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Comparative study of the disposition of levofloxacin, netilmicin and cefepime in the isolated rat lung

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Abstract

An experimental model of artificially perfused and mechanically ventilated lung has been applied to compare the kinetic behaviour of levofloxacin, cefepime and netilmicin in this body tissue. The study has been performed to explore the usefulness of the isolated lung technique in the pharmacokinetic field, particularly to study the disposition of antibiotics in pulmonary tissue. The lung was perfused with Krebs-Henseleit medium containing 3% bovine albumin at a flow rate of 5 mL min^{-1}. It was ventilated at 60 respirations/min with a 2-mL tidal volume of air previously humidified and warmed to 37°C. The concentrations of the above antibiotics were determined by HPLC techniques and the outflow curves were analysed by stochastic, as well as by model-dependent, methods. The results show pharmacokinetic differences among these antibiotics, which are in accordance with previously reported data, levofloxacin being the drug with the highest distribution coefficient in this tissue (1.25 ± 0.14 vs 0.39 ± 0.07 and 0.41 ± 0.06 mL g^{-1} for netilmicin and cefepime, respectively). Accordingly, the isolated lung of the rat, under the experimental conditions used here, constitutes an alternative model to be incorporated to pharmacokinetic studies with a great potential use for those drugs that show a pharmacological or toxicological action depending on the kinetic profile in the lung tissue.

Introduction

Respiratory infections constitute one of the most frequent diseases in clinical practice, affecting hospitalized as well as community patients; antimicrobial agents are the main resource available for the treatment of this pathology. Choice of the most appropriate antibiotic from among the extensive range of this type of drug should be based on a combination of pharmacodynamic and pharmacokinetic criteria (Klutman 1996; Nicolau 2002) to guarantee antimicrobial potency and exposure of the pathogen to the drug at the site of infection. Both potency and exposure determine the clinical response to antimicrobial agents. Pharmacokinetic parameters provide information about the kinetic profile of the drug in the organism and therefore about the degree of exposure, although this is generally restricted to plasma levels and very little information about the kinetic profile in specific body tissues is available. Levofloxacin, netilmicin and cefepime are antimicrobial agents that follow different types of kinetic behaviour regarding distribution processes. The fluoroquinolone shows a broad distribution and reaches concentrations above the corresponding plasma levels in most body tissues (Fish & Chow 1997). Thus, a distribution coefficient of 0.99–1.47 L kg^{-1} has been reported in man for this drug (Chien et al 1997; North et al 1998; Zhanel et al 2002). The aminoglycoside and the cephalosporin, however, show a restricted tissue access, with distribution coefficient values of 0.20–0.33 L kg^{-1} and 0.18–0.31 L kg^{-1}, respectively, reported for man (Chung et al 1980; Campoli-Richards et al 1989; Nye et al 1989; Kovarik et al 1990; Barbhaiya et al 1992). Since all three antibiotics are used in clinical practice to combat respiratory infections (Lane 1984; Conway et al 1985; Hoepelman et al 1993; Leophonte et al 1993; McCabe et al 1996; Zhao et al 2000; Zhanel & Noreddin 2001; Huang et al 2002; Zhanel et al 2002), the kinetic profile in the lung must play

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an important role in the response to these drugs. Although some data about the concentration of levofloxacin, netilmicin and cefepime in lung tissue can be found in the literature (Fraschini et al 1988; Lee et al 1998; Breilh et al 2001; von Baum et al 2001), no comparative studies about the kinetic profile of these antibiotics in this body space are available. Isolated tissue techniques (Mehendale et al 1981; Wolfgang 1998; Tronde et al 2002) offer an alternative to traditional studies of drug distribution based on the sacrifice of animals at different sampling times. The former techniques –initially developed in the field of physiology – have been applied in pharmacokinetics to study the disposition of drugs, particularly in organs or body tissues such as the liver (Svoboda et al 1999; Desmoulin et al 2002), kidney (Zarzuelo et al 2002) and, more recently, the lung (Murata et al 1999; Reinoso et al 1999; Tronde et al 2002; Martínez Martínez et al 2005). The results afforded by these experimental approaches suggest that the isolation and artificial perfusion of tissues have great potential in the characterization of the disposition of drugs, particularly in body spaces of pharmacological or toxicological interest. The aim of this study was to explore the usefulness of these experimental models when applied to drug distribution assays. An experimental model of isolated lung of the rat was used to perform a comparative study on the disposition of levofloxacin, netilmicin and cefepime in the lung and to analyse the relevance of the information provided by this type of experimental model.

