the entrapping efficiency of the emulsion in serum could be evaluated, because PDMS-GB can adsorb quantitatively the protein-bound fraction of the drug displaced by SDS as well as the protein-unbound fraction.

As shown in Fig. 2, the standard curves for [$^3$H]TEI-9090 in HSA solution, dog serum and human serum, assayed after the addition of SDS, were linear up to 20 ng of the drug with the linear regression equations of $Y = 0.392X + 0.066$ [ng] ($r = 0.998$), $Y = 0.156X + 0.099$ [ng] ($r = 0.982$) and $Y = 0.144X + 0.052$ [ng] ($r = 0.998$), respectively. The

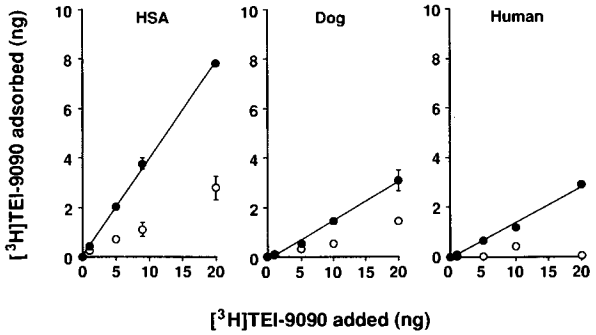



Fig. 2 Standard curves for [$^3$H]TEI-9090 in HSA solution, dog and human sera. HSA solutions and the serum samples were incubated at 37°C for 1 min. PDMS-GB assay was performed for the samples without addition of SDS (○) or 10 sec after addition of SDS (●). Each value represents the mean and range of duplicate assays.

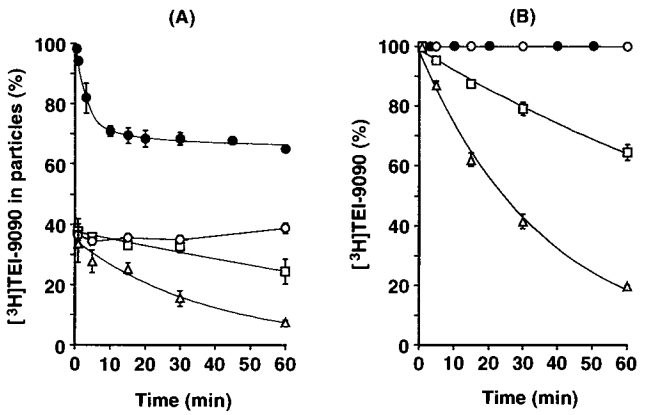



Fig. 3 Entrapping efficiency of the o/w emulsion containing [$^3$H]TEI-9090 (A) and hydrolysis of the drug (B) *in vitro*. The emulsion was diluted 100 times with PBS (●), HSA solution (○), dog (□) and human sera (△). Each point represents the mean ± S.E. of three samples.

slopes were higher than the values obtained by assay without SDS. Using these standard curves, the total concentrations of the protein bound and unbound fractions of [$^3$H]TEI-9090 in sera and HSA solution ($C_{b+f}$) can be calculated by the equation:

$$C_{b+f} = \frac{(C_{tot} - C_{sup}) - a'}{b'} \quad [ng/ml] \qquad (3)$$

where a′ and b′ are the intercepts and slopes of the standard curves. The entrapping efficiency (E) of the o/w emulsion is estimated by the following equation, where the concentration of TEI-7165 ($C_h$), which was generated by hydrolysis of TEI-9090 in the serum, must be reduced, because TEI-7165 had a lower lipophilicity compared with TEI-9090 and was slightly adsorbed to PDMS-GB (4) (Eq. 4).

