DISTRIBUTION OF DIURETICS AND HYPOGLYCEMIC SULFONYLUREAS IN RABBIT ERYTHROCYTES

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The distribution of three sulfonlureas and six diuretics in rabbit erythrocytes was studied in vitro at 37°C. The drugs were taken up by the erythrocyte compartment, and distribution equilibrium was reached within 60 min of incubation. A distribution percentage in erythrocyte compartment was maintained at roughly constant value over the whole concentration range of drugs. Therefore, a linear relationship was established between total concentrations of drug in whole blood or erythrocyte suspension and in the erythrocyte compartment. Bovine serum albumin combined with the erythrocyte suspension appeared to reduce drug distribution in the erythrocyte compartment. Whole blood obtained from renal failure rabbits showed greater distribution of drug in the erythrocyte compartment compared with the whole blood of a normal rabbit. This might be due to a change in plasma protein binding ability related to the progress of renal failure.

Keywords — distribution in erythrocyte; sulfonlurea; diuretic; whole blood; erythrocyte suspension; hematocrit; furosemide; hydroflumethiazide; plasma protein binding; renal failure rabbit

INTRODUCTION

It is known that many drugs in the bloodstream are to some extent bound to plasma proteins, especially plasma albumin, and only unbound drug is freely diffusible into other compartments of the body. Drug binding is usually determined in plasma or purified serum albumin solutions, while few studies have been done on drug binding to erythrocytes. Many pharmacokinetics studies dismiss the erythrocyte compartment of the blood as an insignificant consideration, and the blood and plasma levels of drugs are often assumed to be equivalent. However, many drugs accumulate significantly in erythrocytes. Therefore, the significance of drug-erythrocyte binding in the overall pharmacokinetics picture has to be determined.

Previously, the plasma protein binding of sulfonlurea compounds and various diuretics was studied in detail. This study is an attempt to elucidate the in vitro distribution characteristics of drugs, examined previously, in erythrocytes and to determine the distribution of these drugs in each blood component of whole blood.

MATERIALS AND METHODS

Materials — Chlorothiazide, hydrochlorothiazide, hydroflumethiazide, furosemide, bumetanide and piretanide were used. These diuretics were kindly supplied by Hoechst Japan Ltd. and Sankyo Co., Ltd. The hypoglycemic sulfonlureas used in this study were tolbutamide, chlorpropamide and carbutamide. These sulfonlureas were kindly supplied by Hoechst Japan Ltd. All chemicals and solvents used were of analytical reagent grade. The blood of normal and acute renal failure rabbits was collected and used.

Preparation of Erythrocyte Suspension for in Vitro Distribution Studies — Rabbit blood, ob-
tained from either normal or acute renal failure rabbits, was divided into two equal portions. One portion was used as whole blood. The other portion was centrifuged at 3000 rpm for 10 min. The packed erythrocytes were obtained after removing the plasma water and washed two times with two volumes of chilled 0.9% NaCl solution. And then, the erythrocytes were washed with two volumes of Krebs Ringer phosphate buffer (pH 7.2) two times. Finally, the volume was returned to the initial value using the preceding buffer. Hematocrit values of whole blood and the erythrocyte suspension were determined. Both hematocrit values were adjusted to 0.38 by adding plasma or phosphate buffer solution (pH 7.2). The erythrocyte suspension prepared in this experiment included also leucocytes and platelets.

Distribution of Drugs in the Erythrocyte Compartment — In an appropriate volume of ethanol solution a constant weight of drug was dissolved. The solution was transferred to a 10 ml centrifuge tube with a round bottom and evaporated under reduced pressure. Whole blood or an erythrocyte suspension was added to the dried drugs in the centrifuge tube. After gentle stirring at 37°C for prearranged hours, these tubes were centrifuged for 10 min at 3000 rpm and the upper layer was used for drug analysis.

The distribution percentages (D%) of drug in the erythrocyte compartment are expressed by eq. (1) for whole blood and eq. (2) for erythrocyte suspension, respectively.

\[
D\% = \frac{C_t - C_p (1 - H_t)}{C_t} \quad (1)
\]

\[
D\% = \frac{C_{es} - C_{bs} (1 - H_t)}{C_{es}} \quad (2)
\]

where \(C_t\) is the total concentration of drug in whole blood, \(C_p\) the total concentration in plasma, \(C_{es}\) the total concentration in erythrocyte suspension, \(C_{bs}\) the total concentration in buffer phase and \(H_t\) the hematocrit (fractional volume of erythrocytes per volume of blood). The value of 0.38 was used as the \(H_t\) in this experiment.

Effect of Bovine Serum Albumin (BSA) on the Distribution of Furosemide and Hydroflumethiazide in Erythrocytes — The distribution of furosemide and hydroflumethiazide was measured in erythrocyte suspensions containing various concentrations (0–3 or 3.3%) of BSA. The concentration of furosemide and hydroflumethiazide used in this experiment was 13.3 μg/ml.

