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Short communication

# $^{19}$F NMR spectroscopic study on the binding of trifluprom azine to bovine and human serum albumins

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**Abstract**

The $^{19}$F NMR spectrum of trifluprom azine hydrochloride (TFZ) in a buffer solution (pH 6.8) showed a single sharp signal of the TFZ $CF_3$ group at 13.5 ppm from the external trifluoroacetic acid. The addition of 1 mM HSA or BSA to the sample solution caused a split of the $CF_3$ signal into two broadened signals shifted to slightly lower (0.2 ppm) and higher (0.7 ppm) fields, respectively, from the original position. Denaturation of the albumins by guanidine hydrochloride (3 M) restored the two broadened signals to a slightly broadened single signal, indicating that TFZ has at least two binding sites on HSA and BSA, respectively. From the competitive binding $^{19}$F NMR experiments using Warfarin (Site-I ligand), L-tryptophan (Site-II ligand), NaCl, and oleate, the signal at high field was assigned to the TFZ bound to Site II. Comparison of the signal intensity revealed that the affinity of TFZ for Site II on HSA was considerably higher than that on BSA. The low-field signal could be identified as a weight-averaged signal between nonspecifically bound TFZ to HSA (BSA) and free TFZ in the water phase. In the presence of physiological concentrations of NaCl, major binding of TFZ to HSA and BSA was considered to be nonspecific. The present work indicates that $^{19}$F NMR is very useful for obtaining important detailed information regarding the binding of fluorinated drugs to serum albumins.


*Keywords:* $^{19}$F NMR; Trifluprom azine; Drug–protein binding; BSA; HSA

## 1. Introduction

The binding of drugs to serum albumins has been intensively investigated because of its pharmacokinetic and pharmacological significance [1,2]. However, little has been known about the albumin binding of phenothiazine drugs, which are widely prescribed psychotropic drugs in medical practice [1]. In the present study, we investigated the binding of trifluprom azine (TFZ), a widely used phenothiazine drug, to bovine (BSA) and human (HSA) serum albumins by $^{19}$F nuclear magnetic resonance (NMR) spectroscopy, as TFZ has a trifluoromethyl group ($CF_3$).

$^{19}$F NMR spectroscopy has been used successfully to investigate macromolecule-$^{19}$F-labeled ligand interactions [3–5]. Its usefulness is due first to the natural abundance of the $^{19}$F nucleus being 100% and its relative sensitivity to protons being approximately 83%. Second, the $^{19}$F NMR chemical shift has an approximate range of 250 ppm, which is far larger than the $^{1}$H NMR chemical shift; as such, $^{19}$F NMR signals are much more sensitive to the changes in chemical environment than $^{1}$H signals. Third, as the $^{19}$F nucleus is absent in natural biological substances, it is easily observable without any interfering signals, even at low ligand concentrations.

There are many drugs that have fluorine(s) in their structures, e.g., some psychotropic drugs, some synthetic steroidal hormones, and new quinolone drugs, and they are widely used

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clinically because the introduction of fluorine atom(s) into the drugs improves their pharmaceutical activities or induces new pharmaceutical activities. Our study results indicate that $^{19}$F NMR spectroscopy will be widely applicable to obtaining detailed information regarding the binding interactions of fluorinated drugs with serum albumins.

## 2. Experimental

### 2.1. Chemicals and reagents

BSA (essentially fatty acid free), HSA (essentially fatty acid and globulin free), TFZ hydrochloride, Warfarin sodium salt (War), L-tryptophan (L-Try), and oleic acid sodium salt (OA) were purchased from Sigma. Sodium chloride (NaCl) and guanidine hydrochloride (GU) of analytical grade were purchased from Merck and Kanto Chemical Co., respectively. All reagents were used without further purification.

### 2.2. Preparation of albumin solutions

Stock solutions of BSA and HSA were prepared in potassium phosphate buffer (0.05 M, pH 6.8), and exact albumin concentrations were determined by UV absorption using by their absorptivity at 279 nm [1], respectively.

