BIOPHARMACEUTICS & DRUG DISPOSITION
Biopharm. Drug Dispos. **21**: 313–319 (2000)
DOI: 10.1002/bdd.245

Influence of Dose on the Distribution Kinetics of Ciprofloxacin and Ofloxacin in the Isolated Hindlimb of the Rat

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ABSTRACT: The aim of this study was to determine whether the dose influences the distribution kinetics of ciprofloxacin and ofloxacin in muscle- bone- and skin-tissues included in the isolated hindlimb of the rat. Experiments were carried out in the isolated perfused hindlimb of the rat, administering a single dose of 45, 450 or 900 μg of each quinolone as a bolus injection. Outflow perfusate samples were collected for 20 min and drug levels were determined by an HPLC technique. The mean transit time (MTT) and the distribution volume of ciprofloxacin significantly increased with the dose injected (MTT = 1.47 ± 0.69, 8.74 ± 0.27 and 9.52 ± 2.95 min for 45, 450 and 900 μg, respectively). A similar situation was observed with ofloxacin, although the increase in these parameters was less pronounced (MTT = 3.65 ± 0.86, 7.92 ± 2.03 and 8.32 ± 1.70 min for 45, 450 and 900 μg, respectively). The distribution of ciprofloxacin and ofloxacin in the rat hindlimb appears to be a dose-dependent process, at least for the dose range considered in this study. This might explain the high variability in the distribution coefficients reported for these drugs in literature. Copyright © 2000 John Wiley & Sons, Ltd.

Key words: ciprofloxacin; dose-dependent kinetics; isolated hindlimb; ofloxacin; quinolones distribution

Introduction

The quinolones ciprofloxacin and ofloxacin are widely used in clinical practice for the treatment of infections affecting most body spaces such as the urinary tract [1–3], the respiratory system [4,5] and gynaecologic [6] and prostatic tissues [7] among others. A high steady state distribution coefficient reported for humans—due to a low degree of plasma protein binding (about 20%) and a high lipophilicity [8]—is a useful pharmacokinetic property for antimicrobial agents, since it indicates that they can access most body spaces and consequently be of value in the treatment of infectious processes located in different body tissues. Values of 2–5 L/kg [9–11] and 1–3 L/kg [12,13] have been reported for ciprofloxacin and ofloxacin, respectively, for a human therapeutic dose range of 200–750 mg. The influence of the dose on the pharmacokinetics of these quinolones has been studied regarding the absorption process and a dose-independent absorption with an apparent first-order kinetics for the studied dose range has been reported [14,15]. Regarding literature information about quinolone distribution, most data refer to the distribution volume and the partition coefficient values for different tissues at steady state, no attention having been paid to the influence of the dose on this process.

The aim of this study was to investigate the possible influence of dose on the distribution kinetics of ciprofloxacin and ofloxacin using the isolated hindlimb of the rat as an experimental model for drug distribution [16] since this experimental preparation provides outflow tissue

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Received 20 July 2000
Accepted 10 April 2001

curves, allowing comparative analysis and determination of the effect of dose, if any.

Material and Methods

Surgical Procedure

Experiments were carried out using 3-month-old female Wistar rats (mean body weight = 160 ± 20 g) in compliance with 'Principles of Laboratory Animal Care'. The animals were maintained with access to food until 12 h prior the start of the experiment and with water *ad libitum* until the surgical procedure, which is a modification of the experimental method described by Ruderman [17].

Briefly, the method consists in isolating the left hindlimb of the anaesthetized rat (sodium pentobarbital, 40 mg/kg, i.p.) by placing ligatures in all vessels emerging and flowing into the abdominal aorta and inferior vena cava, respectively; isolation and ligation of the internal-iliac vessels, the inferior epigastric vessels and the pudendal vessels is also necessary to restrict perfusion to the hindlimb. Additional ligatures are placed around the contralateral iliac vessels and the lowest joint of the perfused limb in order to exclude the paw from the perfusion circuit.