Assay of Drugs — Tolbutamide and Chlorpropamide: These drugs were determined by the gas chromatography (GLC) method developed by Prescott11 with some modification. To 0.5 ml of sample in a 10 ml-stoppered centrifuge tube, 0.5 ml of internal standard (chlorpropamide for tolbutamide determination and vice versa), 0.5 ml of 2N HCl and 5 ml of toluene were added. The tube was shaken and centrifuged. Four ml of toluene was transferred to a 10 ml-stoppered centrifuge tube and 1 ml of 10% Na₂CO₃ solution was added. After shaking and centrifuging, the toluene layer was removed. To the water layer, 1 ml of methanol and 0.1 ml of dimethylsulfate were added and mixed. The tubes were allowed to stand for 10 min at 60°C, and 1 ml of 1 M acetate buffer (pH 5.2) and 5 ml of \(n\)-hexane were added after cooling. The mixture was shaken and centrifuged. Four ml of the \(n\)-hexane layer was removed and transferred into another test tube. The solvent was evaporated to dryness under reduced pressure at 40°C. Residues were dissolved in 30–50 μl of chloroform. A 5 μl aliquot was injected into GLC apparatus (Shimadzu GC-6AM). The column was a glass tube 1.5 m × 3 mm in i.d., packed with 3.8% silicone W-98 on Unipor HP 80/100. The column temperature was 245°C and nitrogen was used as a carrier gas at a flow rate of 40 ml/min.

Carbutamide: A colorimetric method developed by Bratton and Marshall13 was used.

Hydroflumethiazide, Furosemide, Bumetanide and Piretanide: These drugs were assayed by a spectrophotofluorimetric method reported previously.10,11

Chlorothiazide and Hydrochlorothiazide: To 0.5 ml of sample in a 10 ml-stoppered centrifuge tube was added 0.5 ml of internal standard (hydroflumethiazide was used), 0.5 ml of 1N HCl and 5 ml of ether. After shaking and centrifug-
Drug Distribution in Erythrocytes

ing, the ether layer (4 ml) was evaporated to dryness under reduced pressure. The residue was dissolved in 50–100 μl of ethanol. The ethanol solution was assayed using HPLC (Gasukuro Kogyo Model 570) equipped with a reversed phase column (Unisil Q C18, 4 × 300 mm) and a spectrophotometric detector at 280 nm. A solvent system of 20% acetonitril in 0.005M phosphoric acid was used and the flow rate was 1.4 ml/min.

Experimental Formation of Acute Renal Failure in Rabbit—The healthy rabbits (Yanahara Rabbit Center) were i.m. injected in the femoral region with HgCl₂ solution in a dose of 5–10 mg/kg per day. Blood urea nitrogen (BUN) was measured on the second day after i.m. injection of HgCl₂. Rabbits with a BUN value above 50 were selected and used for further experiments.

RESULTS

Plasma Protein Binding

Duplicate equilibrium dialysis determinations were carried out and the results agreed roughly with the binding percent as shown previously. The mean values are given in Table I. Distribution in the Erythrocyte Compartment

Each drug concentration range in the medium was decided based on therapeutically useful blood concentrations. Figure 1 shows the distribution profile of furosemide in the erythrocytes as a function of time during incubation of the washed erythrocyte suspension or whole blood containing 66.7 μg/ml furosemide. During the first 30 min of incubation, furosemide was taken up rapidly by the erythrocyte compartment of whole blood. However, a clear explanation related to the above phenomenon has not been made. A distribution equilibrium was attained after one hour in both cases.

The distribution equilibrium of drugs in the erythrocyte compartment of whole blood and the erythrocyte suspension was approximately independent of concentration in the range studied. As a typical example, the distribution of

<table>
<thead>
<tr>
<th>Drug</th>
<th>Plasma protein binding (%)</th>
<th>Distribution in erythrocytes (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>13.3 μg/ml b)</td>
<td>66.7 μg/ml b)</td>
</tr>
<tr>
<td></td>
<td>Whole blood</td>
<td>Suspension</td>
</tr>
<tr>
<td>Chlorothiazide</td>
<td>86.7</td>
<td>26.4±1.0 c)</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>56.9</td>
<td>59.8±1.7</td>
</tr>
<tr>
<td>Hydroflumethiazide</td>
<td>25.2</td>
<td>38.9±2.8</td>
</tr>
<tr>
<td>Furosemide</td>
<td>98.4</td>
<td>8.1±3.0</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>94.2</td>
<td>33.3±2.4</td>
</tr>
<tr>
<td>Piretanide</td>
<td>91.5</td>
<td>13.8±3.8</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>92.9</td>
<td>–</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>90.1</td>
<td>–</td>
</tr>
<tr>
<td>Carbutamide</td>
<td>86.9</td>
<td>–</td>
</tr>
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</table>

a) These values were obtained by in vitro equilibrium dialysis using rabbit plasma. drug concentration: 1×10⁻⁴ M
b) These values represent the drug concentration used in the rabbit erythrocyte experiments.
c) These values represent the mean ± S.D. Three experiments were done for each concentration of drugs.
Furosemide is shown in Fig. 2.

