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Protein Binding of Timiperone, 4'-Fluoro-4-[4-(2-thioxo-1-benzimidazolinyl)piperidino]butyrophenone, a Neuroleptic

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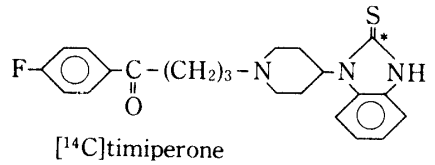
The binding of timiperone to human plasma protein and human serum albumin *in vitro* was studied by using four different analytical procedures. After addition of timiperone to the plasma at concentrations of 10 and 100 ng/ml, more than 95% of timiperone was bound to the plasma protein. The finding that the binding ratio of the drug to human serum albumin was approximately 80% suggested that most of the timiperone in the plasma was bound to human albumin. More than 98% of protein-bound timiperone was extracted with trichloroacetic acid and 80% methanol solution. Thus, it was demonstrated that almost all the binding of the drug to human plasma protein is reversible.

Keywords——protein binding; neuroleptic; ultracentrifugation; gel filtration; equilibrium dialysis; human plasma; human serum albumin

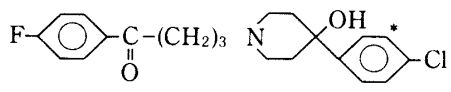
Introduction

It is generally accepted that many drugs are partly bound to the plasma proteins, and that the therapeutic effect of drugs is closely associated with the blood concentrations of unbound drugs.[1,2] Moreover, some drugs are converted *in vivo* to reactive metabolites and bound covalently to proteins of the plasma and tissues, causing adverse reactions[3,4] such as allergic reactions and liver necrosis. From this point of view, it is of great importance to study the protein binding of neuroleptics, which are administered clinically to patients for a long period. However, there are few studies on the protein binding of the butyrophenone neuroleptics.

Timiperone, a new butyrophenone, whose chemical structure is 4'-fluoro-4-[4-(2-thioxo-1-benzimidazolinyl)piperidino]butyrophenone, possesses potent antipsychotic activity with a reduced likelihood of extrapyramidal adverse reactions.[5,6] Its metabolic disposition and affinity to cerebral dopamine receptors in experimental animals have been investigated in detail, as reported previously.[7–9] In this work, we studied the protein binding *in vitro* of timiperone to human plasma protein and human serum albumin, using four different techniques.



[^{14}C]timiperone



[^{3}H]haloperidol

Fig. 1. Structures of Timiperone and Haloperidol
Asterisks denote the positions of ^{14}C and ^{3}H.

Materials and Methods

Materials —— [^{14}C]Timiperone, 4'-fluoro-4-[4-(2-thioxo-1- [2-^{14}C]benzimidazollinyl) piperidino]butyrophenone (36 Ci/mol and 43.1 Ci/mol), was supplied by Daiichi Pure Chemicals Co., Ltd. (Japan). [^{3}H]Haloperidol, 4-(4-)4-[3-^{3}H)chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-butanone (9.7 Ci/mmol), waspurchased from CEA (France). The radio-chemical purity of the labelled drugs was more than 97% (checked by thin-layer chromatography (TLC)). The chemical structures of the drugs are shown in Fig. 1. Other commercial chemicals used were of analytical grade.

Measurement of Radioactivity——Radioactivity was measured with a liquid scintillation spectrometer (Aloka, model LSC-672, Japan). All counts were corrected for quenching by the automatic external channel ratio method.

Measurement of Protein Concentration——Protein concentrations were determined according to the method of Lowry *et al.*[10] with crystalline bovine serum albumin as the protein standard.

Preparation of Incubation Mixtures——An appropriate amount of [^{14}C]timiperone or [^{3}H]haloperidol dissolved in isotonic 10 mM phosphate buffer (pH 7.4) was immediately added to fresh human plasma and human serum albumin (HSA) solution to produce final concentrations of 10 ng/ml and 100 ng/ml.

HAS solutions were prepared as follows: HSA (Sigma Chemical Company, U.S.A.) was dissolved in the same buffer as described above to give a final concentration of 43 mg/ml.[11]

Determination of Protein Binding——Ultracentrifugation: Five ml of plasma or HSA solution was incubated at 37°C for 30 or 120 min, and then ultracentrifuged at $160000 \times g$, 20°C for 18 h in an ultracentrifuge (Hitachi 55P-2, Japan). A 1.5 ml aliquot of the supernatant of each solution was collected, and the radioactivity was determined. The protein binding ratio was calculated according to the following formula: $\% \text{ bound} = \left(\frac{C_i - C_s}{C_i}\right) \times 100$. C_i and C_s denote the concentrations of the drug in the incubation mixture and in the supernatant, respectively.