Materials and Methods

Isolated lung model

The study was performed using 18 adult male Wistar rats with a mean body weight of 262 ± 12.4 g. The rats had not had access to food for the 12 h before the experiment and were allowed free access to water until the moment of anaesthesia, which was induced with sodium thiopental (Penthothal sodico 0.5 g, Abbott) administered by intraperitoneal injection at a dose of 80 mg kg^{-1}. All experiments were carried out in compliance with the Principles of Laboratory Animal Care and according to the laws for animal welfare.

The method used to isolate lungs and to keep them artificially perfused and mechanically ventilated has been previously reported (Martínez Martínez et al 2005). Briefly, it consisted of performing a tracheotomy, followed by tracheal cannulation of the rats and mechanical ventilation under positive pressure. The lungs were isolated by insertion and fixation of inflow and outflow cannulae into the pulmonary artery and left ventricle, respectively. The inflow cannula was connected to the mechanical pump before its insertion to initiate artificial perfusion of the lungs at the same moment as the blood supply to the tissue was interrupted. The criteria used to evaluate the viability of each experimental preparation were: firstly, visualization of the preparation at the start of the infusion and through the experimental procedure to verify that the whole lung was properly perfused and that no tissue oedema was present – the presence of local areas with a slow washout of blood or development of translucent areas as the experiments progressed indicated deficiencies in the procedure and was a criterion for the non-viability of the experimental preparation; secondly, continuous measurement and recording of the flow rate and hydrostatic pressure at arterial level, using a probe and pressure transducer connected to the inflow cannula and to the corresponding data acquisition software – changes in flow rate above 2% were considered to be deficiencies in the procedure and were deemed criteria for the non-viability of the experimental preparation. The same applied to hydrostatic pressures beyond the 5–13 mmHg range.

The following elements were included in the experimental set-up implemented in our laboratory to perform the experiments with isolated rat lung.

A rodent ventilator (7025 Ugo Basile) was pre-set to supply a tidal volume of 2 mL at a respiratory frequency of 60 respirations/min of atmospheric air previously conditioned at 37°C and saturated humidity. Conditioning of the air before its supply to the rats was carried out by connecting the ventilator to a double-jacketed chamber into which atmospheric air was bubbled through water at 37°C; the ventilator took the air from this chamber instead of supplying non-conditioned atmospheric air.

A perfusion pump (Minipuls 3 Gilson) provided a constant flow of 5 mL min^{-1} of the perfusion medium, Krebs-Henseleit bicarbonate (pH 7.4) with glucose (0.9 g L^{-1}) and bovine albumin (fraction V, 30 g L^{-1}).

To oxygenate the perfusion medium, a mixture of 95% of O_2 and 5% of CO_2 was bubbled through the perfusion medium 10 min before starting the perfusion and throughout the experiments. A bubble trap was used to prevent the presence of air bubbles in the medium supplied to the isolated lung.

A thermostatted bath was used to maintain water at 37°C circulating through the double-jacketed elements.

A fraction collector (Helifrac, Pharmacia) was connected to the outflow cannula and was programmed to collect efferent fluid at the following sampling times after injection of the dose: 3-s intervals for the first minute and 6-s intervals over the next minute. Subsequently, sampling-time intervals were: 10 s for the next 2 min; 20 s for the next 2 min; 30 s for the next 2 min and 60 s for the next 2 min (total sampling time 10 min and total samples 54).

The flow and pressure-control device was a probe (Transonic Systems Inc; T106) connected to the inflow cannula to measure the flow rate and the corresponding pressure transducer (Transpac IV; Abbott Critical Care) was fitted to determine the hydrostatic pressure at arterial level.

A data acquisition software (Windaq (DATAQ Instruments WINDAQ) Version 1.91) program was used to record and file all the data concerning flow rate and pressure throughout the experiments.

Drug injection

Drug injection was carried out according to a parallel design in which each antibiotic was assayed in 6 experimental preparations, the injected doses of levofloxacin, netilmicin and cefepime being 500, 250, and 1000 μg, respectively. Five minutes after the start of the artificial perfusion (stabilization period), the drug dissolved in 200 μL of perfusion medium was given through the Y-device of the inflow cannula as a bolus injection.

Analytical techniques

Levofloxacin, cefepime and netilmicin quantification in outflow perfusate was carried out using different techniques of reverse-phase high-performance liquid chromatography (HPLC).

