Kinetic Study on the Mechanism of Tissue Distribution of Vinblastine

Konstanty WIERZBA, Yuichi SUGIYAMA, Tatsuji IGA*, and Manabu HANANO
Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113, Japan
(Received March 8, 1988)

The purpose of present study was to analyze the factors involved in the tissue distribution of vinblastine (VBL). The specific binding of VBL to mouse tissue cytosol determined by a charcoal method correlated well with the tissue concentration of tubulin, a target protein for the pharmacological activity of Vinca alkaloids. The calculated tissue-to-plasma partition coefficients ($K_p$) in various tissues, based on the VBL binding to 100000 × $g$ cytosols showed a good correlation with the corresponding in vivo $K_p$ values of rats reported in the literature, however, the calculated $K_p$ values were greatly underestimated. The total binding (including specific and non-specific bindings) to cytosols from the liver and kidney, determined with the ultrafiltration method, were approximately 5 times higher than those determined with the charcoal method for both tissues. However, the total bindings to cytosol cannot explain the high $K_p$ values in vivo. Considering the intracellular distribution of VBL, it was found that cytosol is not the main binding component for VBL since approximately one-half of the VBL in the liver homogenate was associated with the nuclear fraction. The $K_p$ value in the liver, calculated by considering the intracellular distribution, became close to the in vivo $K_p$ value. It was concluded that the tissue distribution of VBL cannot be accounted for only by the binding to tubulin, and that the bindings to other intracellular components should also be included.

Keywords — vinblastine; tissue distribution; pharmacokinetics; microtubule; tissue tubulin; Vinca drug

Introduction

Vinblastine (VBL) is an antineoplastic Vinca alkaloid compound with a wide range of activity. The antitumor activity of Vinca alkaloids has been related to their binding to spindle proteins resulting in the inhibition of formation of the mitotic spindle and the subsequent process of mitosis.\textsuperscript{1–4} It has been proposed that the Vinca alkaloids cause their cytotoxic effect by interaction with tubulin, the soluble 110000-dalton protein component of microtubules. The in vitro assembly of soluble tubulin into distinctive microtubular forms is prevented by the Vinca alkaloids at the concentrations observed after routine doses in humans.\textsuperscript{5}

VBL binds to two or more classes of binding sites on tubulin with different affinities.\textsuperscript{6} It is suspected that the high affinity site binds VBL at concentrations where antimitotic effects and the disappearance of cytoplasmic microtubules are observed, whereas the low affinity site is associated with various aggregation phenomena.

The localization of Vinca drugs in several animals\textsuperscript{6–9} and human\textsuperscript{10} tissues has been reported.

* To whom all correspondence should be addressed.
e.g. tubulin if one assumes it to be a main binding protein. It was shown that the tubulin concentration differs among tissues. Based on these data, our preliminary estimation indicated the probability of a good correlation between \( K_{\text{p,app}} \) values for the Vinca alkaloids and the tubulin concentrations in corresponding tissues.

Using the unique property of colchicine (CLC) to bind tubulin, we determined the tissue concentration of tubulin in murine organs. The comparison of binding capacities (reflecting tubulin concentration) of the different tissues for CLC and the \( K_{\text{p,app}} \) of VCR obtained from several animal species, revealed a good correlation of these two factors, suggesting that the tubulin may be a major determinant of the tissue distribution of VCR and other Vinca drugs. Therefore, one may suspect that the tubulin is not only a target protein for the pharmacological activity of Vinca drugs but also is responsible for their tissue retention.

To study this hypothesis VBL was chosen to determine its binding properties to the 100000 × \( g \) cytosols (a main source of tubulin) prepared from several mouse tissues. Based on its binding parameters, a trial to reconstruct its tissue-to-plasma partition coefficients was undertaken.