Another expression was used to describe the distribution of drug in whole blood or the erythrocyte suspension. The total amount in whole blood \( (D_r) \) will be the sum of amounts in plasma water \( (D_{pw}) \), plasma protein \( (D_{pp}) \), and the erythrocyte compartment \( (D_e) \). In the case of the erythrocyte suspension, the total amount \( (D_r) \) will be the sum of the amounts in buffer solution \( (D_b) \) and the erythrocyte compartment \( (D_e) \). The fraction of drug distributed in the plasma compartment \( (f_p) \), buffer compartment \( (f_b) \) and erythrocyte compartment \( (f_e) \) can be expressed as eqs. \( (3)-(5) \):

\[
\begin{align*}
  f_p &= (D_{pw} + D_{pp}) / D_r \\
  f_b &= D_b / D_r \\
  f_e &= D_e / D_r
\end{align*}
\]

Therefore, it is evident that

\[
\begin{align*}
  C_p &= C_r f_p / (1 - H_r) \\
  C_b &= C_r f_b / (1 - H_r) \\
  C_e &= C_r f_e / H_r
\end{align*}
\]

where \( C_p \) is the total concentration of drug in the plasma compartment at the equilibrium state, \( C_b \) the total concentration of drug in the buffer compartment at the equilibrium state, \( C_e \) the total concentration of drug in the erythrocyte compartment at the equilibrium state, and \( C_r \) the total concentration of drug in whole blood or the erythrocyte suspension at the initial time. All drugs used in this experiment were relatively stable during experimental times.\(^6,8\) Figures 3 and 4 illustrate the relationship between \( C_e \) and \( C_r \), and a proportional relationship can be observed. Using the least squares method, the slope which represents the value of \( f_e / H_r \), of the regression line was determined, and 0.27 in whole blood and 1.33 in erythrocyte suspension were obtained for furosemide. Hydroflumethiazide has an \( f_e / H_r \) of 1.30 in whole blood and 1.45 in erythrocyte suspension.

The percentage of distribution into the erythrocyte compartment are summarized in Table I. The experiments were done in three concentration levels.

**Effect of BSA on Distribution in Erythrocytes**

The effect of BSA on the distribution of furosemide and hydroflumethiazide was checked. The results are shown in Figs. 5 and 6. BSA dis-

![Graph 1](image1.png)

**FIG. 1.** **Effect of Incubation Time on the Distribution of Furosemide in the Erythrocyte Compartment at 37°C**

Key: ●, in whole blood; ○, in erythrocyte suspension.

Total concentration of furosemide was 66.7 μg/ml.

Each point represents the average of two measurements.

![Graph 2](image2.png)

**FIG. 2.** **Effect of Total Concentration on the Distribution of Furosemide in the Erythrocyte Compartment at 37°C**

Key: ●, in whole blood; ○, in erythrocyte suspension.

The value represents the mean±S.D. (vertical bar) of three experiments.
**Drug Distribution in Erythrocytes**

**FIG. 3.** Relationship between Total Concentration of Furosemide in the Erythrocyte Compartment and Total Concentration of Furosemide in Whole Blood (●) or Erythrocyte Suspension (○).
Each point represents the average of two measurements.

**FIG. 4.** Relationship between Total Concentration of Hydroflumethiazide in the Erythrocyte Compartment and Total Concentration of Hydroflumethiazide in Whole Blood (●) or Erythrocyte Suspension (○).
Each point represents the average of two measurements.

**FIG. 5.** Effect of Various Concentrations of Bovine Serum Albumin (BSA) on the Distribution of Furosemide in the Erythrocyte Compartment

Key: ●, whole blood; ○, erythrocyte suspension containing various concentrations (0—3.3%) of BSA.
The percentage (5.6%) on abscissa represents the serum albumin concentration in whole blood of rabbit used.
The concentration of furosemide was 13.3 μg/ml.
Each point represents the average of two measurements.

**FIG. 6.** Effect of Various Concentrations of Bovine Serum Albumin (BSA) on the Distribution of Hydroflumethiazide in the Erythrocyte Compartment

Key: ●, whole blood; ○, erythrocyte suspension containing various concentrations (0—3%) of BSA.
The percentage (6%) on abscissa represents the serum albumin concentration in whole blood of rabbit used.
The concentration of hydroflumethiazide was 13.3 μg/ml.
Each point represents the average of two measurements.
urbed the distribution of furosemide in the erythrocyte compartment, while hydroflumethiazide had little effect.

