Proposal of Membrane Transport Mechanism of Protein-Unbound Ulifloxacin into Epithelial Lining Fluid Determined by Improved Microdialysis

Makoto AOKI,* a Maki IGUCHI, a Hiroyuki HAYASHI, a Hisashi SUZUKI, a Shigeki SHIBASAKI, a
Tohru KUROSAWA, a and Masahiro HAYASHI b

a Applied Pharmacology Research Laboratories, Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd.; Yokohama 222–8567, Japan; and b School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; Hachioji, Tokyo 192–0392, Japan. Received January 9, 2008; accepted June 26, 2008; published online June 27, 2008

Microdialysis method (MD) is useful for sampling protein-unbound substances in vivo. Generally in the MD, a reference compound is used to correct differences in drug permeation clearance through a dialysis membrane in vivo and in vitro. No reference compound was, however, used for determination of a protein-unbound drug concentration in the epithelial lining fluid (ELF). In this study, we firstly examined the propriety of endogenous urea as a reference compound to determine the protein-unbound ulifloxacin concentrations in rat ELF by MD. Endogenous urea was used to correct differences in the permeation clearance in vivo and in vitro which reflect the differences in the extent of contact between a tip probe and ELF in vivo and in vitro. The results showed that our MD is applicable to determine the various concentrations of ulifloxacin and urea, and that we can use endogenous urea as a reference compound even if the extent of the contact between a tip of the probe and the ELF is small. In addition, use of urea concentrations does not affect drug distribution from plasma to ELF because we used endogenous urea. These results support usefulness of endogenous urea as a reference compound to determine protein-unbound drug concentration in ELF by MD. In addition, our results also suggest the existence of certain distribution mechanisms which cause the high penetration ulifloxacin into ELF. Our MD can help progress in pharmacokinetic-pharmacodynamic analysis of various antibiotics in the case where the concentrations in ELF are not equal to that in plasma.

Key words epithelial lining fluid; microdialysis method; ulifloxacin; urea; rat

There are various kinds of pneumonia causative pathogens such as Staphylococcus aureus, Streptococcus pneumoniae, Chlamydia pneumoniae, and Legionella pneumophila. These pathogens are classified into two categories, intracellular and extracellular infections. Chlamydia pneumoniae and Legionella pneumophila are classified into the intracellular infection group. In the case of pathogen-induced intracellular infection, penetration of antibiotics into macrophages and neutrophils is particularly important because pathogens propagate themselves in the cells.11 On the other hand, Staphylococcus aureus and Streptococcus pneumoniae are classified into the extracellular infection group.22 They invade the lung through the trachea, and are located in the epithelial lining fluid (ELF), which may lead to tissue damages.23 Determination of the protein-unbound drug concentration in ELF is therefore quite important to evaluate therapeutic efficacy of a drug.

If passive diffusion is the sole system on distribution of a drug from plasma to the infection site, the protein-unbound drug concentration profile in the infection site is considered to be almost the same as that in plasma. However, several antibiotic concentration profiles in the human ELF are different from those in plasma.4–6

Microdialysis method is useful for sampling protein-unbound drug concentrations in tissues and blood.7–9 Eisenberg et al.10 determined protein-unbound concentrations of tobramycin and vancomycin in ELF of rats using microdialysis method without a reference compound. However, in order to determine an accurate protein-unbound drug concentration in ELF by microdialysis method, we must correct differences in drug permeation clearance (PA) through a dialysis membrane in vivo and in vitro which reflect differences in the extent of contact between a tip probe and ELF in vivo and in vitro using a reference compound.

In this study, we firstly examined the propriety of endogenous urea as a reference compound to determine protein-unbound concentrations of new prodrug-type quinolone, ulifloxacin, in rat ELF by microdialysis method. In addition, we examined the existence of a distribution mechanism which causes high ulifloxacin penetration into ELF.

MATERIALS AND METHODS

Chemicals Ulifloxacin (Fig. 1) was synthesized in Nippon Shinyaku Co., Ltd. (Kyoto, Japan). Urea was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). As an internal standard, enoxacin was purchased from Sigma-Aldrich Japan (Tokyo, Japan). All the other chemicals were of the analytical grade.

Animal Male Sprague-Dawley (SD) rats, aged 8 weeks, were supplied by Charles River Japan (Tokyo, Japan). They were housed and handled according to the “Principles of

Fig. 1. Structure of Ulifloxacin

* To whom correspondence should be addressed. e-mail: makoto_aoki@meiji.co.jp © 2008 Pharmaceutical Society of Japan
Laboratory Animal Care” (NIH publication #85-23, revised 1985) and the “Guidance for the Care and Use of Laboratory Animals” (Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd.). The rats were kept in the temperature- and light-controlled environment with the standard food and tap water provided ad libitum.