**Materials and Methods**

**Materials** — [\( ^{3}H \)]VBL (11.5 Ci/mmol) was purchased from Amersham International (Amersham, U.K.) and [\( ^{3}H \)]CLC (32.5 Ci/mmol) from New England Nuclear Co., (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma Co., (St. Louis, MO, U.S.A.).

**Animals** — Male ddY mice weighing 22—25 g (Nippon Igaku Dobutsu Co., Ltd., Tokyo, Japan) were used to prepare tissue homogenates and the corresponding 100000 × \( g \) cytosols.

**VBL Binding** — Preparation of 100000 × \( g \) cytosols was previously described. VBL binding was determined by the activated charcoal method on the day of preparation. A tracer amount of [\( ^{3}H \)]VBL was added to an aliquot of 100000 × \( g \) cytosol to a final concentration of radioactivity of 0.2 \( \mu \)Ci/50 \( \mu l \) of cytosol. Then, an aqueous solution of unlabeled VBL (10 \( \mu l \)) was added to give final drug concentration, ranging from \( 10^{-7} \) to \( 10^{-4} \) M. The samples were incubated for 30 min at 37°C to reach an equilibrium. Then, to separate the unbound VBL, 400 \( \mu l \) of 0.5% suspension of activated charcoal in distilled water were added. One minute after the addition of activated charcoal and vigorous mixing, samples were centrifuged in a Beckman tabletop microfuge. The concentration of the bound VBL was determined in the supernatant fluid. This method gives only the specific binding (probably binding to tubulin).

VBL binding to mouse liver and kidney 100000 × \( g \) cytosols and mouse serum protein was also determined with the ultrafiltration method. Four hundred \( \mu l \) of cytosols or mouse serum were supplemented with the tracer [\( ^{3}H \)]VBL (1.6 \( \mu \)Ci) and 80 \( \mu l \) of unlabeled VBL. After incubation for 30 min at 37°C, the samples were transferred to an Amicon ultrafiltration device (MPS-1) and were centrifuged for 90 s (2000 rpm). The unbound concentration of VBL was measured in the ultrafiltrate. Control experiments, in which only the buffer was used instead of the protein preparations, were carried out each time to determine the adsorption of VBL to the membrane filter. The fraction adsorbed depended on the concentrations of VBL, and varied from 0.12 to 0.27, at VBL concentrations of 100 and 0.1 \( \mu \)M, respectively. The adsorption to the membrane filter was then corrected for the calculation of the unbound concentration. This ultrafiltration method allows the determination of total binding, including tight and loose bindings.

**Intracellular Distribution of [\( ^{3}H \)]VBL and [\( ^{3}H \)]CLC** — Mouse livers were homogenized in the buffer used for binding experiments supplemented with 0.34 M sucrose (20% (w/v) homogenates). The homogenates (4 ml) were supplemented with 2 \( \mu \)Ci of [\( ^{3}H \)]VBL or [\( ^{3}H \)]CLC and were incubated for 1 h at 37°C. After incubation, the homogenates were subjected to differential centrifugation at 600 × \( g \) for 10 min, 10000 × \( g \) for 15 min, and at 100000 × \( g \) for 40 min, and the radioactivity
associated with the above fractions was measured and expressed as a percent of the total radioactivity in the homogenate.

Under the same condition, the stability of VBL in the homogenate was examined by high performance liquid chromatography (HPLC) analysis. After incubation, the homogenate was diluted twice with the buffer and the proteins were precipitated with an equal volume of methanol containing 0.1 N HCl. The supernatant fluid obtained by centrifugation was subjected to HPLC analysis, using 20 mM KH₂PO₄-50% MeOH as a mobile phase with MeOH gradient concentration from 0 to 80%. The column system was Milford MA, Radial-PAC Cartridge (10 cm) with Bondapak C18 (Water Associates). During a 1 h incubation period, the formation of a tracer amount of the biotransformation product, with the retention time of 7.72 min, was observed, while the parent VBL appeared with the retention time of 9.1 min. However, this product accounted for less than 5% of the total amount of VBL present.

