Equilibrium Between α- and β-Isomers of Dihydroartemisinine and its Multiple-Peaks in High-Performance Liquid Chromatography

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Key Words
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Summary
The interconversion between the α- and β-isomers and the multiple-peaks of dihydroartemisinine (dihydroqinghaosu) were investigated in reversed-phase, high-performance liquid chromatography. A convenient method using the Gaussian distribution function instead of the Schmidt graphical method for the theoretical calculation of the "diffuse" probability curves versus the fraction of the dead time on column as β form was introduced. Based on the theoretical probability curves and the experimental multiple-peak chromatograms, the effects of temperature and flow velocity on the accuracy and precision of analysis of dihydroartemisinine are indicated and the activation energy of the interconversion between α- and β-isomers of dihydroartemisinine is estimated.

Introduction
Many authors paid attention to multi-zoning observations in thin-layer and paper chromatographies and in electrophoresis. Keller and Giddings [1] summarized the explanations of these observations and developed some new ones. Equilibrium between two species of a solute is one of the explanations. This case can be often encountered in organic chemistry, for instance the equilibrium between cis- and trans-isomers of a compound. It may be predicted that multiple-peaks will appear in column chromatography when the half time of the establishment of the equilibrium is of the order of the retention time.

Multiple-peaks may lead to fault in identification and quantitation of chromatographic peaks because of possible new peaks and peak tailing.

Dihydroartemisinine (dihydroqinghaosu) is an effective antimalaria compound [2]. It exists as two isomers, α and β (Scheme I). Severe peak tailing and lack in analytical precision were found in its column chromatographic experiments. The present work attempts to investigate the multiple-peaks of dihydroartemisinine in reversed-phase, high-performance liquid chromatography and to calculate the theoretical graphs of the multiple-peaks by combining the probability functions introduced by Keller and Giddings [1] with the Gaussian distribution function.

Experimental
Apparatus and Chemicals: The HPLC system consisted of a LC-4A chromatograph, a SPD-2AS UV-detector, a column oven, a 4mm i.d. x 25cm stainless steel column packed with LiChrosorb-RP18 of E. Merck (West Germany) and a CR-2AX Chromatopac microprocessor, all manufactured by Shimadzu (Japan).

The dihydroartemisinine standard was purified by recrystallisation in acetone. Its purity was verified by mass-spectroscopy. The other chemicals were of analytical-reagent grade.

Chromatographic Conditions: Mobile phase, water ethanol = 55:45; detection wavelength, 210nm; temperature, the temperature of the column oven was controlled by electric heating or cooling with ice placed in its column chamber; detector sensitivity (AUFS), 0.32.
Theoretical Calculation of the Multiple-peak Curves

If a solute can exist in two different interconvertible isomers, $\alpha$ and $\beta$, the scheme below may represent the equilibriums which it undergoes during chromatography:

$$\begin{align*}
\alpha &\xrightarrow{K_1} \beta & \text{mobile phase} \\
\alpha' &\xrightarrow{K_2'} \beta' & \text{stationary phase}
\end{align*}$$

where $k_1$ and $k_2'$ are rate constants. We suppose here that they are rate constants of the first order and that $k$ is equal to $k'$ either for $k_1$ or $k_2$.

To calculate the concentration distribution of the solute after travelling through a separation column in terms of retention time, $T_0$, one needs merely to know the probability function, $P(x)$, in terms of the fraction, $x$, of the dead time on column which a solute molecule spent in $\beta$-form because $P(x)$, $x$-coordinate can be converted into $C(T_0)$, $T_0$-coordinate by the relations (1) and (1a) where $C(T_0)$ is the amount of the solute eluted at time $T_0$:

$$C(T_0) = C_s \cdot P(x) \quad (1)$$

where $C_s$ is the amount of the solute injected onto column.

$$T_0 = L/[V_\alpha (1-x) + V_\beta \cdot x] \quad (1a)$$

$x = T_0/(T_0 + T_\beta) \quad (2)$

where $L$ is the length of the column; $V_\alpha$ and $V_\beta$ are the velocities of $\alpha$ and $\beta$, respectively; $T_\alpha$ and $T_\beta$ are the times a molecule spent in $\alpha$- and $\beta$-forms in mobile phase on the column, respectively. The value of $T_\alpha + T_\beta$ is constant on a column when flow rate is constant.

Because the partition ratio between the two phases for both $\alpha$- and $\beta$-isomers is constant the distribution curve in the stationary phase should be the same as that in mobile phase. Therefore, we may consider here only the latter.

