Uptake of Basic Drugs into Rat Lung Granule Fraction in Vitro

Junko Ishizaki, Koichi Yokogawa, Emi Nakashima, Shoji Ohkuma, and Fujiio Ichimura

Hospital Pharmacy, Department of Clinical Pharmaceutics, Graduate School of Pharmaceutical Sciences, and Department of Molecular and Cell Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-8641, Japan. Received January 29, 1998; accepted April 14, 1998

Although basic drugs are distributed widely in various tissues, they are characteristically concentrated in the lung granule fraction. We examined the uptake of seven lipophilic basic drugs into rat lung granule fraction (P2) in vitro and investigated the contributions of drug lipophilicity and lysosomal trapping to the characteristic lung P2 distribution. The uptake of each drug into P2 was examined at various pH values. The drug concentration in P2 was determined by gas chromatography. Biperiden (BP) was rapidly taken up into P2, reaching a maximal concentration within 1 min at pH 7.4 at both 4°C and 37°C. Both BP and chlorpromazine uptake into P2 was biphasic. Though the uptake rates of the seven drugs into P2 increased with rising pH, the rate of increase varied for each drug. There was a good correlation between the octanol–water partition coefficient of the nonionized form (Poni) of each drug and the uptake into P2 in the presence or absence of NH4Cl, which inhibits lysosomal trapping. However, uptake into P2 in the presence of NH4Cl showed a stronger Poni-dependency. We conclude that the distribution of basic drugs into lung P2 is dependent on both drug lipophilicity and lysosomal uptake.

Key words basic drug; rat lung granule fraction; lipophilicity; distribution; lysosome

To predict drug effectiveness and/or side effects, it is important to elucidate the mechanism of drug tissue distribution. We have shown that drug lipophilicity and fat tissue volume influence distribution kinetics, and furthermore, that basic drugs are distributed widely in various tissues, but are concentrated in the lung. We have also studied the characteristic subcellular distribution of anticholinergic basic drugs in the brain, heart and lung and concluded that it is dependent on the protein amount in each fraction, except in the lysosome-containing lung granule fraction (P2), which had an 8-fold greater drug concentration than predicted from the protein amount.

Basic drugs are protonated and accumulate in lysosomes in membrane-impermeable forms. The pulmonary toxicity of amidarone coincides with the intralysosomal accumulation of the drug in the lung and may be a consequence of the drug-induced intracellular storage of phospholipids. Several reports describe the uptake and accumulation in the lung of basic drugs, such as chlorpromazine (CPZ), imipramine (IM) and diazepam. Xie et al. reported that many highly lipophilic benzodiazepine drugs with pK_s values greater than 7 are not stored in adipose tissue, and concluded this is due to lysosomal trapping in lean tissues. Moreover, data on the tissue distribution of basic, highly lipophilic drugs in vivo obtained by distribution dialysis imply that in vitro studies may have grossly underestimated drug distribution in the lung, liver, and kidney, probably as a consequence of the destruction of lysosomes by homogenization and incubation. Recently, we reported that the subcellular distribution of basic drugs decreased after NH4Cl treatment. The largest decrease in distribution was by 30% in lung P2. Lung P2 has a high affinity for basic drugs, so clarification of the distribution kinetics of P2 might enable us to treat patients more effectively and safely.

The purpose of this study was to investigate the lung P2 distribution of seven basic, lipophilic drugs, as well as its relation to drug lipophilicity and lysosomal uptake.

MATERIALS AND METHODS

Materials Biperiden (BP) (Dainippon, Osaka, Japan), pentazocine (PTZ) (Sankyo, Tokyo, Japan) and trihexyphenidyl (TP) (Nippon Lederle, Japan) were used as supplied. Chloroquine (CQ), CPZ, IMP and promethazine (PMT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of reagent grade and were used without purification.

Preparation of P2 Rat lung was fractionated according to the methods of De Robertis et al. Briefly, male Wistar rats (260±22 g; mean±S.D., Sankyo Laboratory Animal Co., Toyama, Japan) were killed, and their lungs were removed and homogenized with ten volumes of ice-cold 0.32 M sucrose. All subsequent steps were performed at 4°C. Homogenates were centrifuged at 1000×g for 10 min and the supernatants were centrifuged at 12300×g for 20 min. Pellets were resuspended in ice-cold tris–HCl buffer (0.25 M sucrose, 3.4 mM tris–HCl, at various pH values), then used.