For determination of levofloxacin, the stationary phase was Nucleosil 120-C_{18} (5 μm) (Teknokroma) packed in a 15-cm column. The mobile phase was 90% phosphate buffer containing 40% of tetrabutyl ammonium hydrogen sulfate (final pH: 3) and 10% acetonitrile; the flow rate was 2 mL min^{-1}. A fluorescence detector was used with $\lambda_{excitation} = 330$ nm and $\lambda_{emission} = 450$ nm (Colino et al 1998).

Separation and quantification of cefepime in perfusate samples were achieved using a 15-cm Nucleosil 120-C_{18} (5 μm) column (Teknokroma). Samples were eluted with a mobile phase containing 100 mM monosodium phosphoric acid (pH 3.0)–methanol (87:13, v/v) at a flow rate of 1 mL min^{-1}. UV detector wavelength was set at 270 nm (Chang et al 2001).

Netilmicin samples were injected into a 15-cm reverse-phase column filled with Kromasil 120-C_{18} (5 μm) (Teknokroma) and were eluted with a mixture of acetonitrile and phase A (48:52, v/v) at a flow rate of 2 mL min^{-1}. Phase A consisted of water–acetic acid–1-heptanesulfonic acid (0.1 M) (80:10:10, v/v/v). A fluorescence detector was used with $\lambda_{excitation} = 337$ nm and $\lambda_{emission} = 437$ nm (Santos et al 1995).

Before injection into the chromatographic system, perfusate samples were treated with trichloroacetic acid solution (10%) to precipitate the proteins. For the quantification of netilmicin, a reaction with a derivatizing reagent, ophthalaldehyde, was carried out.

Using these experimental conditions, the intra- and inter-day coefficients of variation showed values lower than 7.5% for the three antibiotics.

Pharmacokinetic analysis

Drug concentration curves in efferent fluid were analysed by two different mathematical approaches, the statistical moments theory (Yamaoka et al 1978) and the dispersion model (Roberts & Rowland 1986a).

According to the theory of statistical moments, the AUC (area under the curve) and MTT (mean transit time) may be estimated in the isolated lung from the stochastic analysis of the outflow curves using the following equations:

$$AUC_0^{\infty} = \int_0^{\infty} C(t)dt \tag{1}$$

$$MTT = \frac{\int_0^{\infty} t \cdot C(t)dt}{\int_0^{\infty} C(t)dt} \tag{2}$$

Assuming the experimental preparation is a stationary system, the distribution volume of the drug in the lung is calculated as follows:

$$V_d = MTT \cdot Q \tag{3}$$

where Q represents the perfusion flow rate (5 mL min^{-1}).

Nevertheless, the experimental system used here includes tubing, besides the isolated tissue, which makes it necessary to correct the influence of these devices on the MTT estimated from the above equation. Additional experiments, performed under the same experimental conditions as described above but in absence of the tissue, were carried out to quantify the MTT of the drug in the devices. The difference between the former and latter MTT values gives the actual MTT of the drug in the tissue.

The dispersion model developed by Roberts & Rowland (1986a) and applied to the isolated liver of the rat (Roberts & Rowland 1986b, c) has been another kinetic approach used to analyse our data. This considers the axial dispersion of the drug, which experiments convective and diffusive processes after entering the organ as well as interaction or binding to the intracellular tissue components. Dispersion models of two and three compartments were assayed and outflow concentration data were fitted to both options by numerical inversion of the Laplace transform. Statistical criteria, such as the minimum squares, standard deviation of the parameters and the Akaike's criteria, were used for the model selection. Compartment 1 would represent the vascular space and compartments 2 and 3 might be considered as tissue spaces of rapid and slow drug transference, respectively.

Considering the lung as a system without elimination and mixed boundary conditions, the following integrated equation in the Laplace domain is obtained for the perfusate levels curve:

$$\tilde{C}_1(s) = \frac{M}{Q}\tilde{f}_c(s) \cdot \exp\left[\frac{1 - \sqrt{1+4D_N(V_1/Q)\cdot\left(s+K_{12}-\frac{K_{12}\cdot K_{21}}{s+K_{21}+K_{23}-(K_{23}\cdot K_{32}/K_{32}+s)}\right)}}{2 \cdot D_N}\right] \tag{4}$$

Where M is the amount of drug administered, $\tilde{f}_1(s)$ is the Laplace transform of the function corresponding to the experiment in the absence of tissue, Q is the perfusate flow rate through the organ, V_1 is the apparent distribution volume of the vascular compartment, D_N is the dispersion number, K_{12} and K_{21} are the rate constants between vascular and rapid access compartments of the lung, and K_{23} and K_{32} are the rate constants between deep and rapid access compartments.