**Distribution of Furosemide in Erythrocytes in Acute Renal Failure Rabbits**

Table II compares the distribution in the erythrocyte compartment of furosemide between normal and renal failure rabbits. Further furosemide distribution experiments were done similar to that stated in “Materials and Methods”, using the mixture of erythrocytes, from normal rabbit, and plasma from renal failure rabbit, and the other mixture of erythrocytes, from renal failure rabbit, and plasma from normal rabbit. The results are also summarized in Table II.

**DISCUSSION**

An equilibrium state was established 60 min later as shown in Fig. 1. If distribution percentage of furosemide in the erythrocyte compartment at equilibrium is plotted against the total concentration in whole blood or the erythrocyte suspension, roughly constant values are obtained for all furosemide concentrations tested as shown in Fig. 2. The similar tendency have been obtained for other drugs. Furthermore, the relationship between the total concentrations in initial time and the equilibrium concentration in the erythrocyte compartment for furosemide or hydroflumethiazide, are shown in Figs. 3 and 4. The slope represents the $f_e / H_e$ value as is evident from eq. (8). Linearity was likewise observed for all drugs used in this experiment. The difference between the slopes of the two lines obtained for whole blood and the erythrocytes suspension was relatively large for furosemide, and, conversely, quite small for hydroflumethiazide.

The distribution ratio of furosemide and hydroflumethiazide between the erythrocyte compartment and buffer solution (pH 7.3) was determined in the presence of various concentrations of BSA (Figs. 5 and 6). Unfortunately, we could not obtained purified rabbit serum albumin (RSA). Therefore, BSA was used in this experiment for convenience. The results indicate that BSA was responsible for the lowered drug partitioning in the erythrocyte compartment. The displacement effect of BSA on the distribution in erythrocytes compartment of furosemide was larger than that of hydroflumethiazide. The distribution in erythrocyte compartment of furosemide decreased considerably from 69 to 16% as the BSA concentration increased from 0 to 3.3% (w/v). However, for hydroflumethiazide within a concentration range of 0–3% (w/v),

<table>
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<th>TABLE II. Plasma Protein Binding and Distribution of Furosemide a) in Erythrocytes in Normal and Acute Renal Failure Rabbits</th>
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<tr>
<td><strong>Plasma protein binding (%)</strong></td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Renal failure (R.F.)</td>
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<tr>
<td>Normal erythrocyte and R.F. plasma c)</td>
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<tr>
<td>R.F. erythrocyte and normal plasma c)</td>
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<tr>
<td>Whole blood</td>
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<tr>
<td>Suspension</td>
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a) Furosemide concentration: 66.7 μg/ml.

b) These values represent the mean ± S.D.

Three experiments were done for each treatment.

c) Albumin concentration in the both plasma using in this experiment was almost the same (5.81 g/dl for normal and 5.53 g/dl for renal failure). Both hematocrit values were adjusted to 0.38.
Drug Distribution in Erythrocytes

the distribution decreased slightly from 56 to 49%. Therefore, such a fact suggests that the distribution in erythrocyte compartment of drug is influenced by the capacity for plasma protein-drug binding. There is some inaccuracy, but the above suggestion may be applied to drugs tested except a few drugs, for example, bumetanide. The distribution percentages in erythrocyte compartment and the percentages of plasma protein binding are summarized in Table I for all drugs used in this experiment.

The plasma protein binding of furosemide in the whole blood of acute renal failure rabbits decreased compared with normal rabbits and the erythrocyte distribution conversely increased. The distribution percentages in erythrocyte compartment was apparently unchanged in the suspension of erythrocyte obtained from renal failure rabbit compared with normal rabbit. When the mixture (adjusted to hematocrit value 0.38) of normal rabbit erythrocyte and renal failure rabbit plasma was used, the distribution percentages in erythrocyte compartment was in agreement with that using the whole blood of acute renal failure rabbit. On the other hand, when the mixture (adjusted to hematocrit value 0.38) of renal failure rabbit erythrocyte and the normal rabbit plasma was used, the distribution percentages was much the same with that using the whole blood of normal rabbit.

The cause of the decreased binding of plasma protein may be due to either endogenous inhibitor, produced by acute renal failure, competition for the drug binding site or a conformational change in the albumin molecule. The distribution pattern of furosemide in the body of acute renal failure rabbit is considered to be very different from that in normal rabbit in the same manner as distribution in erythrocyte compartment. Therefore, the accumulation of these drugs in the specific tissues and organs in acute renal failure will become a serious problem. 10)

REFERENCES