Enzyme and Protein Assay The specific activity of cytochrome c oxidase was determined as described previously. The specific activity of acid phosphatase was determined by a p-nitrophenyl phosphate method using a commercial kit (Acid phospha B-test, Wako, Tokyo, Japan). Protein concentration was determined with a commercial kit (Protein Assay Kit, Bio-Rad Laboratories, Ltd., Osaka, Japan).

Determination of Basic Drugs The concentrations of basic drugs were determined by gas chromatography (GC), as described previously, with slight modifications. pK_s and Lipophilicity pK_s values were determined by potentiometric titration at 37°C. The organic solvent–water partition coefficient of each drug was determined experimentally at 37°C, as described previously, with slight modifications. Octanol was used as the organic solvent, and isonicotinic acid hydrochloride buffer (pH 7.4) as the aqueous solution. The octanol–water partition coefficient of the nonionized form (Poni) was calculated from the apparent partition coefficient accord-

* To whom correspondence should be addressed.

© 1998 Pharmaceutical Society of Japan
ing to the Henderson–Hasselbalch equation.

Uptake of Basic Drugs into \( P_2 \) The uptake of basic drugs into the \( P_2 \) fraction was determined in ice-cold tris-HCl buffer (0.25 \( \text{m} \) sucrose, 3.4 \( \text{mm} \) tris-HCl, at various pH values) containing 1 ml of \( P_2 \) (230—300 \( \mu \text{g} \) protein containing 1—200 \( \mu \text{M} \) basic drug). After incubation at 4°C for 10 min, samples were centrifuged at 12300 \( \times g \) for 20 min at 4°C. Drug concentrations in the supernatant were determined by GC.

Inhibition of Basic Drug Uptake To test the effect of \( \text{NH}_4\text{Cl} \) on the uptake of basic drugs, fractions were incubated in the presence or absence of \( \text{NH}_4\text{Cl} \) (10 \( \text{mm} \)) at 4°C for 10 min, then a basic drug (1 \( \mu \text{M} \)) was added. After 10 min of incubation at 4°C, the uptake of basic drugs was determined as described above.

Data Analysis The parameters of the Scatchard plots were estimated by the non-linear least-squares method using the NONLIN program on a FACOM-M360AP digital computer at the Information Processing Center, Kanazawa University. Data were analyzed using Student’s \( t \) test for comparison of the unpaired means of two sets of data.

RESULTS

Seven commonly used basic, lipophilic drugs were investigated, as listed in Table 1. Their values of apparent \( P_{oc} \) vary widely, from 26 to 1900.

Uptake of Basic Drugs into \( P_2 \) Figure 1 shows the time courses of BP (1 \( \mu \text{M} \)) uptake into \( P_2 \) at pH 7.4 at 4 and 37°C. In both cases, BP was rapidly taken up by \( P_2 \) to approximately the same maximal value. However, the BP concentration at 37°C started to decrease slowly at 10 min and was 30% (\( p<0.1 \)) lower than that at 4°C by 30 min.

Figure 2 shows Scatchard plots of BP and CPZ uptake into \( P_2 \) in the concentration range of 0.01—200 \( \mu \text{M} \) at pH 7.4 at 4°C. At least two types of binding sites for these drugs are evident in \( P_2 \), namely high-affinity/low-capacity sites and low-affinity/high-capacity sites. The dissociation constants (\( K_d \), \( \mu \text{M} \)) and maximum number of binding sites (\( B_{max} \), nmol/mg protein) calculated using the NONLIN program for the uptake of these drugs into \( P_2 \) are listed in Table 2. The \( K_d \) value of BP in the high-affinity/low-capacity sites was similar to that of CPZ. However, the \( K_d \) value of BP in the low-affinity/high-capacity sites was about 3 times that of CPZ. On the other hand, the \( B_{max} \) values of CPZ at both sites were about 5—8 times those of BP.

Influence of Medium pH Figure 3 shows the influence

![Fig. 1. Time Courses of Biperiden (BP) Uptake into Rat Lung Granule Fraction (\( P_2 \))](image)
P, was incubated with 1 \( \mu \text{M} \) BP at pH 7.4 at 4°C (●) and at 37°C (○). Each point represents the mean±S.E.M. of three experiments.