The parameters of the dispersion model were optimized from equation 4, using the different experimental data sets with the MULTI(FILT) program, which combines non-linear regression with the numerical inversion of the Laplace transform (Yano et al 1989).

Calculation of apparent distribution volumes of rapid (V_2) and slow access (V_3) compartments of the lung was done using the following expressions:

$$V_2 = V_1 \cdot \frac{K_{12}}{K_{21}} \tag{5}$$

$$V_3 = V_2 \cdot \frac{K_{23}}{K_{32}} \tag{6}$$

$$Vdss = V_1 + V_2 + V_3 \tag{7}$$

Statistical analysis

Statistical comparison of parameters obtained for levofloxacin, netilmicin and cefepime was performed by an analysis of variance using the STATGRAPHICS Plus 4.0 program. Post-hoc multiple comparison was made using the Tukey multiple range test.

Results

Figure 1 shows the mean concentration curves (normalized by the injected dose) of levofloxacin, netilmicin and cefepime in efferent fluid. The kinetic profile of levofloxacin in the isolated lung differs from the profiles observed for netilmicin and cefepime, both the latter showing very similar concentration curves in the outflow fluid. Table 1 includes the statistical moments (AUC/D, MTT) and the derived parameter (Vd) estimated from these curves for each antibiotic by stochastic methods. As shown in Table 1, the mean transit time in the lung was statistically longer for levofloxacin ($P < 0.001$) than for netilmicin or cefepime, implying a longer and more intense exposure of the tissue to the fluoroquinolone than to the aminoglycoside or the cephalosporin. The MTT, and consequently the distribution volume of levofloxacin, were over twice the corresponding values obtained for netilmicin and cefepime (0.31 ± 0.03 min vs 0.10 ± 0.02 min and 0.11 ± 0.02 min; 1.25 ± 0.14 mL g^{-1} vs 0.39 ± 0.07 mL g^{-1} and 0.41 ± 0.06 mL g^{-1}, respectively). These values also showed that, in the tissue studied, levofloxacin behaves differently from the other two antibiotics.

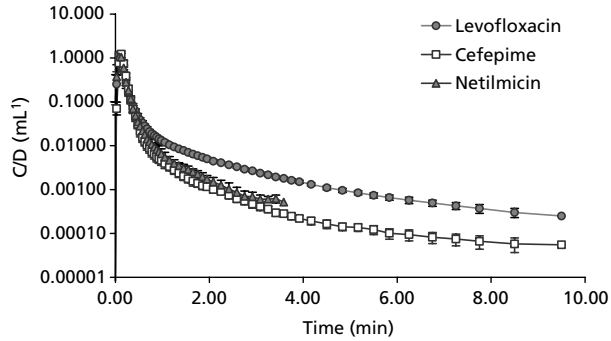

Figure 1 Mean concentration curves (normalized by the injected dose) of levofloxacin, netilmicin and cefepime in efferent fluid after its injection in the rat isolated lung preparation as a bolus injection at 500, 250 and 1000 μg, respectively.

Table 1 Values of statistical moments (AUC/D, MTT) and derived parameter (Vd) estimated for the three antibiotics by stochastic methods

Parameter	Levofloxacin	Netilmicin	Cefepime
AUC/D (min mL^{-1})	0.20 ± 0.09	0.20 ± 0.01	0.19 ± 0.01
MTT (min)	0.31 ± 0.03***	0.10 ± 0.02	0.11 ± 0.02
Vd (mL g^{-1})	1.25 ± 0.14***	0.39 ± 0.07	0.41 ± 0.06

Values are means $\pm$ s.d.; ***$P < 0.001$, levofloxacin vs netilmicin or cefepime.

