Hydrogen–Deuterium (H–D) Exchange Reaction of Warfarin in D_2O Solution

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To prove the presence of a hydrogen–deuterium (H–D) exchange reaction, ^{1}H- and ^{13}C-NMR spectra of warfarin were measured in solvents containing D_2O and H_2O. In D_2O or D_2O/dimethyl sulfoxide (DMSO)-d_6 solvent, signal pattern changes were observed on H12 and H11 as well as 14 methyl protons over time while no changes were observed on H_2O or H_2O/DMSO-d_6 solvent. The observed changes in the solvents containing D_2O were concluded to be caused by the H–D exchange reaction on H12, the process of $CH_2 \rightarrow CHD \rightarrow CD_2$. MS spectroscopy also confirmed these H–D exchanges. The kinetics of this reaction were analyzed as the successive reaction, and the mechanism was also proposed.

Key words warfarin; hydrogen–deuterium exchange reaction; ^{1}H-NMR; ^{13}C-NMR; successive reaction

Warfarin (**1**) is a common coumarin-type anticoagulant used widely in hematological diseases, and is well known to be highly bound in circulation, with most of this binding taking place with the protein human serum albumin (HSA).[1)] In solution, **1** is present as the equilibrium between an open chain type and a cyclic hemiketal type, as shown in Chart 1.[2)] Since the position of this equilibrium depends on the solvent polarity, hydrogen donating/accepting ability, and pH,[3,4)] the NMR spectrum pattern varies with solution conditions. For example, **1** exists as a cyclic hemiketal form in dimethyl sulfoxide (DMSO)-d_6 solution[3)] and, moreover, **1** preferably adopts the cyclic hemiketal type rather than the open chain type in low dielectric constant solvents.[5)] However, in general, lower pH shifts the equilibrium to the cyclic type, and the open chain type exists at neutral and higher pH, and this nature refers to not only H_2O solution but D_2O and mixture of H_2O or D_2O and DMSO-d_6 solution.[6)]

Although, NMR measurement of this drug often uses D_2O as a solvent for studies related to biological fluids, some hydrogen–deuterium (H–D) exchange reactions have often been overlooked. Recently, a study of the stability of **1** was reported in which the ^{1}H-NMR spectrum changes on H12 in deuterated phosphate buffer were discussed as resulting from molecular decomposition.[7)] This conclusion has been a question for us and we have regarded these spectrum changes to be an exchange with deuterium.

In this work, ^{1}H- and ^{13}C-NMR of **1** were measured in D_2O, H_2O, D_2O/DMSO-d_6, and H_2O/DMSO-d_6 over many days, the presence of the H–D exchange was proved, and a mechanism of this reaction was further discussed.

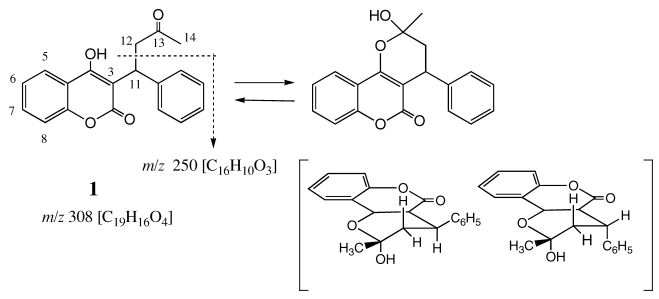

Chart 1. Equilibrium of **1** in Solution

Two kinds of diastereomers exist for a cyclic hemiketal due to two chiral centers.

Experimental

Racemic warfarin (**1**) was used for all experiments reported here.