**Determination of Radioactivity** — The radioactivity was determined in a Tri-Carb Counter (model 3255, Packard Instruments Corp., Downers Grove, IL). One hundred μl samples were added to 10 ml of liquid scintillation cocktail (0.1 g 1,4-bis-2-(5-phenoxazolyl)-benzene (P0POP), 4.0 g DPO, 500 ml of Triton X-100 and 1 liter of toluene).

**Kinetic Analysis of VBL Binding** — The binding parameters of VBL to mouse tissue 100000 × g cytosols was calculated by a nonlinear iterative least squares method. The relationship between the bound (Cₜ) and unbound (C₀) concentrations of VBL was fitted to the following Eqs. 1 and 2:

For the activated charcoal method:

\[ C_b = \frac{nP \cdot C_f}{K_d + C_f} + \alpha \cdot C_f \]  

(1)

And for the ultrafiltration method:

\[ C_b = \frac{nP_1 \cdot C_f}{K_{d,1} + C_f} + \frac{nP_2 \cdot C_f}{K_{d,2} + C_f} \]  

(2)

where \( nP \) and \( K_d \) are the binding capacity and the dissociation constant, respectively, and \( \alpha \) is the proportionality constant for the non-specific binding.

The unbound fraction \( f_u \) of VBL in mouse serum was calculated over a wide range of VBL concentrations by the following equation:

\[ f_u = \frac{C_f}{C_b + C_f} \]  

(3)

**Calculation of Tissue-to-Plasma Partition Coefficients \( (K_p) \) Based on VBL Binding to Mouse Tissue 100000 × g Cytosols** — In the estimation of the \( K_p \) value of VBL from the results of in vitro experiments, the following assumptions were made: 1) the cytosol is a main binder of VBL, 2) only the un-ionized form which is unbound in plasma can diffuse through the plasma membranes.

From the aforementioned assumptions regarding the distribution of VBL in the organs, the following equations were derived to estimate the \( K_p \) values, when only the binding to tubulin was considered:

\[ K_p = q \cdot f_u \cdot (1 + nP/K_d + \alpha) \]  

(4)

and when all the binding to cytosol components was considered

\[ K_p = q \cdot f_u \cdot (1 + nP_1/K_{d,1} + nP_2/K_{d,2}) \]  

(5)

where

\[ q = \frac{1 + 10^{pK_a - pHi}}{1 + 10^{pK_a - pHe}} \]  

(6)

\( q \) is the ratio of the unbound drug in the cell to that in the plasma, which was calculated to be 1.43, based on the pH partition hypothesis (\( pK_a = 7.0 \), intracellular pH\( i = 7.0 \), extracellular pH\( e = 7.4 \)).

**Calculation of the \( K_p \) Value in the Liver Based on the Participation of Other Subcellular Components in VBL Binding** — Considering 20% homogenate, and the linear binding, the following equations hold:

\[ A_{b,1} = 0.2 \times aP_1 \times C_f \]  

(7)
\[ A_{b,2} = 0.2 \times aP_2 \times C_f \] (8)
\[ A_{b,3} = 0.2 \times aP_3 \times C_f \] (9)
\[ A_{b,4} = 0.2 \times aP_4 \times C_f \] (10)

where \( A_{b,i} \) is the amount of VBL bound to the \( i \)-th component (1: 600 \( \times \) g pellets, 2: 10000 \( \times \) g pellets, 3: 100000 \( \times \) g pellets, 4: 100000 \( \times \) g cytosol), \( aP_i \) is the binding parameter (corresponding to undiluted homogenate) and \( C_f \) is the unbound concentration of VBL. The parameters, \( aP_i \), have a dimension of ml/g liver, and if we assume the cytosol volume (\( V \)) of 1 ml/g liver, this parameter can be considered dimensionless.