Table 2 Values of pharmacokinetic parameters obtained from data fitting to the three-compartment dispersion model

Parameter	Levofloxacin	Netilmicin	Cefepime
$D_N$$^{\#}$	0.32 ± 0.07	0.23 ± 0.05	0.10 ± 0.05
K_{12} (min^{-1})$^{\#}$	1.20 ± 0.14	0.19 ± 0.18	0.06 ± 0.04
K_{21} (min^{-1})$^{\#}$	0.52 ± 0.09	1.22 ± 0.51	0.19 ± 0.08
K_{12}/K_{21}	2.37 ± 0.60***	0.17 ± 0.21	0.37 ± 0.31
K_{23} (min^{-1})	0.14 ± 0.04	0.08 ± 0.03	0.14 ± 0.07
K_{32} (min^{-1})	0.08 ± 0.03**	0.22 ± 0.09	0.43 ± 0.21
K_{23}/K_{32}	1.92 ± 0.83***	0.48 ± 0.33	0.43 ± 0.29
V_1 (mL g^{-1})	0.44 ± 0.07	0.38 ± 0.05	0.35 ± 0.09
V_2 (mL g^{-1})	1.02 ± 0.161***	0.05 ± 0.08	0.12 ± 0.09
V_3 (mL g^{-1})	1.97 ± 0.09***	0.04 ± 0.06	0.05 ± 0.04
V_{DSS} (mL g^{-1})	3.44 ± 0.97***	0.48 ± 0.12	0.53 ± 0.13

Values are means $\pm$ s.d.; $^{\#}P < 0.001$ values for each antibiotic vs the others; **$P < 0.01$, ***$P < 0.001$ levofloxacin vs netilmicin or cefepime.

According to statistical criteria, the dispersion model of three compartments is the one which best fits the data. Table 2 shows the parameter values estimated for levofloxacin, netilmicin and cefepime corresponding to this model. These confirm the differences in the kinetic behaviour of levofloxacin as compared with netilmicin

and cefepime. The most relevant differences are observed for the distribution volumes in peripheral compartments 2 and 3, as well as their transference constants (K_{12}, K_{21}, K_{32}), showing values that point to a higher capacity of levofloxacin to access and persist in the extravascular spaces of the isolated rat lung. The dispersion number shows statistical differences ($P < 0.001$) for all three antibiotics included in the study, the highest value corresponding to levofloxacin. This also points to the existence of a deeper space inside the tissue for the fluoroquinolone as compared with the other two drugs.

The differences in the parameter values obtained for the distribution volume by the two methods used are due to the different assumptions of each approach. The dispersion model assumes the existence of three spaces into the tissue, while the stochastic analysis considers only one space in which the drug stays for a given time. Although the absolute values of Vd obtained by both methods are not the same, these values are correlated, leading to the same conclusion regarding the comparative behaviour of the three drugs studied.

Discussion

The characterization of the kinetic profile of drugs in specific organs or tissues is becoming increasingly interesting since pharmacological and toxicological responses are more closely related to drug concentrations at target sites than in plasma. This is even more important in the case of antimicrobial agents because inadequate levels at the site of infection lead not only to clinical failure but also to the development of drug resistance (Cazzola et al 2000; Andes 2001). Despite the general consensus concerning the relevance of this issue, most pharmacokinetic studies only provide information about the kinetic profile of drugs in plasma, whereas distribution data are generally reduced to a few drug concentrations in the main body tissues. This is a logical consequence of the methodological difficulties involved in tissue sampling, particularly in man, although also in laboratory animals. Traditional methods for the study of drug distribution based on the sacrifice of animals at different sampling times involve the use of large numbers of animals, since each animal provides a single point on the tissue concentration curve and many replicates must be performed owing to the existence of inter-individual variability in each curve. Thus, in this type of methodology the characterization of the kinetic profile of a drug in a given tissue involves the sacrifice of 8–10 animals per curve, which must be replicated at least 6–8 times, leading to a total of 50–80 animals per body tissue. The techniques of tissue isolation and perfusion offer an excellent alternative to traditional methodology when wide information about the drug distribution in a particular body tissue is wanted. The former allows the characterization of the kinetic profile for a tissue in a single animal and avoids the inter-individual variability in each single curve, leading to a corresponding reduction in curve replicates and hence a drastic reduction in the number of animals sacrificed (5–8 vs 50–80 per tissue kinetic profile). Furthermore, the outflow concentration curves obtained by this method may be fitted to physiological or other types of mechanistic models requiring a high number of experimental data per curve. The results of our comparative study on the disposition of levofloxacin, netilmicin and cefepime in the isolated lung of the rat confirm these advantages and show that the data provided by this experimental model afford very useful information about the kinetic behaviour of drugs in this tissue. The values of the distribution coefficients show that levofloxacin ($1.25 \pm 0.14\,mL\,g^{-1}$) enters the intracellular space and probably binds to some tissue structure, while the access of netilmicin and cefepime is more restricted with distribution coefficient values of $0.39 \pm 0.07\,mL\,g^{-1}$ and $0.41 \pm 0.06\,mL\,g^{-1}$, respectively. Additionally, the fitting of outflow curves to a pseudo-mechanistic model, such as the three-compartment dispersion model, was possible owing to the very high number of experimental points defining each curve.