Equilibrium Dialysis: One ml plasma samples were incubated at 37°C for 30 or 120 min. Cellophane membrane (Visking Company, U.S.A.) soaked in 10 mM phosphate buffer (pH 7.4) before use was placed between the two halves of a small dialysis cell to separate the two compartments. Plasma sample (700 μl) was added to one compartment and the same volume of dialysis buffer (isotonic, 10 mM phosphate buffer, pH 7.4) to the other. The cell was allowed to stand at 4°C for 5 d until equilibrium was attained. After dialysis, the radioactivity in the sample recovered from each compartment was determined. The protein binding ratio was calculated according to the following formula: $\% \text{ bound} = \frac{C_p - C_d}{C_p} \times 100$

C_p and C_d denote the concentrations of radioactivity in the plasma sample and dialysis buffer after dialysis, respectively.

Gel Filtration: Three ml of plasma was incubated at 37°C for 30 or 120 min. After the incubation, 1.5 ml was applied to a column packed with Sephadex G-25 and eluted with isotonic 10 mM phosphate buffer (pH 7.4). Fifty μl aliquots from each 1 ml eluate were analyzed for radioactivity or protein. The protein inding ratio was calculated according to the following formula: $\% \text{ bound} = \frac{R_p}{R_e} \times 100$

R_e and R_p denote the total radioactivities recovered from all eluates and from all protein fractions, respectively.

Extraction with Trichloroacetic Acid and 80% Methanol Solution: One ml plasma samples were incubated at 37°C for 30 min. Each incubation mixture was then mixed with 2 ml of 0.9 M trichloroacetic acid (TCA) and centrifuged at room temperature for 15 min at $1000 \times g$. The supernatants were discarded, and the protein precipitates were resuspended in 3 ml of 0.6 M TCA for 3 min by the use of a vortex mixer. After centrifugation at $1000 \times g$ for 15 min, the supernatants were discarded, and the precipitates were washed again with 0.6 M TCA. The residual protein was resuspended in 3 ml of 80% methanol for 3 min by the use of the vortex mixer. The resuspensions were centrifuged as described above and the supernatants were discarded again. These procedures were repeated until no further radioactivity could be removed (usually six extractions). The extracted protein was dissolved in 1 ml of 1 N NaOH and then diluted to 10 ml with distilled water. An aliquot adsorbed on a strip of paper was burnt in an automatic sample combustion system (Aloka, ASC-113, Japan) and the radioactivity was determined. The protein binding ratio was calculated according to the following formula: $\% \text{ bound} = \frac{R_e}{R_p} \times 100$

R_p and R_e denote the radioactivities in 1 ml of plasma and in the extracted protein solutions, respectively.

Results

Binding of [^{14}C]Timiperone to Human Plasma Protein

[^{14}C]Timiperone was added to fresh human plasma at a final concentration of 10 or 100 ng/ml, because the plasma levels of the drug were less than 10 ng/ml in schizophrenic patients who were receiving repeated oral clinical doses of 6 to 9 mg of timiperone. The ratios of free or bound timiperone were determined by three different methods. As shown in Table I, plasma protein binding ratios of [^{14}C]timiperone determined by the ultracentrifugation method were more than 95% at both concentrations. Similarly, binding ratios obtained by the equilibrium dialysis method ranged from 89.6 to 96.3%. By the gel filtration method, the ratios at the two incubation times were 76.5 and 79.4% at the concentration of 100 ng/ml. Incubation

time and drug concentration had no influence on the protein binding ratios of the drug.

TABLE I. Binding of [^{14}C]Timiperone to Human Plasma Protein

Method	Concentration (ng/ml)	Protein binding ratios (%) *In vitro* incubation time (h) 0.5	2
Ultracentrifugation	10	95.0±0.1	95.3±0.3
	100	96.1±0.2	96.4±0.2
Equilibrium dialysis	10	95.7±0.3	96.3±0.5
	100	95.9±0.1	89.6±6.3
Gel filtration	100	76.5±0.8	79.4±0.9

Each value represents the mean ± standard error of triplicate determinations.
Human plasma was incubated with the drugs at 37 °C for 30 or 120 min.