After ligation, a cannula is first inserted into the inferior vena cava, pulled down until the bifurcation of the common iliac vessels and tied. Then, another cannula is placed into the abdominal aorta at the same level as the previous one and fixed by tying. The artificial perfusion is started immediately using a modified Tyrode medium, with 4% dextran and 3% albumin, kept at 25°C, oxygenated with carbogen and supplied at a flow rate of 3 mL/min. Cannulation of the artery after the vein reduces the ischaemic period to 2–4 s and avoids reperfusion injury in the preparation. The animals were then overdosed with pentobarbitone administered as an intrathoracic injection. After a 20 min stabilization period, a single dose of drug (45, 450 or 900 μg) was injected through the cannula in the aorta as bolus injection and outflow sampling was begun at programmed times, using a fraction collector connected to the cannula in the vena cava. Samples were collected for 20 min at different frequency intervals. Five experiments were carried out for each drug dose.

Analytical Methods

Quantification of ciprofloxacin and ofloxacin in outflow perfusate samples was carried out by a HPLC, ion paired and reverse phase technique, widely used for these drugs [18]. The stationary phase was Nucleosil 120–C_{18} (5 μm) packed in a 15 cm column. The mobile phase was 90% phosphate buffer containing 40% of hydrogen sulphate tetrabutil ammonium (final pH 3) and 10% acetronitrile and the flow rate was 2 mL/min. A fluorescence detector was used with $\lambda_{excitation}$ = 277 nm, $\lambda_{emission} = 445$ nm and $\lambda_{excitation} = 330$ nm; $\lambda_{emission} = 450$ nm for ciprofloxacin and ofloxacin, respectively.

The internal standard method was used for quantification, with ciprofloxacin as internal standard for ofloxacin samples and *vice versa*. Two calibration curves with standard solutions were prepared for each quinolone in Sorensen buffer (pH 7.4) at concentration ranges of 1–0.01 and 1–50 μg/mL.

Standard solutions and samples underwent the same procedure: 50 μL of internal standard solution and 100 μL of tricloroacetic acid solution (20%) were added to 100 μL of sample of standard; the mixture was vigorously shaken for 15 s and centrifuged at 410 × *g* for 5 min; then, 100 μL of the supernatant was injected into the chromatograph for analytical determination. Using these experimental conditions, the analytical technique proved to have a detection limit of 0.01 μg/mL for both drugs and the intra- and inter-day coefficients of variation showed values lower than 5% and 6% for ciprofloxacin and ofloxacin, respectively.

Determination of Drug Protein Binding

The fraction of ciprofloxacin bound to albumin in the perfusate was determined by equilibrium dialysis and microdialysis techniques.

Equilibrium dialysis was performed using an equilibrium dialysis apparatus (Dianorm®) with two-chambered Teflon dialysis cells (1 mL each) separated by a dialysis membrane (Diachema®, type 10.16). Prior to use, these membranes were

rinsed and washed with distilled water followed by conditioning in the dialysing buffer. Preliminary studies showed no adsorption of ciprofloxacin to membrane and cell material used in the equilibrium dialysis. One millilitre of perfusate was placed in one chamber of the dialysis cell and this was dialysed against an equal volume of ciprofloxacin solution ($C_i = 25$ μg/mL) of the same composition as the perfusate without albumin. After a 4-h equilibrium period, the concentration of ciprofloxacin in the albumin-free solution was determined.

In vitro microdialysis was also carried out to determine the unbound fraction of ciprofloxacin in the perfusate. Probes were made in our laboratory using silica and polyethylene tubing and HOSPALAN 69 dialysis membranes. The probe was introduced into a 5 μg/mL ciprofloxacin solution in Tyrode (3% albumin) medium and it was perfused with ciprofloxacin Ringer solutions of 1, 7.5 and 10 μg/mL at a flow rate of 2 μL/min. After a 1-h probe stabilization period for each initial concentration, three 10 min outflow replicate samples (20 μL) were obtained and directly injected into the HPLC system connected on line to the microdialysis equipment.