In case of the liver cytosol (if one considers the linear binding to the two binding sites):

\[ aP_4 = \frac{nP_1}{K_{d,1}} + \frac{nP_2}{K_{d,2}} \] (11)

where \( nP_i \) and \( K_{d,i} \) represent the binding capacity and the dissociation constant, respectively, for the liver cytosol which are determined by the ultrafiltration method. Furthermore, from Eqs. 7–11

\[ \frac{A_{b,i}}{A_{t,\text{cyt}}} = \frac{A_{b,i}}{A_{t,\text{cyt}}} \]

\[ = \frac{0.2 \times aP_i \times C_f}{0.2 \times aP_i \times C_f + V \times C_f} \]

\[ = \frac{0.2 \times aP_i}{0.2 \times aP_i + V} \] (12)

where \( V \) is the volume (ml) of the liver cytosol and is kept constant (1 ml), \( A_{t,\text{cyt}} \) is the total amount of the drug present in the liver cytosol. The \( A_{b,i}/A_{t,\text{cyt}} \) ratio is determined experimentally from the intracellular distribution of VBL. Therefore, \( aP_i \) can be calculated from this ratio according to Eq. 12. Substituting Eq. 11 into 12 yields

\[ \frac{A_{b,i}}{A_{t,\text{cyt}}} = \frac{0.2 \times aP_i}{0.2 \times (nP_1/K_{d,1} + nP_2/K_{d,2}) + 1} \] (13)

Considering the case for 100% homogenate, the ratio of the amount of VBL bound to one gram liver (\( A_{b,T} \)) to the unbound concentration in the cytosol (\( C_f \)) can be expressed by

\[ \frac{A_{b,T}}{C_f} = \frac{(aP_1 + aP_2 + aP_3 + aP_4) \times C_f}{C_f} \]

\[ = aP_1 + aP_2 + aP_3 + aP_4 \] (14)

Assuming the \( V \) value of 1 ml/g liver, the \( K_p \) value (\( K_{p,T} \)) can be given by

\[ K_{p,T} = \frac{C_f + C_{b,T}}{C_{f,p} + C_{b,p}} = \frac{C_f + A_{b,T}}{C_{f,p} + C_{b,p}} \] (15)

where \( C_{f,p} \) and \( C_{b,p} \) are the unbound and bound concentrations in plasma, respectively, and \( C_{b,T} \) is the concentration in the liver. Rearranging Eq. 15 yields

\[ K_{p,T} = \frac{C_f}{C_{f,p}} \times \frac{C_{f,p}}{C_{f,p} + C_{b,p}} \times (1 + \frac{A_{b,T}}{C_f}) \] (16)

In the absence of active transport, \( C_{f} \) \( / \) \( C_{f,p} \) is equal to the pH partition coefficient (\( q \)) for VBL, and \( C_{f,p} / (C_{f,p} + C_{b,p}) \) is equal to the \( f_u \) of VBL in plasma.

Substituting Eq. 14 into 16 and rearranging gives

\[ K_{p,T} = q \times f_u \times (1 + aP_1 + aP_2 + aP_3 + aP_4) \] (17)

The liver-to-plasma partition coefficient for VBL was calculated by Eq. 17 using the experimental data. Protein concentration was determined with a Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin was used as the standard. The apparent tissue-to-blood partition coefficients of VBL in vivo were taken from the literature\(^6\) and were transformed to the tissue-to-plasma partition coefficients (\( K_{p,\text{app}} \)) based on the blood-to-plasma concentration ratio (\( R_B = 2.62 \)).\(^7\)

**Results**

Determination of VBL Binding with Activated
Tissue Distribution of Vinblastine

Fig. 1. Reversibility of VBL Binding to 100000 × g Mouse Liver Cytosols

The cytosols were incubated for 30 min at 37°C with a tracer amount of VBL. Incubation was terminated by the addition of activated charcoal suspension followed by incubation at room temperature (●) or on an ice-bath (○). At indicated time intervals, the samples were centrifuged to separate the activated charcoal.