$$E = \frac{C_{tot} - C_h - C_{b+f}}{C_{tot}} \times 100 \quad [\%] \qquad (4)$$

Time-dependent alterations of the entrapping efficiency of the o/w emulsion containing [$^3$H]TEI-9090, which was diluted 100 times with aqueous solution (PBS), HSA solution, and human and dog sera, are shown in Fig. 3(A). In PBS, [$^3$H]TEI-9090 was rapidly released from the emulsion particles and an equilibrium was established at 60 min showing an entrapping efficiency of 68%. We had concluded previously that TEI-9090 was not released into the aqueous phase of the diluted emulsion but to the surface area of the particles (3). On the other hand, the entrapping efficiency of the emulsion in HSA solution was lower than that in the aqueous solution, and was shown to be 34–39% up to 60 min after dilution. These results suggest that [$^3$H]TEI-9090 was released to the surface of the emulsion particles by dilution and was removed by HSA molecules. In dog and human sera, the entrapping efficiency of the emulsion was 38% and 34% at 1 min, and decreased with half-lives of 100 min and 27 min, respectively. With parallel phenomena of decrease of the entrapping efficiency, hydrolysis of [$^3$H]TEI-9090 in the samples was observed (Fig. 3B). Whereas [$^3$H]TEI-9090 was stable in PBS and HSA solutions, the drug was hydrolyzed in dog and human sera, with half-lives of 96 min and 25 min, respectively. These results revealed that the entrapping efficiency of the o/w emulsion containing [$^3$H]TEI-9090 was reduced by hydrolysis as well as by protein binding of the drug in serum, in addition to the dilution of the emulsion.

While a number of studies of the o/w emulsion formulations for drug delivery have been reported, interactions between the drugs, the emulsion particles and the components in blood have not been clearly defined yet. Previously, we have reported that the phase distribution of [$^3$H]TEI-9090 in the o/w emulsion changed in a time-dependent manner after dilution with saline (3). In this study, we have demonstrated using PDMS-GB assay combined with the technique of protein-binding displacement by SDS, that the entrapping efficiency was reduced by protein binding and hydrolysis of the drug, as well as by dilution of the emulsion in serum *in vitro*. These observations help elucidate phenomena occurring in the body fluid after intravenous dose of the o/w emulsion formulation containing TEI-9090.

## REFERENCES

1. M. Shibasaki, Y. Torisawa and S. Ikegami. Synthesis of 9(0)-methano-$\Delta^{6(9a)}$-$PGI_1$: the highly potent carbon analog of prostacyclin. *Tetrahedron Lett.* 24:3493–3496 (1983).
2. Y. Mizushima, R. Igarashi, K. Hoshi, A. K. Sim, M. E. Cleland, H. Hayashi and J. Goto. Marked enhancement in antithrombotic activity of isocarbacyclin following its incorporation into lipid microspheres. *Prostaglandins* 33:161–168 (1987).
3. T. Minagawa, Y. Kohno, T. Suwa and A. Tsuji. Entrapping efficiency and drug release profile of an oil-in-water (o/w) emulsion formulation using a polydimethylsiloxane-coated glass bead assay. *Pharm. Res.* 11:503–507 (1994).
4. T. Minagawa, Y. Kohno, T. Suwa and A. Tsuji. Determination of protein binding of a highly lipophilic drug using a polydimethylsiloxane-coated glass beads assay. *J. Pharm. Pharmacol.* in press (1994).
5. R. Takeshita and H. Yoshida. Studies on anion active surfactants. I. Determination of anion active surfactants in natural and waste waters using cleanup by Amberlite XAD 2 column chromatography. *Jpn. J. Toxicol. Environ. Health* 11:209–215 (1975).
6. M. L. Corrin and W. D. Harkins. The effect of salts on the critical concentration for the formulation of micelles in colloidal electrolytes. *J. Am. Chem. Soc.* 69:683–688 (1945).
7. J. Reynolds, S. Herbert and J. Steinhardt. The binding of some long-chain fatty acid anions and alcohols by bovine serum albumin. *Biochemistry* 7:1357–1361 (1968).