### 2.3. $^{19}$F NMR experiments

To a 2-mL volumetric flask, 40 μL of TFZ aqueous stock solution (50 mM) and a suitable aliquot of albumin solution to give a final albumin concentration of 1.0 mM were added, and then buffer was added to volume. In competitive binding experiments, before buffer was finally added to volume in the above procedure, each 200 μL of War, L-Try, NaCl, and OA aqueous stock solution (10 mM, 10 nM, 1.5 M, and 60 mM, respectively) was added to the flask, and then buffer was added to the intended volume, so that the final competing ligand concentrations became 1.0 mM, 1.0 mM, 0.15 M, and 6.0 mM, respectively. It has previously been shown [6] that mixing the OA and albumin solutions as described above can give an OA-bound albumin (6:1 molar ratio) solution. Approximately 1 mL of the sample solution was then transferred in a 5-mm NMR tube, and a glass capillary containing trifluoroacetic acid (TFA) $D_2O$ solution was inserted into the NMR tube. The $CF_3$ signal of TFA in the capillary tube was used as an external reference for chemical shift and relative peak intensity, and $D_2O$ was used as a source of the lock signal. All $^{19}$F NMR spectra were measured on a Varian XL-300 operating at 282.203 MHz. Measurements were performed at $21 \pm 2\,^{\circ}C$. The number of FID accumulations to improve the signal-to-noise ratio was 5000.

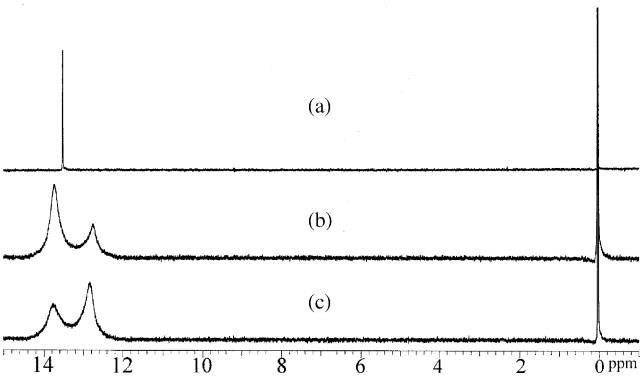


Fig. 1. $^{19}$F NMR spectra of 1.0 mM TFZ in buffer: (a) without serum albumin, (b) with 1.0 mM BSA, (c) with 1.0 mM HSA.

## 3. Results and discussion

### 3.1. $^{19}$F NMR spectra of TFZ bound to BSA and HSA

TFZ in a buffer solution showed a single sharp $^{19}$F NMR signal of its $CF_3$ group at 13.5 ppm, as depicted in Fig. 1(a). Upon the addition of 1.0 mM BSA or HSA to the sample solution, the $CF_3$ signal changed to two broadened signals shifted to high and low fields, 12.8 and 13.7 ppm, respectively, as illustrated in Fig. 1(b) or (c), indicating that both BSA and HSA have at least two binding sites for TFZ. In both Fig. 1(b) and (c), no signal can be seen at 13.5 ppm where the $CF_3$ signal of TFZ in buffer solution is located (Fig. 1(a)); the exchange rates of TFZ between the bound and unbound free states in BSA or HSA solution can therefore be considered to be fast enough in the $^{19}$F NMR (282 MHz) time scale. The appearance of two separate signals (12.8 and 13.7 ppm) indicates that the rate of direct exchange between the two bound states is a slow process in the $^{19}$F NMR (282 MHz) time scale for both BSA and HSA.

A comparison of Fig. 1(b) and (c) reveals that there is a large difference in the signal intensity ratio of high-field and low-field signals between BSA and HSA, i.e., the ratio is approximately 3:7 for BSA and 6:4 for HSA, respectively, as listed in Table 1. This result indicates that the binding affinity of TFZ for each site is different between BSA and HSA.

When 3 M GU, a typical protein denaturant, was added to each of the sample solutions of Fig. 1(b) and (c), respectively, the observed high- and low-field signals of each sample solution reverted to the slightly broadened original single signal, as shown in Fig. 2(a) and (b), respectively. The reverted signal

Table 1
Effects of competing ligands on the relative intensity of low- and high-field $^{19}$F NMR signals of TFZ interacting with BSA and HSA

| Competing ligand | BSA | | HSA | |
|---|---|---|---|---|
| | Low-field | High-field | Low-field | High-field |
| Non | 0.675 | 0.325 | 0.406 | 0.594 |
| War | 0.682 | 0.318 | 0.436 | 0.564 |
| L-Try | 0.888 | 0.112 | 0.756 | 0.244 |
| NaCl | 0.831 | 0.169 | 0.780 | 0.220 |
| OA | 0.914 | 0.086 | 0.840 | 0.160 |