Data Analysis

Statistical moments ($M_n = \int_0^\infty t^n C(t)\,dt$) were estimated by numerical integration up to the last time point, using the slope value of the terminal linear phase of the curve for extrapolation to infinity. The relative dispersion of transit times is defined as $CV^2 = VTT/MTT^2 = (M_2M_0/M_1^2) - 1$, where VTT denotes the variance of the transit time distribution and M_0, M_1 and M_2 are estimated from the general equation with $n = 0$, 1 and 2, respectively [19,20]. The distribution volume, V_d, is then obtained as the product of the mean transit time ($MTT = M_1/M_0$) and the flow rate. Additional experiments were carried out in the same experimental conditions as described above but in the absence of the hindlimb to estimate the influence of the catheter for the correction of statistical moments obtained from the outflow curves.

The fraction of unbound drug (f_u) was estimated from equilibrium dialysis data according to the following expression:

$$f_u = \frac{C_f}{C_i - C_f}$$

where C_i and C_f represent the initial concentration of ciprofloxacin in the Tyrode (3% albumin) medium and the final ciprofloxacin concentration determined in the albumin-free solution, respectively.

The zero net flux method was applied to the microdialysis data [21,22]; accordingly, the net change in the ciprofloxacin concentration in the dialysate ($C_{out} - C_{in}$) was plotted against the initial probe perfusate concentration (C_{in}) and the statistical analysis of the linear regression was performed. The intercept of the plot with the x-axis is the point of no net flux, which is equal to the unbound concentration of ciprofloxacin in the Tyrode (3%) albumin medium.

Statistical comparison of parameter values was carried out by a two-way ANOVA test, at a 0.05 significant level.

Results

Figure 1 shows the results corresponding to ciprofloxacin and Figure 2 includes those obtained with ofloxacin. These curves represent the means of the experimental drug levels normalized by the corresponding injected dose; that is, the mean unit disposition functions (UDF) obtained with each dose. Differences in these curve

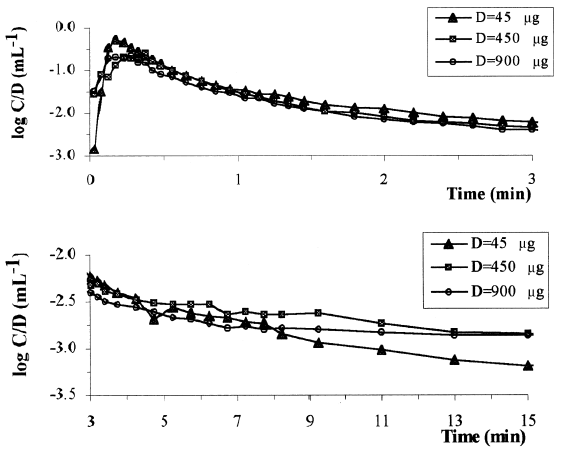

Figure 1. Mean outflow curves normalized by the injected dose (UDF) corresponding to ciprofloxacin

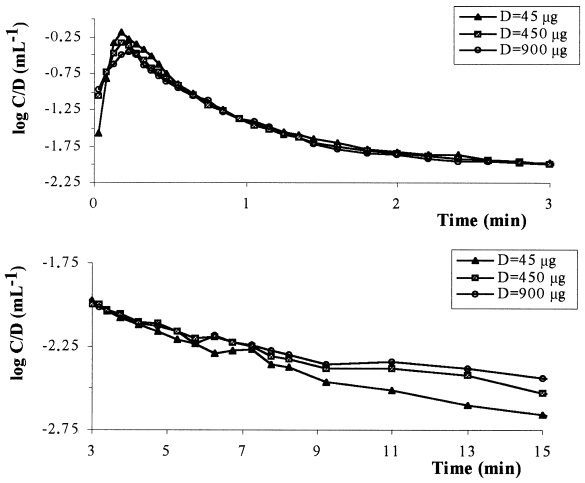

Figure 2. Mean outflow curves normalized by the injected dose (UDF) corresponding to ofloxacin

profiles directly reflect the modifications in the kinetic behaviour of the drug when administered at different doses. The outflow curve corresponding to the lowest dose of ciprofloxacin shows relevant differences in comparison to the curve corresponding to the higher doses, which exhibit a similar profile. The differences between the dose of 45 µg and the higher doses affect both the initial and final phases of the curve. For ofloxacin, the differences between the curve corresponding to the lowest dose and the profiles obtained with 450 and 900 µg are not so relevant. Nevertheless, a progressive decrease in the peak value is observed with the increase of dose and differences among the slope values of the final linear phase of each dose curve are also seen.