Interpretation of the values of the model parameters obtained for each antibiotic leads to the same conclusions as those drawn from the statistical analysis of the curves and also provides additional information about the intrinsic mechanisms involved in the distribution of the drugs in the lung. Again, levofloxacin shows statistically significant differences in the parameter values related to tissue access as compared with netilmicin and cefepime, both the latter showing fairly similar values for most of the model parameters. The mechanistic value of this model is clearly seen in Figure 2, which shows the distribution volumes of compartments 1, 2 and 3 for the three drugs studied. No differences were observed in the values of V_1, representing the vascular space of the lung, while V_2 and V_3 – representing extravascular spaces of rapid and slow access or intracellular binding sites – were significantly higher for levofloxacin than for netilmicin or cefepime. The values of the transference constants, K_{12}, K_{21} and K_{32}, also lead to the same conclusions, since the leaving rate constants from compartment 1 to compartment 2 (K_{12}) and from compartment 2 to compartment 3 (K_{23}) show higher values for levofloxacin, although the latter one is without statistical significance. Therefore, the K_{12}/K_{21} and K_{23}/K_{32} ratios have higher values for the fluoroquinolone than for netilmicin and cefepime, the differences between the former and the other two antibiotics being statistically significant ($P < 0.001$).

Our results are in complete agreement with data found in the literature concerning the distribution of these antibiotics; such studies reported a wider tissue distribution, a greater capacity to enter the intracellular space and a higher tissue accumulation for levofloxacin (Fish & Chow 1997; Garcia et al 2000), while a more restricted distribution tissue space, with no binding processes, has been described for netilmicin and cefepime (Lanao et al 1991; Okamoto et al 1993; Casquero-Dorado & Sánchez-Navarro 2000). Thus, the results obtained in this study seem to validate the experimental model of the isolated rat lung, at least under the experimental conditions used, and show that this methodology offers an excellent alternative to traditional tissue distribution assays. Once tested and

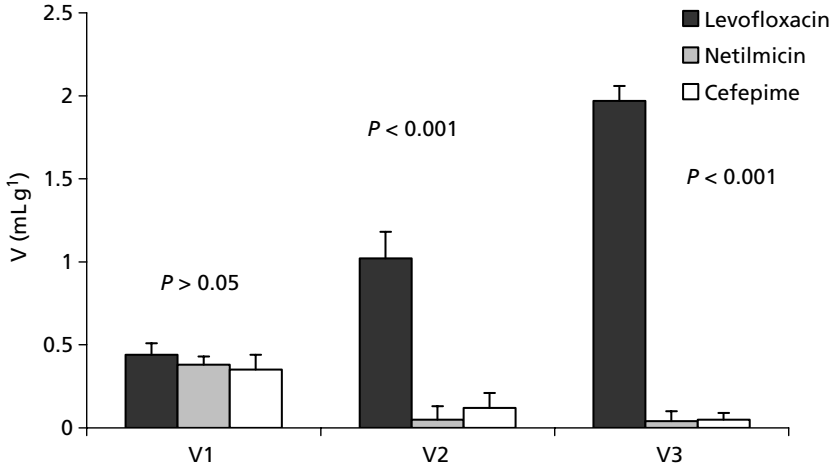

Figure 2 Values of the distribution coefficients (mean ± s.d.) estimated for the central (V1) and peripheral compartments (V2 and V3) for levofloxacin, cefepime and netilmicin when using the three-compartments dispersion model for outflow data fitting.

validated with antibiotics of known distribution characteristics, the proposed model might be used to investigate the behaviour of new drugs in this tissue. Since outflow sampling may be as frequent as wished, the kinetic profile of the drug in the tissue may be more precisely defined, allowing data fitting to mechanistic models that provide information about the mechanisms involved in the drug disposition in the isolated tissue. In sum, the above-described experimental model of the isolated rat lung permitted us to establish the differences in the kinetic profile of levofloxacin, netilmicin and cefepime in the lung tissue and therefore the proposed experimental model provides a viable and interesting methodology to determine the kinetic behaviour of drugs whose target site for pharmacological or toxicological responses is the lung. Besides, with this type of study, a further step in the application of pharmacokinetic/pharmacodynamic (PK/PD) principles based on drug levels in the infection site instead of plasma concentrations might be possible, since data on the pkarmaokinetics in the biophase would be available.

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