Keller and Giddings [1] introduced the probability functions in terms of $x$. In the probability functions several parameters, $a$, $b$, $\alpha$ and $\beta$, are involved where $a$ and $b$ are $k_1(T_\alpha + T_\beta)$ and $k_2(T_\alpha + T_\beta)$, respectively; $\alpha$ and $\beta$ are the initial fractions of the solute as $\alpha$- and $\beta$-isomers, respectively, in the sample solution injected onto the column. In the following, $f_1$ and $f_2$ will be used instead of $\alpha$ and $\beta$ to avoid confusion with the isomer symbols.

To get a probability curve versus $x$, we may calculate the probabilities at a sufficiently large number of points over the $x$ range each of which is to represent the mean probability in the interval $x \pm \Delta x$ and then draw a line graph of $P(x)$.

Giddings' probability functions mentioned above have not considered the actual peak broadening due to such as distribution kinetics between the two phases, molecular diffusion and eddy diffusion. Giddings and co-worker [1] resolved the problem by using the Schmidt graphical method [3] and obtained the "diffuse" probability curves by adding the "diffuse" $P(x)$ profile to the Gaussian curves representing the end zones.

The author used the Gaussian distribution function for the calculation of the "diffuse" probability curves versus $x$. This calculation method is much more simple and convenient than using the Schmidt graphical method. Supposing that the fraction of the solute distributed at point $x$ is one of the "components" of the solute and is distributed in the Gaussian curve around $x$, then the "diffuse" probability, $P(x)_{\text{diff}}$, at the point $x$ is the sum of those of all the "components" over the range from $x = 0$ to $x = 1$. It can be calculated by the equation

$$P(x)_{\text{diff}} = \sum_{n=1}^{N} \frac{1}{\sqrt{2\pi} \cdot \sigma} \cdot e^{-\frac{1}{2} \left( \frac{x-x_n}{\sigma} \right)^2} \cdot P(x_n) \quad (3)$$

where $P(x_n)$ are the probabilities given by Giddings probability functions over the whole range from $x = 0$ to $x = 1$.

Before the calculation of the probability curves and the "diffuse" probability curves, the parameters $a$, $b$, $f_1$, $f_2$ and $\sigma$ as well as the number of the intervals in the $x$ range involved in Giddings probability functions and in equation (3) were to be ascertained.

$f_1$ and $f_2$ can be determined according to the peak areas of the $\alpha$- and $\beta$-isomers under such conditions that both $k_1$ and $k_2$ are near zero. Figure 2A, with no peak tailing, should meet that case. Therefore, 0.75 and 0.25 were selected to be $f_1$ and $f_2$ according to the areas of the two isomer peaks in this figure, respectively.

According to chemical kinetics, there exists the relations among $a$, $b$, $f_1$ and $f_2$:

$$a = \frac{k_1}{k_2} \quad (4)$$

$a$ and $b$ depend on reaction temperature, but the ratio, $a/b$, is independent of temperature. To gain the ratio, $a/b$, in the mobile phase used, $f_2/f_1$ in the mobile phase was measured by means of injecting sample solution prepared with the mobile phase as solvent and performing the separation of $\alpha$- and $\beta$-isomers at very low temperature ($15^\circ C$), obtaining a chromatogram without peak tailing as Fig. 2A. The ratio, $a/b$, in the mobile phase was about 1/3 like that in ethanolic solution. In the selections of every set of $a$ and $b$, this ratio was kept.

The standard deviation $\sigma$ is a combined factor of various peak broadening effects and may be estimated by the basic equations connected with peak broadening in HPLC [4]. In consideration of the HPLC system used, 0.08 was selected to be $\sigma$.

The range in $x$ was divided into 100 equal intervals. "Diffuse" probabilities were computed from the point $x = -0.2$ to the point $x = 1.2$.

Inserting all the parameters selected above into eq. (3) yields

$$P(x)_{\text{diff}} = \sum_{n=0}^{100} \frac{1}{\sqrt{2\pi} \cdot 0.08} \cdot e^{-\frac{1}{2} \left( \frac{x-x_n-0.01}{0.08} \right)^2} \cdot P(x_n) \quad (5)$$

$\sigma$ changes with retention time. Because $\Delta \sigma T_r = \Delta T_r / N^{1/2}$ [4] we have $\Delta \sigma_x = \Delta x / N^{1/2}$ where $\Delta \sigma_x$ and $\Delta x$ are increases in retention time and in $x$, respectively; $N$ is the