Binding of [^{14}C]Timiperone to Human Serum Albumin

As shown in Table II, the HSA binding ratios of [^{14}C]timiperone obtained by the ultracentrifugation method were also high, like the plasma protein binding ratios, and were approximately 80% at both concentrations of the drug.

TABLE II. Binding of [^{14}C]Timiperone to Human Serum Albumin

Method	Concentration (ng/ml)	Protein binding ratios (%) *In vitro* incubation time (h) 0.5	2
Ultracentrifugation	10	78.7±0.8	82.6±3.1
	100	77.5 ± 0.5	78.5±0.4

Each value represents the mean ± standard error of triplicate determinations.
HSA solution was incubated with the drugs at 37 °C for 30 or 120 min.

Comparison of Plasma Protein Binding of [^{14}C]Timiperone and [^{3}H]Haloperidol

Plasma protein binding of [^{14}C]timiperone determined by two different methods was compared with that of [^{3}H]haloperidol, a typical neuroleptic. As shown in Table III, binding ratios of [^{14}C]timiperone and [^{3}H]haloperidol determined by the ultracentrifugation method were 96.3 and 92.7%, respectively. Since both drugs showed extremely high binding ratios, we examined whether covalent binding of the drugs to plasma protein occurs. Human plasma incubated with the drugs was extracted stepwise with trichloroacetic acid and methanol. After the extraction, the protein binding ratios of [^{14}C]timiperone and [^{3}H]haloperidol were 1.7 and 7.6%, respectively, as presented in Table III. More than 98% of incubated [^{14}C]-timiperone was released, whereas 7.6% of incubated [^{3}H]haloperidol was still bound to the protein. Accordingly, there was no evidence of covalent binding of [^{14}C]timiperone to human plasma protein.

TABLE III. Comparison of Binding of [^{14}C]Timiperone and [^{3}H] Haloperidol to Human Plasma Protein

Method	Concentration (ng/ml)	Protein binding ratios (%) [^{14}C]timiperone	[^{3}H]haloperidol
Ultracentrifugation	100	96.3±0.3	92.7±0.5
Extraction with trichloroacetic acid and 80% methanol	100	1.7±0.4	7.6±0.5

Each value represents the mean ± standard error of triplicate determinations.
Human plasma was incubated with the drugs at 37 °C for 30 min.

Discussion

There are several methods for estimating the protein binding of drugs, and the binding ratios determined vary remarkably depending on the methods used, since different conditions in the individual methods influence the equilibrium between free and bound forms of the drugs. In this study, plasma protein binding of timiperone was, therefore, evaluated by four different methods.

The results obtained with different incubation times indicated that equilibrium in the plasma protein binding of timiperone was attained within 30 min. It is well-known that the concentration of unbound drug in human plasma is closely related to its therapeutic effect,[1,2] and it was also reported that the protein binding ratios of some neuroleptics including chlorpromazine decreased with increasing plasma concentrations of the drugs.[12] In this regard, the present results indicated that the protein binding ratios of timiperone were not affected by a 10-fold increase in the plasma concentration of the drug. This finding suggests that concentrations of unbound timiperone in the plasma of schizophrenic patients who receive repeated clinical doses may not be increased to an abnormal extent.

As shown in Table I, the results obtained by the ultracentrifugation and equilibrium dialysis methods, which are suitable for the estimation of weak and strong binding of drugs, indicated that the plasma protein binding of timiperone was extremely high. For this reason, the reversibility of timiperone binding was examined by gel filtration. A comparison of the binding ratios obtained by three methods clarified that approximately 17% of timiperone bound to human plasma protein was released by gel filtration.

It is well-known that albumin is the major binding protein for most drugs. In this connection, the results on the binding of timiperone to human serum albumin showed that timiperone was also mainly bound to albumin in human plasma protein.

It has been reported that the covalent binding of many drugs to endogenous macromolecules induces untoward adverse reactions, including allergy.[4] From this point of view, we investigated whether or not timiperone was bound covalently to plasma protein in comparison with haloperidol as a representative of neuroleptics used clinically. Our data indicated that essentially no covalent binding of timiperone to plasma protein occurs, whereas a significant amount of haloperidol appeared to be bound covalently to plasma protein.

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