From the statistical analysis of the raw data, a series of parameters was calculated taking into account, and consequently correcting for the influence of the catheter (MTT $= 0.35 \pm 0.07$ min) on the experimental data. Tables 1 and 2 include the mean values of the statistical moments and distribution volumes (V_d) obtained for ciprofloxacin and ofloxacin at different doses, respectively, the mean values of the slope of the linear phase of the curves are also included. The above-mentioned differences affecting the curve profiles are quantified in the parameter values. For both quinolones, the MTT increased with the dose; accordingly the distribution volume also increases. By contrast, the value of the slope decreased with the dose injected in all cases.

The unbound fraction of ciprofloxacin in the perfusate determined from equilibrium dialysis data was 0.78 ± 0.11 and the corresponding value obtained by microdialysis was 0.88, calculated from the x-axis intercept of the regression line included in Figure 4.

Discussion

Studies on drug distribution kinetics face the difficulty of the tissue sampling required to characterize the tissue curve profile. In this sense, the experimental techniques of isolation and perfusion of body tissues provides a methodology suitable for the study of distribution kinetics and this has recently been used in pharmacokinetics for this purpose [23–26]. The isolated hindlimb of the rat is a particular example of this type of experimental model that is very useful for the study of quinolone distribution since the tissues included in this preparation (muscle, bone and skin) show a high affinity for these drugs.

Our results concerning the influence of the dose injected on the distribution kinetics of ciprofloxacin and ofloxacin reveals that this process depends on the dose. In the case of ciprofloxacin, the dose of 45 µg afforded a curve profile that was significantly different from that

Table 1. Mean values and standard deviation of parameters estimated from the outflow curves of ciprofloxacin

Dose (µg)	AUC_0^∞ (µg min/mL)	MTT (min)	CV^2	V_d (mL)	Slope (1/min)
45	9.98 ± 2.71	1.47 ± 0.69	7.37 ± 1.94	4.42 ± 2.07	0.17 ± 0.07
450	83.74 ± 7.84	8.74 ± 0.27	3.93 ± 1.22	26.23 ± 0.8	0.045 ± 0.007
900	154.24 ± 40.06	9.52 ± 2.95	5.16 ± 1.92	28.56 ± 8.84	0.037 ± 0.007

Table 2. Mean values and standard deviation of parameters estimated from the outflow curves of ofloxacin

Dose (μg)	AUC_0^∞ (μg min/mL)	MTT (min)	CV^2	V_d (mL)	Slope (1/min)
45	15.37 ± 1.45	3.65 ± 0.86	5.03 ± 1.59	10.96 ± 2.58	0.09 ± 0.011
450	147.45 ± 13.50	7.92 ± 2.03	3.61 ± 0.54	23.76 ± 6.09	0.056 ± 0.011
900	289.48 ± 24.26	8.32 ± 1.70	3.10 ± 0.56	24.96 ± 5.09	0.06 ± 0.013

corresponding to the other two doses, which showed *quasi*-superimposed curves. Thus, MTT and the V_d had mean values of 1.47 min and 4.42 mL for the lower dose versus 8.74 or 9.52 min and 26.23 or 28.56 mL for the doses of 450 and 900 μg, respectively. Similarly, the slope of the linear phase of the curve showed a significantly higher value for the dose of 45 μg (0.17/min) as compared to the doses of 450 and 900 μg (0.045 and 0.037/min, respectively). All these parameter values point to a more restrictive access of ciprofloxacin to hindlimb tissues (a lower distribution coefficient value) as well as a more rapid tissue washout (higher slope value) when administered at the lowest dose.