![Fig. 2. Scatchard Plots of BP (●) and Chlorpromazine (CPZ) (○) Uptake into \( P_2 \)](image)
P, was incubated at pH 7.4 at 4°C for 10 min. Each point represents the mean±S.E.M. of three experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>High-affinity/low-capacity sites</th>
<th>Low-affinity/high-capacity sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d ) (( \mu \text{M} ))</td>
<td>( B_{max} ) (nmol/mg protein)</td>
</tr>
<tr>
<td>Biperiden</td>
<td>0.183±0.148</td>
<td>1.10±0.52</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>0.197±0.101</td>
<td>8.48±3.13*</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. of three experiments. \( a \) The dissociation constant. \( b \) The maximum number of binding sites. Significantly different (* \( p<0.05 \), ** \( p<0.01 \)) from biperiden.
of pH on BP (1 μM) uptake into P2 at 4 °C. BP uptake increased gradually with rising buffer pH, the uptake at pH 8.5 being about 3 times that at pH 5.5.

Figure 4(a) shows the uptake of the seven basic drugs (1 μM) into P2 at pH 6.0, 7.4 and 8.5 at 4 °C. At pH 6.0, the uptake of these drugs tended to increase gradually with drug lipophilicity. The uptake of each drug in P2 increased with rising pH, and the uptakes of all the drugs were similar at pH 8.5. Lower drug lipophilicity correlated well with an increased uptake ratio at higher pH values. Figure 4(b) shows the uptake of 200 μM CPZ, BP or CQ. The uptakes of these drugs increased gradually with rising pH. However, when the medium pH was changed from 6.0 to 8.5, CPZ uptake at concentrations of 1 and 200 μM increased by 10 and 70%, respectively. The corresponding increases in BP uptake under the same conditions were 140 and 160%, while those for CQ were 150 and 60%, respectively.

Influence of NH4Cl Figure 5 shows the inhibitory effect of NH4Cl (10 mM), a lysosomal inhibitor, on basic drug (1 μM) uptake into P2 at pH 7.4. The uptakes of all drugs were inhibited by NH4Cl, but to widely differing extents (6—87%). Lower drug lipophilicity correlated with stronger NH4Cl inhibition.

Figure 6 shows the relationships between basic drug (1 μM) uptake into P2 at pH 7.4 in the control and NH4Cl-treated fractions, and drug lipophilicity. Uptake into P2 increased with an increase in lipophilicity. The relationship between uptake and P oct for six drugs, excluding CQ, is described by the following equation.

\[ X = \alpha \cdot P_{\text{oct}}^\beta \]

where \( X \) is the uptake into P2, \( \beta \) is the slope of the logarithmic plot and \( \alpha \) is the value of \( X \) when \( P_{\text{oct}} \) is 1. The regression equations for \( X_{\text{control}} \) and \( X_{\text{NH4Cl}} \) are given by Eqs. 2 and 3, respectively.

\[ X_{\text{control}} = 0.163 \cdot P_{\text{oct}}^{0.265} \]  

\[ X_{\text{NH4Cl}} = 0.00274 \cdot P_{\text{oct}}^{0.602} \]

The correlation coefficients were 0.965 and 0.976, respectively.

Fig. 3. Effect of Medium pH on BP (1 μM) Uptake into P2 at 4 °C
Bars represent the mean ± S.E.M. of three experiments.

Fig. 4. Effect of Medium pH on Uptake of Various Basic Drugs into P2 at 4 °C
Bars represent the mean ± S.E.M. of three experiments. Drug concentrations were 1 μM (a) and 200 μM (b). ■, pH 6.0; ■, pH 7.4; ■, pH 8.5.

Fig. 5. The Percent Inhibition of Various Basic Drugs (1 μM) by 10 mM NH4Cl Treatment at pH 7.4 at 4 °C
Bars represent the mean ± S.E.M. of three experiments.

Fig. 6. The Relationship between Uptake into P2 and log \( P_{\text{oct}} \) for Various Basic Drugs at pH 7.4 at 4 °C
Each point represents the mean of three experiments. ●, control; ○, 10 mM NH4Cl treatment.
DISCUSSION

In this study, P₂ was obtained by centrifugation at 4 °C for 20 min. Since the uptake of BP into P₂ reached a maximum within 1 min, and the effect of temperature on uptake is not clear (Fig. 1), the possibility that P₂ uptake occurred during the 4 °C centrifugation period must be considered. However, when P₂ was rapidly separated by aspirated filtration, similar results were obtained. We chose to routinely use centrifugation because basic drugs are adsorbed on glass fiber filters.