Both the deuterated phosphate buffer (D_2O) and phosphate buffer (H_2O) used here were prepared at pH (pD) 7.4. For the NMR measurements, five types of **1** samples were prepared: each 0.6 mg of **1** in deuterated phosphate buffer 0.8 ml and phosphate buffer (H_2O) 0.8 ml (Samples 1 and 2), 2.9 mg of **1** in a mixture of deuterated phosphate buffer and DMSO-d_6(1/1) 0.8 ml (Sample 3), 3.4 mg of **1** in a mixture of phosphate buffer (H_2O) and DMSO-d_6(1/1) 0.8 ml (Sample 4), and 4.7 mg of **1** in a mixture of deuterated phosphate buffer and DMSO-d_6(3/5) 0.8 ml (Sample 5). The concentrations of the samples were 2.4 mmol/l (Samples 1 and 2), 11.8 mmol/l (Sample 3), 13.8 mmol/l (Sample 4), and 19.1 mmol (Sample 5). DMSO-d_6 was added to the phosphate buffer to increase the solubility of **1**.

^{1}H- and ^{13}C-NMR spectra were measured with a Varian Unity-INOVA 500 and/or VNMRS 500 (^{1}H: 499.8 MHz, ^{13}C: 125 MHz) at room temperature. The large H_2O or HOD signal was suppressed by presaturation or with WET sequence.[8)] ^{1}H spectra were measured 16 times and 10 times for Samples 1 and 2, respectively, during 0—125 d, and 26 times for Samples 3 and 4 during 0—192 d after sample preparation. For Sample 5, ^{1}H spectra were measured 20 times and ^{13}C spectra were measured 12 times during 0—106 d.

All MS data were measured in the positive ion mode with an Applied Biosystems API3000 LC-MS/MS system equipped with an electronspray ionization (ESI) interface.

All samples were stored at room temperature during the measurement period.

Results and Discussion

H–D Exchange Reaction of Warfarin The ^{1}H spectrum for Sample 1 immediately after sample preparation is shown in Fig. 1, which indicated that an open chain type mainly exists in this solution. Other samples (Samples 2—5) also show the similar spectrum pattern of an open chain type, although the signal patterns were ABX for H12 and H11 on Samples 3, 4 and 5 of the solvents mixed with DMSO-d_6. The series of ^{1}H spectra over times are shown in Fig. 2 for Samples 1—4, in which spectrum patterns changes were observed in Figs. 2A and C. On Fig. 2A, for Sample 1, the doublet signal of H12 rapidly diminished and, after 3 d, a new doublet and slightly broaden signal appeared at a higher field. And this doublet signal increased gradually for about 11 d and then decreased slowly to a trace. The total integral scale of these two peaks decayed rapidly from two to one portion and diminished gradually, approaching zero. Attending the change

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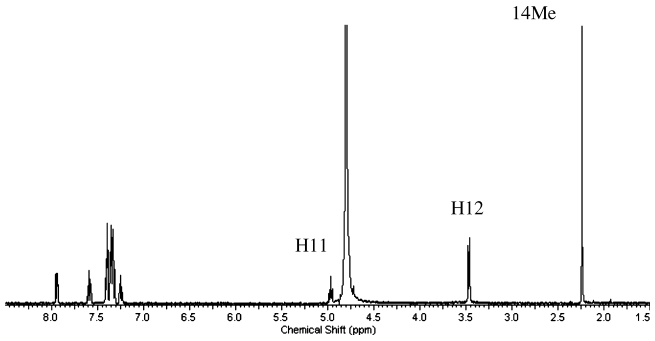

Fig. 1. ^{1}H-NMR Spectrum for Sample 1 Immediately after Sample Preparation

of the H12 signal, the triplet signal of H11 became a doublet and the overlapped pattern of the doublet with the singlet became a singlet. The similar changes were observed on Sample 3 of the DMSO-d_6/D_2O solvent mixture (Fig. 2C).

In contrast, for Sample 2 of H_2O and Sample 4 of the DMSO-d_6/H_2O solvent mixture, no changes in the spectrum patterns of H12 and H11 were observed, as shown in Figs. 2B and D. No change in the solvent containing H_2O implied that the changes of H12 of Sample 1 and Sample 3 were due to the H–D exchange reaction, that is, the process of $CH_2 \rightarrow CHD \rightarrow CD_2$ on H12. The new doublet signal of H12 can be regarded as CHD signal and its broadening may be due to the quadrupole effect of deuterium.