Charcoal Method

The absorption to activated charcoal changed the equilibrium between the unbound and bound fraction of VBL in a time dependent manner as shown in Fig. 1. However, lowering the temperature little affected the dissociation rate of VBL from the binding site on the liver cytosol. Therefore, to determine the bound fraction of VBL, it was necessary to carefully adhere to a procedure in terms of the time and concentration of activated charcoal required to separate the unbound and bound VBL. The optimal conditions were found to be 0.50% suspension of activated charcoal and 1 min absorption time. This modification was used in all our experimental procedures to determine the VBL binding.

VBL Binding to 100000 × g Mouse Tissue Cytosols

The VBL binding to 100000 × g mouse tissue cytosols measured with the activated charcoal method (presumably, the specific binding to tubulin) showed the presence of a single class of the binding site and the non-specific binding, as presented in Fig. 2.

Based on the relationship between the bound ($C_b$) and unbound ($C_p$) VBL concentrations, the basic parameters of the binding kinetics were calculated (Table I). There was a 6-fold difference in the binding affinity of VBL to the tissue cytosols. VBL showed the highest affinity to 100000 × g cytosols prepared from the spleen and liver ($K_d = 0.74$) and 1.13 μM, respectively). The lowest affinity was observed in the case of the brain ($K_d = 4.49$ μM), whereas

Fig. 2. Scatchard Plot of VBL Binding to 100000 × g Mouse Tissue Supernatant Fluids

The binding was determined with the activated charcoal method. (a): (●) brain, (□) liver, (○) lung, and (△) spleen. (b): (●) heart, (○) kidney, and (□) muscle.
Table I. Parameters of VBL Binding to Mouse Tissue 100000 × g Supernatant Fluids a)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dissociation constant (μM)</th>
<th>Binding capacity b) (nmol/g)</th>
<th>Non-specific binding coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>4.49 ± 0.96</td>
<td>20.42 ± 0.79</td>
<td>0.129 ± 0.034</td>
</tr>
<tr>
<td>Liver</td>
<td>1.13 ± 0.09</td>
<td>4.76 ± 0.19</td>
<td>0.151 ± 0.003</td>
</tr>
<tr>
<td>Lung</td>
<td>2.44 ± 0.29</td>
<td>4.51 ± 0.23</td>
<td>0.125 ± 0.004</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.41 ± 1.13</td>
<td>1.63 ± 0.35</td>
<td>0.047 ± 0.007</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.02 ± 0.55</td>
<td>1.59 ± 0.22</td>
<td>0.081 ± 0.003</td>
</tr>
<tr>
<td>Heart</td>
<td>2.95 ± 0.46</td>
<td>2.48 ± 0.18</td>
<td>0.029 ± 0.003</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.74 ± 0.06</td>
<td>2.11 ± 0.07</td>
<td>0.161 ± 0.002</td>
</tr>
</tbody>
</table>

a) Each value of the data is shown as the mean ± computer calculated S.D. Tissues obtained from more than 10 mice were pooled and the binding determinations were made in duplicates.  b) The values correspond to 1 g of tissue.

The affinity of VBL to other tissues were very similar (the values of $K_d$ ranged from 2 to 3 μM). The binding capacities of mouse tissue 100000 × g cytosols differed greatly among tissues examined (Table I). The highest binding capacity was observed in the brain ($nP = 20$ nmol/g), whereas the lowest one was observed in the muscle and kidney ($nP = 1.6$ nmol/g).

The binding capacities for VBL seem to be closely related to that of CLC, reflecting a specific binding to tubulin as shown in Fig. 3. Comparison of the binding capacities of mouse tissues for VBL and those for CLC revealed a good correlation ($r = 0.975$). Except for the kidney and spleen, in general, the binding capacity for VBL is larger, and the slope of the regression line (1.99) indicates that one dimer molecule of tubulin binds more than one molecule of VBL. Taking into account experimental error and the possibility of the presence of inhibitory substance(s), one may assume the stoichiometry of 2 mol of VBL per mol of tubulin.