Microdialysis Method Microdialysis probes of 70 mm length with 2 mm regenerated cellulose membrane tip were used (A-L-70-02, Eicom, Japan). For equilibration, these length with 2 mm regenerated cellulose membrane tip were infused to the rats to maintain a steady state concentration (ulifloxacin 0.2—5 μg/ml in distilled water), 0.1 ml of internal standard solution (2.5 μg/ml of enoxacin in 0.01 mol/l sodium hydroxide), and 2.7 ml of 10 mmol/l phosphate buffered solution (pH 6.0). The diluted sample was loaded on Waters Oasis HLB Extraction Cartridge (60 mg/3 ml). Each cartridge was washed with 3 ml of distilled water and 2 ml of 10% (v/v) methanol, and eluted with 2 ml of acetonitrile. The eluate was evaporated to dryness under nitrogen stream at 50 °C. The residue was dissolved in 0.1 ml of solvent mixture A (0.05 mol/l sodium hydroxide: methanol=1:2) prior to analysis. The portion (0.02 ml) of the sample was injected into HPLC.

A portion (0.05 ml) of dialysate, the Ringer’s solution or standard solutions (0.05—1 μg/ml) was diluted with 0.02 ml of solvent mixture A and 0.02 ml of internal standard solution (0.5 μg/ml of enoxacin in solvent mixture A). The solution was evaporated to dryness under nitrogen stream at 50 °C, and the residue was dissolved in 0.07 ml of solvent mixture A prior to analysis. The portion (0.05 ml) of the sample was injected into HPLC.

The concentrations of ulifloxacin in plasma, the Ringer’s solution containing ulifloxacin, dialysate, and BALF were determined by Shimadzu HPLC system (Kyoto, Japan), consisting of a pump (LC 10AD), an automatic sampler (SIL 10 AX L), a wavelength UV detector (SPD 10AV) and a software system (CLASS-LC10). Chromatographic separation was carried out with TSK-gel ODS-120T 5-μm (4.6 mm i.d.×250 mm, TOSOH, Tokyo, Japan). The mobile phase was composed of 0.05 mol/l KH2PO4—H2PO4 (pH 4) and acetonitrile (80:20, v/v). The flow rate was 1.0 ml/min. The column temperature was kept at 40 °C, and the UV detector wavelength was set at 275 nm.

All standard curves for ulifloxacin were linear (r2>0.995) in the concentration range 0.2—10 μg/ml (plasma), 0.05—1 μg/ml (dialysate) and 0.01—0.5 μg/ml (BALF). The inter- and intra-day precisions in the measurement of quality control (QC) samples were in the range 1.53—14.7% relative standard deviation (RSD) (plasma), 2.77—14.1% RSD (dialysate) and 2.75—13.7% RSD (BALF). The inter- and intra-day accuracies in the measurement of QC sample were in the range 93.0—98.8% (plasma), 91.7—102.4% (dialysate) and 96.2—112.2% (BALF). Over a period of 24 h injection time in the auto-sampler at 4 °C, accuracy and precision of ulifloxacin and urea in all samples were confirmed to be within 15% as the assay variability.

2. Urea: The urea concentrations in plasma, the Ringer’s solution, BALF, and dialysate were determined by urease-indophenol method.

Pharmacokinetic Analysis The R.R. of ulifloxacin and urea in vitro and R.R. of urea in vivo were calculated by the following equations:
RESULTS

1. Effect of Flow Rate on the Recovery of Ulifloxacin and Urea: The endogenous urea concentrations in plasma and dialysate at each flow rate were determined by our assay, and PA of ulifloxacin and urea, which were calculated by Eq. 3, were constant at the flow rates of 0.5—2.8 µl/min. These results showed that each flow rate of perfusate was concluded to be suitable for this experiment. We selected 0.75 µl/min of flow rate for the below experiments.

2. Effect of Ulifloxacin or Urea Concentration on the Recovery of Ulifloxacin and Urea: In order to examine the effect of ulifloxacin or urea concentrations on their recoveries, the R.R. were determined at various concentrations of them. The R.R. of ulifloxacin and urea, which were calculated by Eq. 1, were constant in 0.5—20 µg/ml and 50—1000 µg/ml, respectively (Figs. 2, 3). It was therefore demonstrated that the concentration of ulifloxacin or urea does not affect their R.R.