For ofloxacin, a similar situation was observed since the MTT and the V_d increased with the dose and the slope of the curve also had higher values for the lower doses. Nevertheless, interesting differences between ciprofloxacin and ofloxacin appear as regards the way the dose influences drug distribution. For the latter quinolone, the UDF profile changed progressively with the dose while the former showed no progressive differences, however, the UDF profiles for the lowest dose differed from the profiles for the two higher doses which were similar. The high values of CV^2 (a parameter which provides information about the dispersion of the drug in the system), obtained for all doses and both quinolones can be attributed to two main reasons; first, the hindlimb is a complex preparation with a heterogeneous capillary network and solute trapping zones [23], leading to high dispersion of transit times and second, the quinolones bind to tissue structures, which also leads to high CV^2 values.

A mechanistic interpretation of these results should be based on the physiological process controlling the access and permanence of drugs in body tissues. These include convection depending on flow rate, vascular dispersion, membrane diffusion and binding kinetics. Since flow rate remained constant in all our experiments (3 mL/min), changes in the other three processes might be responsible for the observed differences. According to Rowland *et al*. [27,28], vascular dispersion depends on the characteristics of the capillary network, which also remained constant for all doses. Consequently, the differences could be attributed to changes in membrane diffusion or tissue binding, since an increase of the free fraction of the drug in the perfusate is not likely for drugs whose plasma protein binding reported value is about 20%. Our results from equilibrium dialysis and microdialysis experiments ($f_u = 0.78 \pm 0.11$ and $f_u = 0.88$, respectively) reveal that the unbound fraction of ciprofloxacin in artificial perfusate is not different from the reported data for plasma. For ofloxacin f_u was not determined in the perfusion medium but it was assumed to behave in a similar manner to ciprofloxacin and therefore the literature plasma value ($f_u = 0.8$) was used.

A slower diffusion process would explain the lower V_d value corresponding to the dose of 45 μg. Although the diffusion coefficient is not expected to show changes with concentration, such a concentration-dependence exists in most systems, but often, e.g. in dilute solutions, the dependence is slight and the diffusion coefficient can be assumed constant for practical purposes; accordingly, exponential and linear concentration dependent diffusion have been considered by Wagner and Stokes, respectively [29]. Under this assumption, a slower diffusion for the lowest dose might contribute to the lower V_d value. Another possible hypothesis is based on the findings of Wu *et al*. [30], who showed that interstitial volume of the isolated hindlimb is affected by perfusate composition. An increase of the osmotic pressure due to an increase of dose might elicit physiological changes.

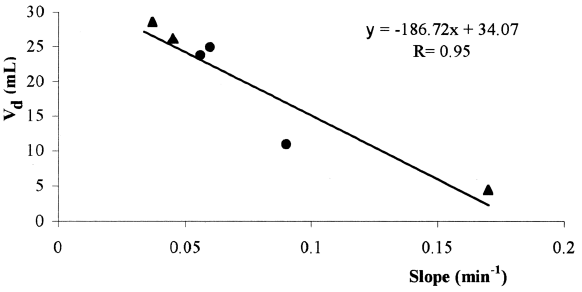

Figure 3. Linear relationship (significant level for the slope, $p = 0.0045$) established between the distribution volume and the slope of the final phase of the curves obtained for the different doses of ciprofloxacin (□) and ofloxacin (●)

Nevertheless, this is not very likely because no reduction in outflow volume recovery was detected.

The modifications in the tissue binding processes (tissue washout) quantified by the slope of the final linear phase of the curves was the most evident finding related to dose changes observed. Figure 3 depicts the linear relationship established between the distribution volume and the slope of the final phase of the curves showing that for these drugs tissue binding strongly influences the distribution volume values. This relationship is mechanistically related to the equation defined for the elimination rate constant ($K_e = CL/V_d$) and implies an inverse relationship between both parameters.

The importance of tissue binding in quinolone distribution has been reported previously [28] after finding that a model considering the kinetics of this process is the most suitable one for fitting this type of experimental data.