Considering all tissues, there is a lack of cor-

Fig. 3. Correlation between Capacities of VBL and CLC Binding to Mouse Tissue Cytosols
The binding was determined with the activated charcoal method. $r = 0.975$.

Fig. 4. Correlation between the Dissociation Constants of VBL and CLC Binding to Mouse Tissue Cytosols
The binding was determined with the activated charcoal method. $r = 0.120$. 
relation between the binding affinities ($K_d$) of VBL and CLC as shown in Fig. 4. Figure 3, however, showed that the specific binding of VBL to mouse tissue cytosols is closely related to that of CLC because of the good correlation between the two binding capacities.  

**VBL Organ Distribution and Tissue Tubulin Concentrations**

Figure 5a shows the direct comparison of the apparent tissue-to-plasma partition coefficients ($K_{p,app}$) observed in vivo in some tissues and their binding capacities for VBL. A good correlation was observed when the spleen was excluded. Because the capability of the tissue to bind VBL is a function of both $nP$ and $K_d$, it is reasonable to compare $K_{p,app}$ with the tissue capability which is expressed as the ratio of $nP$ and $K_d$ ($nP/K_d$). The relationship between these two

Fig. 5. Correlation between VBL Binding Capacity ($nP$) (a) or Binding Activity ($nP/K_d$) (b) to $100000 \times g$ Supernatant Fluids from Mouse Tissues, and $K_{p,app}$ Values Obtained from Rat Tissues in Vivo

The dashed regression line represents all analyzed tissues, while the solid line represents all tissues except the spleen. The number, 170 in the parenthesis is the $K_{p,app}$ value for the spleen.

Fig. 6. Scatchard Plot of VBL Binding to Mouse Serum Proteins (a) and Unbound Fraction ($f_u$) of VBL as a Function of the Total Serum Concentration (b).

The solid line in (a) is a computer-fitted curve using observed data.
Fig. 7. Relationship between Calculated in Vitro $K_p$ Values and Those Obtained in Vivo from Rats

The in vitro $K_p$ values were calculated based on the specific binding to cytosol, according to Eq. 4. The solid line represents the regression line. $r = 0.934$, slope = 82.27.

parameters, ($K_{p,app}$ vs. $nP/K_d$) appeared to be improved when all tissues, including the spleen, were analyzed. As shown in Fig. 5b, the correlation coefficient increased from 0.165 to 0.684.

**VBL Binding to Mouse Serum Proteins**

It was necessary to determine the unbound fraction ($f_u$) of VBL in serum since only the free VBL can penetrate through the biological membranes. Kinetic analysis of VBL binding to mouse serum proteins revealed the presence of two classes of binding sites as shown in Fig. 6a. The values of $K_{d,1}$ and $nP_1$ for the high affinity site were 0.58 and 0.93 $\mu$M, respectively, whereas those of $K_{d,2}$ and $nP_2$ for the low affinity site were 61.7 and 247 $\mu$M, respectively. There was an approximate 100-fold difference in the affinity of VBL for two binding sites on serum proteins. The values of $f_u$ for VBL were almost constant over a 0.01–1.0 $\mu$M range of the VBL concentration (Fig. 6b). When the total serum concentration of VBL increased, the saturation of binding occurred leading to an increase in $f_u$.