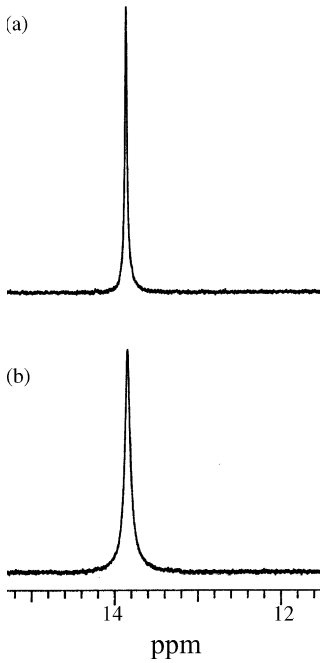


Fig. 2. $^{19}$F NMR spectra of 1.0 mM TFZ in buffer with (a) 1.0 mM BSA and (b) 1.0 mM HSA; after addition of 3 M GU.

showed a small downfield shift of 0.3 ppm from the original position of 13.5 ppm. This shift can be considered to be derived from the change in susceptibility of the sample solution due to the addition of 3 M GU, since the addition of 3 M GU to a TFZ buffer solution without albumins also caused a 0.3 ppm downfield shift of the $CF_3$ signal. The results of the GU denaturation experiment indicate that when the albumins were denatured and lost their folded states, the bound TFZ was liberated into the aqueous phase and retained its unbound state to show a sharp signal. These results confirm that the binding is reversible and that the appearance of two $CF_3$ signals, as seen in Fig. 1(b) and (c), respectively, is not caused by decomposition of TFZ.

### 3.2. Competitive binding

The $^{19}$F NMR spectra of the competitive binding experiments are shown in Figs. 3 and 4 for BSA and HSA, respectively, and the relative intensity of each signal is summarized in Table 1.

At first, addition of a typical Site I (domain IIA) binding ligand [7–9] War (1.0 mM) to the 1.0 mM TFZ sample solutions containing 1.0 mM BSA (Fig. 3(a)) or HSA (Fig. 4(a)) was examined. The results shown in Figs. 3(b) and 4(b), respectively, and listed in Table 1 reveal that War caused little change to either of the high- and low-field signals. It can therefore be considered that TFZ does not bind to Site I of BSA and HSA.

On the other hand, the addition of 1.0 mM L-Try, which has been known to bind to Site II (domain IIIA) [10–12], strongly reduced the intensity of the high-field signal, as seen in Figs.

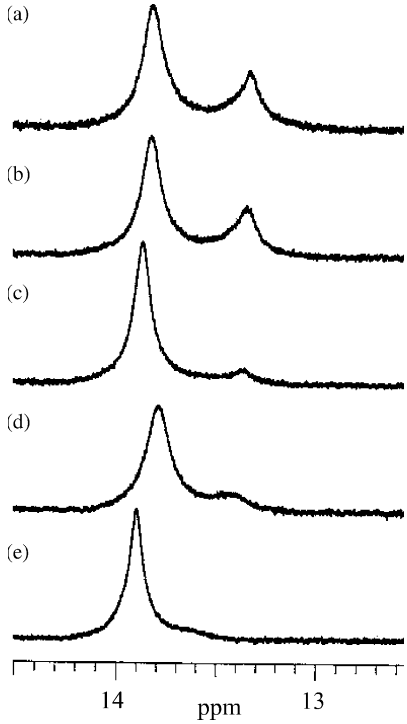


Fig. 3. $^{19}$F NMR spectra of 1.0 mM TFZ with 1.0 mM BSA in the presence of several competing ligands: (a) without any competing ligands, (b) with 1.0 mM War, (c) with 1.0 mM L-Try, (d) with 0.15 M NaCl, (e) with 6.0 mM OA.