Figure 3 shows the ^{13}C spectrum of Sample 5 immediately after preparation and changes in the spectrum over time. The ^{1}H spectrum changes of this sample were similar to those of

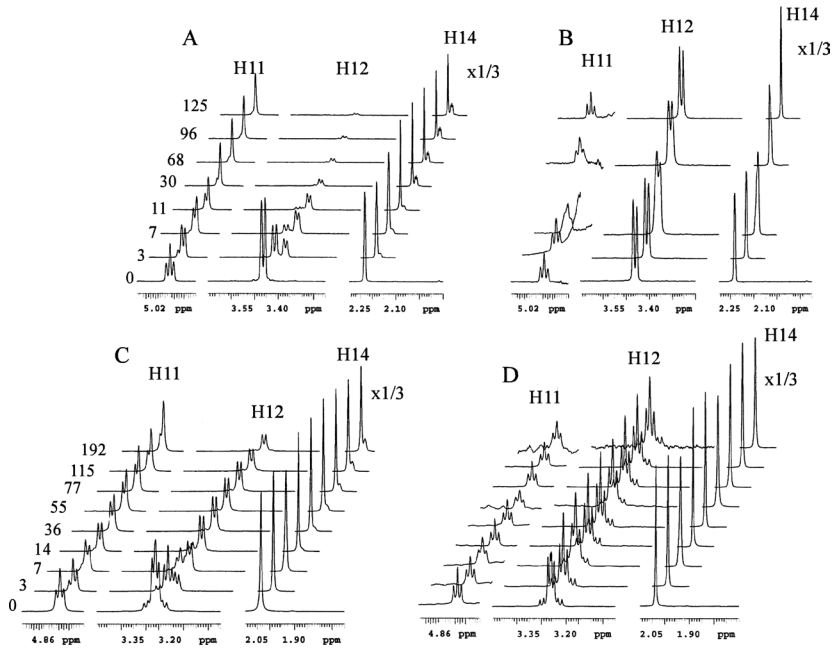

Fig. 2. Changes in ^{1}H Spectrum Pattern of H12, H11 and 14 Methyl of Samples 1 (A), 2 (B), 3 (C) and 4(D) over Time

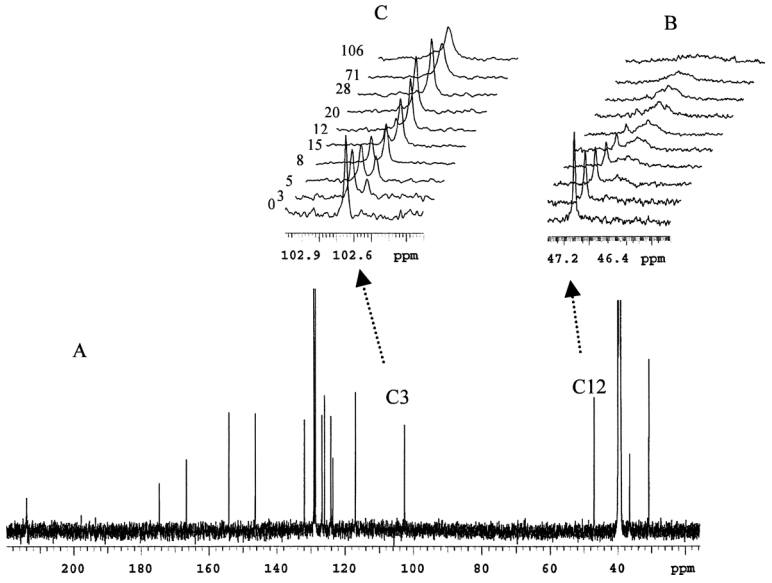

Fig. 3. ^{13}C-NMR Spectrum for Sample 5 Immediately after Sample Preparation (A) and Spectrum Changes of C12 (B) and C3 (C) over Time

Sample 3 although the rate of change was slower because of the decreasing D_2O portion. Figure 3B shows the change of the C12 signal, the sharp signal of C12 of 47.04 ppm becoming smaller, and the appearance of a new broader signal at a higher field. These observations agree with a decrease in CH_2 and the appearance of CHD and CD_2. The higher field shift of the new signal may be due to the heavy atom effect of deuterium[9—11] and the quadrupole effect to broaden the signal.