**$K_p$ Values Estimated Based on the Specific Binding of VBL to Mouse Tissue 100000 × g Cytosols**

The $K_p$ values were calculated according to Eq. 4 taking into account the serum protein binding ($f_u$), the specific binding to tubulin ($nP$, $K_{p,app}$).
**Table II.** Subcellular Distribution of VBL and CLC in Mouse Liver \(a,b\)

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Amount of radioactivity (% of total radioactivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei (600 × g pellets)</td>
<td>53.6</td>
</tr>
<tr>
<td>Mitochondria (10000 × g pellets)</td>
<td>18.7</td>
</tr>
<tr>
<td>Microsome (100000 × g pellets)</td>
<td>5.8</td>
</tr>
<tr>
<td>Cytosol (100000 × g supernatant)</td>
<td>21.9</td>
</tr>
<tr>
<td>VBL</td>
<td>CLC</td>
</tr>
<tr>
<td>23.9</td>
<td>59.5</td>
</tr>
</tbody>
</table>

\(a\) The experiment was performed using 20% homogenates, which were subjected to differential centrifugation.

\(b\) Each value of the data is shown as the mean value of two independent experiments.

**K_d and α** and the pH partition hypothesis. The calculated \(K_p\) values and those observed *in vivo* \((K_{p\text{,app}})\) correlated well with each other \((r = 0.934)\) (Fig. 7). However, the slope of the regression lines \((slope = 82.3)\) indicated a huge underestimation of \(K_p\) values, if the specific binding to the cytosols was only the main binding component.

**Total Binding of VBL to Liver and Kidney 100000 × g Cytosol Fractions**

The good correlation between \(K_{p\text{,app}}\) and \(nP\) of VBL, and the underestimated \(K_p\) values, based on the determination of the specific binding, suggested that the 100000 × g cytosol fraction possesses another type of VBL binding which cannot be detected with the activated charcoal method. Therefore, the total binding ability of the cytosol was determined for the liver and kidney. Figure 8 shows Scatchard plots of the VBL binding to the liver and kidney cytosol fractions. In both cytosols, the presence of the two binding sites was observed. The estimated value of \(nP\) \((3.43 \text{ nmol/g})\) for the high affinity site in the liver was similar to that determined with the activated charcoal method, while the \(K_d\) value was lower than that determined with the same method. Similar results were also observed in the kidney. Based on these data, including both binding sites of the liver and kidney cytosols for VBL, the calculated binding capability \((nP_1/K_{d,1} + nP_2/K_{d,2})\) of the liver cytosol was 10.8 and that of the kidney was 3.5, and the corresponding \(K_p\) values were 2.6 and 0.9, respectively. Thus, the \(K_p\) values, determined by the ultrafiltration method, were higher than those determined by the activated charcoal method (Fig. 7). However, the calculated values of \(K_p\) for the liver and kidney were still greatly underestimated.

**Subcellular Distribution of VBL and CLC**

Subcellular distributions of VBL and CLC are presented in Table II. A large difference in the distribution of these two compounds was observed. Most VBL was localized in the nuclear fraction \((ca. 50\% of the total amount)\), while the cytosol contained approximately 20%. The distribution of both VBL and CLC in the microsomal fraction was small \((ca. 6\% and 2\%)\). The distribution of VBL in the mitochondrial fraction was approximately 3 times higher than that in the microsome. The localization of VBL in the cytosolic fraction was 3 times lower than that of CLC. The extensive accumulation of CLC in the cytosol was in agreement with subcellular distribution of tubulin. Both compounds exhibited an affinity for the nuclear fraction, and in case of VBL this was the main site of its accumulation in the liver cell. Subcellular distribution of VBL appeared to be a crucial point in the calculation of the \(K_p\) of VBL since it is necessary to consider the whole intracellular binding. Kinetic analysis, according to theoretical assumptions presented in Material and Methods (Eqs. 7—16), made it possible to reconstruct the \(K_p\) value of VBL in the liver. The \(K_p\) values calculated based on the binding to tubulin by Eq. 4, those on the total binding to cytosol by Eq. 5, and those on the binding to subcellular components by Eqs. 12 and 17 were 1.1, 2.6, and 15.2, respectively. The \(K_p\) value thus reconstructed, considering the binding to whole components in the liver, became as large as 15, which is still lower than the value of approximately 75 reported *in vivo* for the rat liver (Fig.
Discussion