3. Determination of Protein-Unbound Concentrations of Ulifloxacin in ELF: The endogenous urea concentrations in dialysate and plasma were 61 and 184 µg/ml, respectively, and the R.R. of endogenous urea in vivo was 30.7% (Eq. 2) which was not significantly different from that in vitro. The concentration of ulifloxacin in the dialysate was 0.119 g/ml. The protein-unbound ulifloxacin concentration in ELF was calculated using the ulifloxacin concentration in the dialysate and plasma by the following equation (4).

\[
\text{Total ulifloxacin concentration in ELF} = \frac{\text{ulifloxacin concn. in dialysate}}{\text{R.R. of ulifloxacin in vitro} \times (\text{R.R. of urea in vivo} - \text{R.R. of urea in vitro})} 
\]

Total ulifloxacin concentration in ELF was calculated by the following equation\(^1\):

\[
\frac{\text{total ulifloxacin concn. in ELF}}{\text{ulifloxacin concn. in dialysate/external concentration of ulifloxacin in ELF}} = \frac{\text{ulifloxacin concn. in dialysate}}{\text{R.R. of ulifloxacin in vitro} \times (\text{R.R. of urea in vivo} - \text{R.R. of urea in vitro})} 
\]

where Ratio\(_{MD}\) is the ratio of protein-unbound ulifloxacin concentration in ELF to that in plasma (the data obtained by microdialysis method), and Ratio\(_{BAL}\) is the ratio of total ulifloxacin concentration in ELF to protein-unbound ulifloxacin concentration in plasma (the data obtained by BAL method). The mean ulifloxacin concentration in plasma which was sampled at 2, 3 and 4 h was used for the calculation of protein-unbound ulifloxacin concentration in plasma.

We calculated protein-unbound ulifloxacin concentration in plasma using the protein-binding ratio of ulifloxacin in plasma (49.5%).\(^{13}\)

Fig. 2. Effect of Ulifloxacin Concentration on the Relative Recovery of Ulifloxacin
Ulifloxacin concn.: 0.5, 1, 2, 10 and 20 µg/ml, flow rate: 0.75 µl/min. Values are expressed as the mean±S.E., n=3.

Fig. 3. Effect of Urea Concentration on the Relative Recovery of Urea
Urea concn.: 50, 135, 300 and 1000 µg/ml, flow rate: 0.75 µl/min. Values are expressed as the mean±S.E., n=3.

**Microdialysis Method**

1. Effect of Flow Rate on the Recovery of Ulifloxacin and Urea: The ulifloxacin and urea concentrations in dialysate obtained at each flow rate were determined by our assay, and PA of ulifloxacin and urea, which were calculated by Eq. 3, were constant at the flow rates of 0.5—2.8 µl/min. These results showed that each flow rate of perfusate was concluded to be suitable for this experiment. We selected 0.75 µl/min of flow rate for the below experiments.

2. Effect of Ulifloxacin or Urea Concentration on the Recovery of Ulifloxacin and Urea: In order to examine the effect of ulifloxacin or urea concentrations on their recoveries, the R.R. were determined at various concentrations of them. The R.R. of ulifloxacin and urea, which were calculated by Eq. 1, were constant in 0.5—20 µg/ml and 50—1000 µg/ml, respectively (Figs. 2, 3). It was therefore demonstrated that the concentration of ulifloxacin or urea does not affect their R.R.

3. Determination of Protein-Unbound Concentrations of Ulifloxacin in ELF: The endogenous urea concentrations in dialysate and plasma were 61 and 184 µg/ml, respectively, and the R.R. of endogenous urea in vivo was 30.7% (Eq. 2) which was not significantly different from that in vitro. The concentration of ulifloxacin in the dialysate was 0.119 g/ml. The protein-unbound ulifloxacin concentration in ELF was calculated using the ulifloxacin concentration in the dialysate and plasma by the following equation (4).

\[
\text{Total ulifloxacin concentration in ELF} = \frac{\text{ulifloxacin concn. in dialysate}}{\text{R.R. of ulifloxacin in vitro} \times (\text{R.R. of urea in vivo} - \text{R.R. of urea in vitro})} 
\]

Total ulifloxacin concentration in ELF was calculated by the following equation\(^1\):

\[
\frac{\text{total ulifloxacin concn. in ELF}}{\text{ulifloxacin concn. in dialysate/external concentration of ulifloxacin in ELF}} = \frac{\text{ulifloxacin concn. in dialysate}}{\text{R.R. of ulifloxacin in vitro} \times (\text{R.R. of urea in vivo} - \text{R.R. of urea in vitro})} 
\]

where Ratio\(_{MD}\) is the ratio of protein-unbound ulifloxacin concentration in ELF to that in plasma (the data obtained by microdialysis method), and Ratio\(_{BAL}\) is the ratio of total ulifloxacin concentration in ELF to protein-unbound ulifloxacin concentration in plasma (the data obtained by BAL method). The mean ulifloxacin concentration in plasma which was sampled at 2, 3 and 4 h was used for the calculation of protein-unbound ulifloxacin concentration in plasma.