The multiple editing heteronuclear single-quantum correlation (HSQC) spectra were also measured several times during 0—27 d after sample preparation of Sample 5. As shown in Fig. 4, these spectra visually showed the changing of $CH_2 \rightarrow CHD$ on H/C12. At first, the down peak indicating CH_2 was observed for H/C12 followed by the appearance of small up peak close to the down peak, implying the presence of CH on H/C12. The up peak at H/C12 became larger and the down peak gradually decreased. At 27th day, the down peak replaced the up one for H/C12, completely.

In the ^{13}C spectrum, although several signal broadenings were observed, the most interesting observation was the changes of the C3 signal, as shown in Fig. 3C, which showed similar changes to those of the C12 signal. The original peak gradually shortened and a new broad peak appeared shifted to a higher field, which seemed to be due to the heavy atom effect of CHD or CD_2 on C12.[9—11]

Figures 2A and C show changes in the signal pattern on 14 methyl protons, in which the new higher shifted signals seem to be triplet. These changes were also due to the H–D exchange reaction of CH_3, $CH_3 \rightarrow CH_2D \rightarrow CHD_2 \rightarrow CD_3$ and signal splittings to triplet were due to a coupling with deuterium. However, the rate of this reaction might be slower than that on H12.

These H–D exchange reactions were confirmed by MS spectrometry. The Sample 2 at 125th day after preparation gave *m/z* 309 $[M(C_{19}H_{16}O_4)+H]^+$ and product ion *m/z* 251 $[C_{16}H_{10}O_3+H]^+$ on positive mode MS or MS/MS spectra, which were similar to these of standard sample. On the other hand, *m/z* 311 $[C_{19}H_{14}D_2O_4+H]^+$ and 312 $[C_{19}H_{13}D_3O_4+H]^+$, and product ion *m/z* 252 $[C_{16}H_9DO_3+H]^+$ were observed on Sample 1 at 125th day after preparation. These observations suggest that two or three of hydrogens were displaced by deuterium for the whole molecule, in which one or two of hydrogens were H12 and/or 14-methyle (see Chart 1).

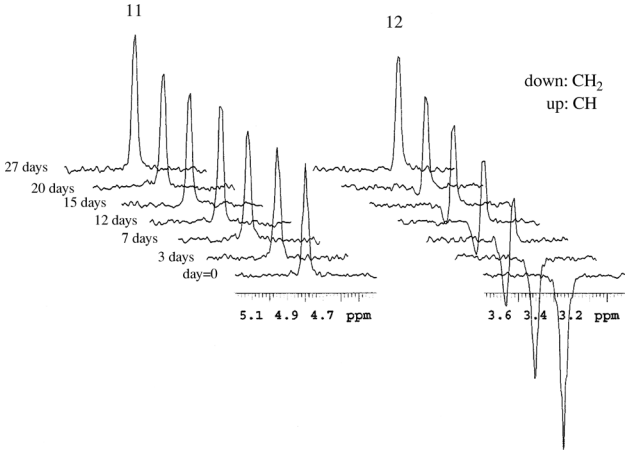

Fig. 4. Spectrum Pattern Changes on a Part of the Multiple Editing HSQC Spectra for Sample 4 over Time

1D projections to $F_2(^1H)$ directions. Down peak depicts CH_2 and up depicts CH.

Mechanism and Kinetics of H–D Exchange Reaction Figure 5 shows plots of the total integrated peak area of the H12 signals normalized to the number of proton *vs.* the passage of time for Sample 3. The rapid reductions from 2 to 1 proton portions were observed, followed by reductions slowly approaching zero. Similar plots were drawn for Samples 1 and 5, although the rates of these changes were slightly different.