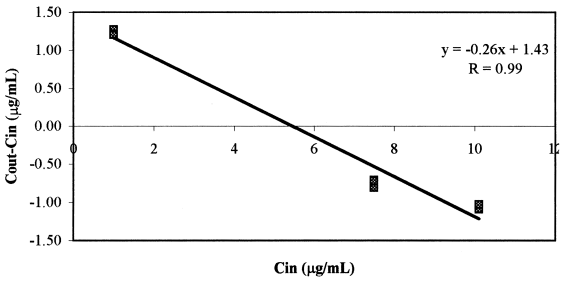

Figure 4. Linear relationship between the net change in the dyalisate concentration and the initial ciprofloxacin concentrations used to estimate, by microdialysis, the unbound fraction of the drug in the Tyrodes (3% albumin) medium

The apparent dose-dependent distribution kinetics shown by ciprofloxacin and ofloxacin might have implications for the administration of these drugs in clinical practice; administration of the lowest therapeutic doses might lead to tissue levels lower than those expected, with the corresponding risk of clinical failure. On the other hand, the slower tissue washout shown by the higher doses may suggest the possibility of extending the dosage interval when high doses are administered. Although these implications need to be confirmed with live animals and additional studies carried out in humans, our results evidence that distribution is a complex kinetic process depending on several factors, particularly in the case of drugs with a high affinity for body tissues. In this sense, the techniques of isolation and artificial perfusion of body tissues facilitate the study of the intrinsic mechanisms underlying drug distribution, revealing the major factors that influence the kinetics of the process and, consequently, those which should be considered in the more restricted human experiments.

Acknowledgements

This work was supported by the DGICYT (Spanish Ministry of Sciences and Education) Research Project PB92-0280.

References

1. Hooton TM, Latham RH, Wong ES, Johnson C, Roberts PL, Stamm WE. Ofloxacin versus trimetoprim-sulfamethoxazole for treatment of acute cystitis. *Antimicrob Agents Chemother* 1989; **33**: 1308–1312.
2. Blomer R, Bruch K, Zahlten RN. Zussamengefasste ergebnise der clinischen phase II und III studien mit ofloxacin (HOE 280) in Europa. *Infection* 1986; **14**(Suppl 1): 102–107.
3. Cox CE, Caller SV, Track KJ. Clinical experience with ofloxacin in urinary tract infection. *Infection* 1986; **14**(Suppl): S303–S304.
4. Paton JH, Reeves DS. Fluoroquinolone antibiotics: microbiology, pharmacokinetics and clinical use. *Drugs* 1988; **36**: 193–228.
5. Todd PA, Faulds D. Ofloxacin: a reappraisal of its antimicrobial activity, pharmacology and therapeutic use. *Drugs* 1991; **42**: 825–876.
6. Hooper DC, Wolfson JS. Treatment of genitourinary tract infections with fluoroquinolones: clinical efficacy in genital infections and adverse effects. *Antimicrob Agents Chemother* 1989; **33**: 1662–1667.