We calculated protein-unbound ulifloxacin concentration in plasma using the protein-binding ratio of ulifloxacin in plasma (49.5%).\(^{13}\)

Peak area ratio of ulifloxacin to the internal standard (enoxacin) was used to obtain the ulifloxacin concentration in each sample.

The \(p\) values were computed using two-tailed Student’s \(t\)-tests. The \(p\) values <0.05 were considered a significant difference. All data are expressed as the mean±standard error (S.E.).
1.87

the dilution of ELF in BALF, was 5.2% at 3 h after the start
concentration in BALF to that in plasma, which represents
was determined by BAL method. Ratio of endogenous urea
range of constant R.R. in the
stant at the ulifloxacin concentration of 0.5—20 \( \mu \text{g/ml} \)
and 4 h after start of the infusion, which showed achievement
of the steady state, and the mean protein-unbound ulifloxacin
were 0.96 ± 0.073, 1.0 ± 0.095 and 0.82 ± 0.027 \( \mu \text{g/ml} \) at 2, 3
and 4 h after start of the infusion, which showed achievement
of the steady state, and the mean protein-unbound ulifloxacin
concentration in plasma was 0.476 \( \mu \text{g/ml} \). Accordingly the
ratio of protein-unbound ulifloxacin concentration in ELF to
that in plasma was 2.90 (Table 1).

**BAL Method** The total ulifloxacin concentration in ELF
was determined by BAL method. Ratio of endogenous urea
concentration in BALF to that in plasma, which represents
the dilution of ELF in BALF, was 5.2% at 3 h after the start
of infusion. The total ulifloxacin concentration in ELF was
1.87 \( \mu \text{g/ml} \) and the protein-unbound ulifloxacin concentration
in plasma was 0.407 \( \mu \text{g/ml} \). The ratio of the total ulifloxacin concentration in ELF to the protein-unbound ulifloxacin concentration in plasma was 4.59 (Eq. 5, Table 2).

**Protein-Unbound Ratio of Ulifloxacin in ELF** The protein-unbound ratio of ulifloxacin in ELF was calculated to be 63.2 ± 5.0% from the data obtained by the microdialysis method and the BAL method (Eq. 6).

**DISCUSSION**
In this study, we showed that our microdialysis method is applicable to determine the various concentrations of ulifloxacin and urea, and that endogenous urea can be used as a reference compound to determine protein-unbound drug concentrations in ELF by the microdialysis method. Our results also suggest the existence of certain distribution mechanisms which cause the high ulifloxacin penetration into ELF.

The protein-unbound concentrations of ulifloxacin in ELF and endogenous urea concentrations in plasma were 1.35—
1.45 \( \mu \text{g/ml} \) and 141—253 \( \mu \text{g/ml} \), respectively. The in vitro
data showed that the R.R. of ulifloxacin and urea were con-
stant at the ulifloxacin concentration of 0.5—20 \( \mu \text{g/ml} \)
and the urea concentration of 50—1000 \( \mu \text{g/ml} \), respectively. Each
range of constant R.R. in the in vitro study was much wider
compared with that in the in vivo study. These results showed
that our microdialysis method is applicable to determine the
various concentrations of ulifloxacin and urea in ELF.

In this study, we maintained anesthesia assiduously to fix a probe which can move as a rat breathes. Actually, there was not significant difference of R.R. between in vivo and in vitro, suggesting that the ELF most always soaked the tip of a probe. A reference compound is necessary to correct differences in drug permeation clearance through a dialysis membrane in vivo and in vitro. In addition, if no reference compound was used in a microdialysis method when ulifloxacin was not detected in the ELF, we cannot conclude whether there is not ulifloxacin or the ELF does not soak the tip of a probe. Therefore, a reference compound is necessary to determine protein-unbound ulifloxacin concentrations in the rat ELF.