The mechanism for this H–D exchange reaction can be proposed as shown in Chart 2. Considering this mechanism, the changes in H12 of Sample 3 over time are regarded as the successive reaction of $CH_2 \rightarrow CHD \rightarrow CD_2$. The exchanges of CH_2 and CHD are represented as Eqs. 1 and 2,

$$-d[CH_2]/dt = k_1[CH_2] \quad (1)$$

$$d[CHD]/dt = k_1[CH_2] - k_2[CHD] \quad (2)$$

where k_1 and k_2 are kinetic constants for $CH_2 \rightarrow CHD$ and $CHD \rightarrow CD_2$, respectively, and $[CH_2]$ and [CHD] are the amount of CH_2 and CHD, respectively. Equation 1 leads to Eq. 3 and combination of Eqs. 2 and 3 leads to Eq. 4.

$$[CH_2] = [CH_2]_0 \exp(-k_1 t) \quad (3)$$

$$[CHD] = \{k_1/(k_2-k_1)\}[CH_2]_0\{\exp(-k_1 t) - \exp(-k_2 t)\} \quad (4)$$

Since, the total integrated peak area of the observed H12 signal of Sample 3 should be $[CH_2]+1/2[CHD]$ and $[CH_2]_0 = 2$, the observed plots on Fig. 5 should decay according to Eq. 5.

$$2\exp(-k_1 t) + \{k_1/(k_2-k_1)\}\{\exp(-k_1 t) - \exp(-k_2 t)\} \quad (5)$$

The observed plots on Fig. 5 were analyzed with a nonlinear

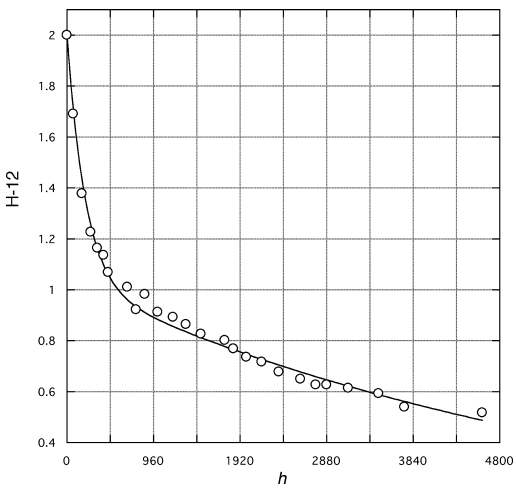

Fig. 5. Plots of the Total Integrated Peak Area of H12 Peaks *vs.* Time for Sample 3

Solid line represents the kinetics of Eq. 5 in which $k_1 = 4.9\times10^{-3}\,h^{-1}$ and $k_2 = 1.6\times10^{-4}\,h^{-1}$ with $R^2 = 0.993$.

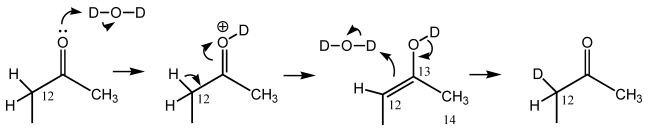

Chart 2. Proposed Mechanism for H–D Exchange Reaction on C12 of **1**

least squares fit to Eq. 5, resulting $4.9\times10^{-3}\,h^{-1}$ for k_1 and $1.6\times10^{-4}\,h^{-1}$ for k_2. Correlation coefficient of 0.993 was obtained, giving a good fit.

If, in the mechanism shown in Chart 2, the double bond on the transition state forms on C13–C14 and not on C12–C13, the 14 methyl protons would be deuterated, as can be observed on Samples 1 and 3.

Conclusion

^{1}H- and ^{13}C-NMR spectra of warfarin were measured in solvents containing D_2O and H_2O. Mostly, the H12 signal pattern changed in D_2O, but no changes could be observed on the H_2O samples. The observed changes on H12 were concluded to be due to the H–D exchange reaction, the process of $CH_2\rightarrow CHD\rightarrow CD_2$. MS spectroscopy also confirmed these H–D exchanges.

The decrease of the total H12 signals over time was analyzed as the successive reaction, as shown in Fig. 5. This analysis supports the proposed mechanism shown in Chart 2.

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