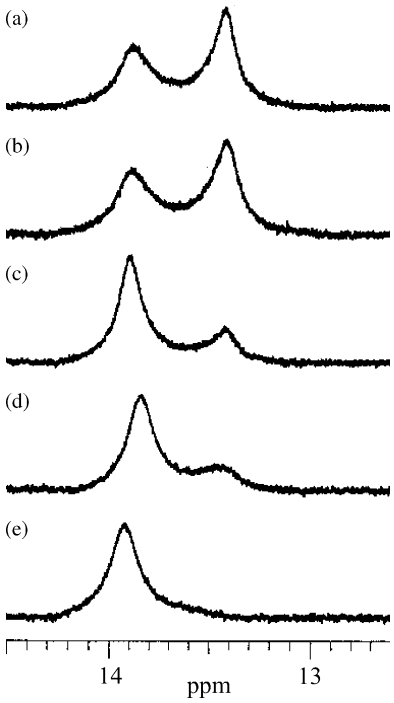


Fig. 4. $^{19}$F NMR spectra of 1.0 mM TFZ with 1.0 mM HSA in the presence of several competing ligands: (a) without any competing ligands, (b) with 1.0 mM War, (c) with 1.0 mM L-Try, (d) with 0.15 M NaCl, (e) with 6.0 mM OA.

3(c) and 4(c), respectively. Therefore, the high-field signals in Figs. 3(a) and 4(a) could be considered to be derived from TFZ bound to Site II in BSA and HSA, respectively. The decrease in the intensity of each high-field signal resulted in an increase in the intensity of each low-field signal, as seen in Figs. 3(c) and 4(c) (Table 1) without the appearance of the unbound free TFZ signal. It can therefore be concluded that TFZ liberated from Site II by the binding of L-Try binds to the other site, which induces a low-field shift to bound TFZ.

The addition of 0.15 M NaCl induced a signal change similar to that with L-Try, as shown in Figs. 3(d) and 4(d), respectively, and in Table 1. However, the lower-field signal slightly shifted to higher field, and this may be considered to be derived from the change in susceptibility of the sample solutions due to the 0.15 M NaCl. The chloride ion, $Cl^-$, has been reported to bind HSA at 7–8 molecules per HSA molecule and competes with tryptophan [13–15] at Site II (domain IIIA) on HSA [1,2]. Therefore, the addition of 0.15 M NaCl showed similar results in Figs. 3(d) and 4(d), respectively, as L-Try in Figs. 3(c) and 4(c), respectively, confirming that TFZ binds to Site II. This result is very important; since the physiological concentration of $Cl^-$ in blood is approximately 0.1 M, though TFZ binds to Site II of BSA and HSA in phosphate buffer solutions, the amount of TFZ bound to Site II under physiological condition is likely very small and TFZ binds mostly to the site(s) which induces the low-field shift to bound TFZ, as seen in Figs. 3(d) and 4(d), respectively.

The effect of oleate, a long-chain fatty acid (LCFA) that primarily binds to domain III on albumin [6,16–19], was also examined. The binding of six oleate molecules per albumin molecule reduced the high-field signal more than L-Try or NaCl for BSA and HSA, as shown in Figs. 3(e) and 4(e), respectively, and in Table 1. It has been reported based on a $^{13}C$ NMR study [6] that two oleate molecules strongly bind to domain III and one to domain I of BSA. Further, X-ray crystallographic studies [17,18] have shown the binding of six LCFA (myristate) molecules to one HSA molecule, and five binding sites, including domain III, in which two myristate molecules are bound as oleate. Previous reports [18,19] have suggested that the binding of six molecules of myristate causes conformational changes in the crystal structure of HSA.

In Figs. 3(e) and 4(e), each low-field signal shows a small downfield shift and an increase in its line width. This observation indicates that though oleate binds to six different sites, it does not compete with TFZ at the site(s) that induced the downfield shift to bound TFZ. These results do not suggest a specific binding for the low-field signals.

In our previous second derivative spectrophotometric study attempting to determine the binding constant of TFZ to BSA [20], a Scatchard analysis showed a nonspecific binding mode for TFZ binding to BSA. As for BSA, as shown in Fig. 1(b) and Table 1, the intensity of the high-field signal corresponding to TFZ bound to Site II is one-third that of the low-field signal; therefore in the Scatchard analysis the low field-signal might primarily be reflected. Along with the results of the above oleate binding experiments, it can be suggested that the low-field signal is a weight-averaged signal of nonspecifically bound TFZ to BSA or HSA, and free TFZ in the water phase.

In conclusion, the results of the present work suggest that TFZ binds to Site II and exhibits nonspecific binding on BSA and HSA. In addition, the affinity of TFZ for Site II on HSA appears to be much higher than that on BSA. In the presence of physiological concentrations of NaCl, major binding of TFZ to HSA and BSA is nonspecific. Our results also confirm that $^{19}F$ NMR is very useful for investigating the interactions between serum albumin and drugs containing $^{19}F$ nuclei.

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