The P₁ fraction includes lysosomes, which are destroyed by homogenization and prolonged incubation: about 96% of the total content of acid phosphatase was accumulated in P₂ in a control study (incubation for 0 min, at 4 °C and pH 7.4). However, the release of acid phosphatase was less than 4% at 4 °C and only about 8% at 37 °C, and the specific activity of cytochrome c oxidase did not change under any of these conditions. On the other hand, intralysosomal pH increases at 37 °C but not at 4 °C (manuscript in preparation). Since the increase in intralysosomal pH may cause a decreased uptake of the basic drugs in the P₁ fraction, all incubations were performed at 4 °C for 10 min.

De Duve reported that the concentration ratio (accumulation ratio) for lysosomotropic substances is equal to the ratio of the hydrogen ion concentration in lysosomes to that in the fluid outside. In our study, BP uptake into P₂ increased gradually with rising medium pH (Fig. 3), in agreement with the results of De Duve.

We previously reported that IMP uptake into lysosomes was biphasic, which indicates that lysosomes have at least two types of binding sites. Similarly, we found here that there are at least two types of binding sites for BP and CPZ in P₂ (Fig. 2 and Table 2). The Kᵢ values of the two drugs for the high-affinity/low-capacity sites were approximately equal, whereas the Kᵢ value of CPZ for the low-affinity/high-capacity sites was significantly smaller than that of BP. These data suggest that the high-affinity/low-capacity binding reflects accumulation in lysosomes, since drug uptake was dependent on the intra- and extralysosomal pH gradient, and the Kᵢ values of these sites were fairly constant. The data also suggest that low-affinity/high-capacity site binding depends on drug lipophilicity, since the Kᵢ values of these sites decreased as the Pₜₑₒₜ values increased. On the other hand, the Bₘₜₜ values of CPZ for both the high-affinity/low-capacity and low-affinity/high-capacity sites were significantly larger than those of BP, suggesting that the Bₘₜₜ values depend on drug lipophilicity.

To identify factors which affect P₂ uptake, the inhibitory effects of NH₄Cl (10 mm) were examined. As drug lipophilicity increased, the inhibitory effect of NH₄Cl decreased (Fig. 5). Similar results were obtained using CQ (25 μM) as a lysosomal inhibitor (r = 0.920) (data not shown).

Basic drug uptake in the presence of NH₄Cl, which inhibits lysosome uptake, might reflect lipophilicity-dependent binding. As shown in Fig. 6, strong correlations were found either in the presence or absence of NH₄Cl. The β value in the presence of NH₄Cl was 2.3-fold larger, suggesting that the uptake, excluding the lysosomal contribution, is more strongly dependent on drug lipophilicity. These results demonstrate that the distribution of basic drugs in lung P₂ is determined by both drug lipophilicity and lysosomal trapping. Since CQ is a diacidic base and has two pKₐ values, the log Pₜₑₒₜ for CQ was excluded from this analysis.

Though pH influenced the uptake of every drug, some drugs showed different patterns of uptake. The uptake ratio of 1 μM of the basic drugs into P₂ at higher pH values increased as drug lipophilicity decreased (Fig. 4(a)). For highly lipophilic drugs, more than 80% of each drug (1 μM) was found in P₂ at pH 6.0, so the increase in uptake ratio at higher pH values was small. However, at a higher concentration (200 μM) of CPZ, a very lipophilic drug, the uptake did increase markedly at higher pH values (Fig. 4(b)). In contrast, for CQ, a weakly lipophilic drug, the increase in uptake ratio at a higher pH was smaller at a CQ concentration of 200 μM than at 1 μM. These results indicate that the protons in the lysosomes are consumed at the higher concentration of CQ, leading to saturation of the uptake of CQ into P₂.

In conclusion, we found that the two major factors which control the distribution of basic drugs in the P₂ fraction of the lung are drug lipophilicity and lysosomal uptake. At a clinical plasma level (1 μM), the distribution of drugs with log Pₜₑₒₜ values greater than 4.5 is predominantly dependent on lipophilicity, whereas the distribution of drugs with Pₜₑₒₜ less than 4.5 is predominantly by lysosomal uptake. Side effects arising from drug accumulation in the lung may be avoided by concomitant administration of nontoxic basic drugs, with a log Pₜₑₒₜ of less than 4.5, as competitive uptake inhibitors.

REFERENCES AND NOTES

1) Present address: Department of Pharmaceutics, Kyoritsu College of Pharmacy, 1–5–30 Shiba-koen, Minato-ku, Tokyo 105, Japan.