7. Weidner W, Schiefer HG, Dalhoff A. Treatment of chronic bacterial prostatitis with ciprofloxacin. Results of one year follow-up study. *Am J Med* 1987; **82**(Suppl 4A): 280–283.
8. Ichikawa N, Naora K, Iwamoto K. Comparative study of permeability into rat cerebrospinal fluid of the quinolones: dependency on their lipophilicities. *Biol Pharm Bull* 1994; **17**: 152–155.
9. Drusano GL, Plaisance KI, Forrest A, Standiford HC. Dose ranging study and constant infusion evaluation of ciprofloxacin. *Antimicrob Agents Chemother* 1986; **30**: 440–443.
10. Allard S, Kinzig M, Boivin G, Sörgel F, LeBel M. Intravenous ciprofloxacin disposition in obesity. *Clin Pharmacol Ther* 1993; **54**: 368–373.
11. Dudley MN, Mandler HD, Gilbert D, Ericson J, Mayer KH, Zinner SH. Pharmacokinetics and pharmacodynamics of intravenous ciprofloxacin. Studies *in vivo* and in an *in vitro* dynamic model. *Am J Med* 1987; **82**: 363–368.
12. Leroy A, Borsa F, Humbert G, Bernadet P, Fillastre JP. The pharmacokinetics of ofloxacin in healthy adult male volunteers. *Eur J Clin Pharmacol* 1987; **31**: 629–630.
13. Yuk JH, Nightingale CH, Quintiliani R, Sweeney KR. Bioavailability and pharmacokinetics of ofloxacin in healthy volunteers. *Antimicrob Agents Chemother* 1991; **35**: 384–386.
14. Tartaglione TA, Raffalovich AC, Poynor WJ, Espinel-Ingroff A, Kerkering TM. Pharmacokinetics and tolerance of ciprofloxacin after sequential increasing oral doses. *Antimicrob Agents Chemother* 1986; **29**: 62–66.
15. Verho M, Malerczyk V, Dragosa E, Korn A. Dose linearity and other pharmacokinetics of ofloxacin: a new, broad spectrum antimicrobial agent. *Pharmatherapeutica* 1985; **4**: 376–382.
16. Casquero-Dorado AC, Sánchez-Navarro A. The isolated hindlimb preparation as a drug distribution model. *Methods Find Exp Clin Pharmacol* 1996; **18**: 453–458.
17. Ruderman NH, Houghton CRS, Hems R. Evaluation of the isolated perfused rat hindquarters for the study of muscle metabolism. *Biochem J* 1971; **124**: 639–651.
18. Colino CI, García Turiño A, Sánchez Navarro A, Lanao JM. A comparative study of ofloxacin and ciprofloxacin erythrocyte distribution. *Biopharm Drug Dispos* 1998; **19**: 71–77.
19. Yamaoka K, Nakagawa T, Uno T. Statistical moments in pharmacokinetics. *J Pharmacokin Biopharm* 1978; **6**: 547–558.
20. Weiss M. Distribution kinetics in the body and single organs: moment analysis. In *Advanced Methods of Pharmacokinetic and Pharmacodynamic Systems Analysis*, vol. 2, D'Argenio DZ (ed.). Plenum Press: New York, 1995; 89–100.
21. Stahle L, Segersvard S, Ungersted U. A comparison between three methods for estimation of extracellular concentrations of exogenous and endogenous compounds by microdialysis. *J Pharmacol Methods* 1990; **25**: 41–52.
22. Nolting A, Dalla Costa T, Vistelle R, Rand KH, Derendorf H. Determination of free extracellular concentrations of piperacillin by microdialysis. *J Pharm Sci* 1996; **85**: 369–372.
23. Oliver RE, Heatherington AC, Jones AF, Rowland M. A physiologically based model incorporating dispersion principles to describe solute distribution in the perfused rat hindlimb preparation. *J Pharmacokin Biopharm* 1997; **25**: 389–412.
24. Weiss M, Koester A, Wu Z, Roberts MS. Distribution kinetics of diazepam, lidocaine and antipyrine in the isolated perfused rat hindlimb. *Pharm Res* 1997; **14**: 1640–1643.
25. Reinoso RF, Sánchez-Navarro A, Lanao JM. Distribution of ciprofoxacin in the isolated rat lung in the presence and absence of tissue oedema. *Eur J Pharm Sci* 1999; **8**: 203–209.
26. Sánchez-Navarro A, Casquero-Dorado AC, Weiss M. Distribution and binding kinetics of ciprofloxacin and ofloxacin in the hindlimb of the rat. *Pharm Res* 1999; **16**: 587–599.
27. Roberts MS, Rowland M. A dispersion model of hepatic elimination: 1. Formulation of the model and bolus consideration. *J Pharmacokinet Biopharm* 1986; **14**: 227–260.
28. Roberts MS, Rowland M. A dispersion model of hepatic elimination: 2. Steady-state considerations—influence of hepatic blood flow, binding within blood and hepatocellular enzyme activity. *J Pharmacokinet Biopharm* 1986; **14**: 261–288.
29. Crank J. Concentration-dependent diffusion: methods of solution. In *The Mathematics of Diffusion* (2nd edn). Oxford Science Publications, Clarendon Press: Oxford, 1992; 104–136.
30. Wu ZY, Rivory LP, Roberts MS. Physiological pharmacokinetics of solutes in the isolated perfused rat hindlimb: characterization of the physiology with changing perfusate flow, protein content and temperature using statistical moment analysis. *J Pharmacokin Biopharm* 1993; **21**: 653–688.