As a reference compound, we used endogenous urea which is generally used as a marker of dilution in BAL method. Since urea has a low molecular mass and diffuses across the lung, urea in ELF is in equilibrium with urea in plasma, thus urea concentration in ELF is assumed to be equal to that in plasma. Practically, direct measurements of urea in ELF of fetal sheep demonstrated agreement between urea concentrations both in plasma and ELF. As in the case of the fetal sheep, the ELF volume (0.26 ml/rat), which was calculated from the ratio of urea concentration in BALF to that in plasma (5.2%) and volume of Ringer’s solution (5 ml), was similar to ELF volume in a previous report (0.75 ml/kg body weight). In addition, the epithelial basement membrane surface area (3107 \( \text{cm}^2/\text{rat} \)) and thickness of ELF (0.02—1.3 \( \mu \text{m} \)) suggests the ELF volume is the range of 0.00621 to 0.404 ml/rat. These results support that urea concentration in ELF is equal to that in plasma.

In the present study, constant R.R. of urea in 50—
1000 \( \mu \text{g/ml} \) and small variation in in vivo urea recoveries demonstrated that the concentration of urea has no effect on the R.R. (Fig. 3). In the in vivo study, the lowest urea concentrations in the dialysate was 61 \( \mu \text{g/ml} \), and the lower limit of quantification (LLOQ) of urea was 2 \( \mu \text{g/ml} \), suggesting that we can use endogenous urea as a reference compound even if the extent of the contact between a tip of the probe and the ELF in other in vivo study is smaller than that in our in vivo study. In addition, use of urea concentrations does not affect distribution of a drug from plasma to ELF because we used endogenous urea. These results support that endogenous urea can be used as a reference compound to determine protein-unbound drug concentrations in ELF by the microdialysis method.

Protein concentrations in rat ELF are 4.78 mg/ml and rat serum protein binding ratio of ulifloxacin is 49.5%, suggesting that a part of ulifloxacin binds to proteins in rat ELF.

The ratio of protein-unbound ulifloxacin concentration in ELF to that in plasma was 2.90, suggesting the existence of certain distribution mechanisms (an active uptake from plasma to lung, an active efflux from lung to ELF, saturation of active uptake from ELF to lung and saturation of active efflux from lung to plasma) which cause the high ulifloxacin penetration into ELF. In the lung, there are some transporters such as peptide transporter (PEPT2), multidrug resistance-associated protein (MRP), and glucose transporter (Glut-1,
Quinolone is a substrate of multidrug resistance (MDR1), MRPL, and MRPB organic cation transporter (OCT2, human), and organic anion transporter (OAT1). azithromycin highly distributes that binding of grepafloxacin to phosphatidylserine is in rat ELF and alveolar macrophages are higher than that levofloxacin, ciprofloxacin and moxifloxacin concentrations (OCT2, human), organic anion transporter (OAT1), organic cation transporter (OCT1), and organic cation transporter (OCT2). The distribution of ulifloxacin from plasma to ELF therefore reflects the antibiotic efficacy in treatment of extracellular pathogens because the alveolar macrophages deliver azithromycin to ELF. Grepafloxacin, levofloxacin, ciprofloxacin and moxifloxacin concentrations in ELF and alveolar macrophages are higher than that in rat plasma, suggesting the possibility that alveolar macrophages are important vehicles for delivering new quinolones to ELF. Ulifloxacin is one of new quinolones, so delivery by alveolar macrophages may involve penetration process of ulifloxacin into ELF. Ulifloxacin is one of new quinolones, so delivery by alveolar macrophages may involve penetration process of ulifloxacin into ELF. Transporters, phosphatidylserine and/or alveolar macrophages may contribute to the distribution of ulifloxacin from plasma to ELF. The permeability of ulifloxacin from plasma to ELF is therefore necessary to clarify the distribution mechanism of ulifloxacin from plasma to ELF.

Water-soluble β-lactam antibiotics are mainly distributed in the extracellular component. The β-lactam antibiotics have good efficacy against pathogens in the extracellular component. The protein-unbound drug concentration in ELF therefore reflects the antibiotic efficacy in treatment of extracellular infections in the lower respiratory tract. Our microdialysis method can help progress in pharmacokinetic-pharmacodynamic analysis of various antibiotics which concentrations in ELF are not equal to that in plasma.

In conclusion, we showed that the protein-unbound concentration of ulifloxacin in ELF can be determined by using our microdialysis method, and that endogenous urea can be used as a reference compound to determine protein-unbound drug concentrations in ELF by the microdialysis method. The ratio of protein-unbound ulifloxacin concentration in ELF to that in plasma suggests the existence of certain distribution mechanisms which cause the high ulifloxacin penetration into ELF. Our microdialysis method can be of much help to proceed in pharmacokinetic-pharmacodynamic analysis of various antibiotics.

REFERENCES