In this study, the mechanism of in vivo tissue distribution of VBL was examined in mice in an attempt to elucidate the mechanism of interorgan variation in the distribution of Vinca drugs. Our preliminary experiments have shown a rapid, even at low temperature, binding of VBL to 100000 × g mouse tissue cytosols, and equilibrium was reached within 10 min incubation (data not shown). The direct application of the assay of VBL binding, utilizing the activated charcoal method for CLC,12,14 appeared to be troublesome. We faced two important problems, i.e. the adsorption of VBL to charcoal was time-dependent, and the bound VBL was relatively quickly removed by the activated charcoal. To quickly remove the unbound VBL, it was necessary to increase the concentration of activated charcoal from 0.25 to 0.50%. This amount of charcoal was sufficient to remove the unbound VBL within 1 min, independently of VBL concentrations. This adsorption time (1 min) was kept constant because, if increased, the bound fraction would be underestimated due to the reversibility of VBL binding to tissue cytosols.

The in vivo values of VBL were characteristically large.6,7 There are several possible mechanisms for this variation, the active transport against the concentration gradient or strong tissue binding. Observed in vivo values of VBL were also seen in vitro in the case of isolated rat hepatocytes.23 However, such large values in rat hepatocytes could not only be explained by the presence of the active transport system for VBL,23 since the membrane transport (initial uptake) of VBL appeared to be insensitive to metabolic inhibitors.

Analysis of VBL binding to rat hepatocytes showed that a total cellular binding is involved in extensive VBL distribution. The contribution of tubulin is rather minor. On the other hand, CLC, which is claimed to be solely bound to tubulin, should have low values, as one can predict from its binding parameters. CLC appeared to have a value in the liver that is several times higher than that predicted from its binding to liver tubulin, suggesting the presence of an active transport system in rat hepatocytes.24

When the binding to tubulin or the total binding to cytosol is considered, the lack of the active transport system and the underestimation of value (Fig. 7) prompted us to study the intracellular distribution of VBL. Additional experiments, using the cellular fractionation, showed a significant participation of the nuclear fraction in the tissue distribution of VBL. The analysis of the total subcellular distribution made it possible to calculate the value. The calculated value approximated the reported value (Kp = 70), although it was still smaller than that reported.6,7 This difference may be due to the use of different animal species. By what mechanism can one explain the good correlation between the Kp values of VBL and tubulin concentrations in various tissues when the binding to tubulin contributes little to the total intracellular binding? There is a possibility of incomplete separation of tubulin during the fractionation procedure or to sedimentation of microtubules which might be associated with nuclei. Assuming that tubulin is a major binder of both compounds (CLC and VBL), similar subcellular distribution patterns should be observed. The data, however, indicated completely different subcellular localization. This is probably the main reason why, considering only the binding to tubulin, it was impossible to reconstruct the Kp values for VBL.

The value of VBL in the brain is as low as 0.3 at 2 h after administration,8 although the tubulin content in the brain is the highest among all the tissues. This is probably because of the high resistance in the uptake of VBL through the blood-brain barrier. Considering the high lipophilicity of VBL, the relatively high molecular weight (M, 814) of the compound may account for such a low value in the brain. In fact, it is accepted that the blood-brain barrier hampers the uptake of the compounds of molecular weights greater than 450—650,25 unless the compounds can utilize a carrier (receptor)-mediated uptake mechanism.

In conclusion, the contribution of tubulin in the tissue binding is minor, but its role in the
regulation of the intracellular binding of Vinca drugs can not be excluded. From the analysis that the tubulin contents in the tissues and the \( K_{\text{p,app}} \) values of several Vinca alkaloids showed a significant correlation, we can only speculate that the concentration of the binding proteins may parallel that of tubulin.

References


