J. Pharm. Pharmacol. 2000, 52: 1491–1497
Received June 9, 2000
Accepted August 23, 2000

Determination of Tissue Spaces of the Isolated Hindlimb of the Rat Using Netilmicin as an Extracellular Space Indicator

ANA CRISTINA CASQUERO-DORADO AND AMPARO SÁNCHEZ-NAVARRO

Department of Pharmacy and Pharmaceutical Technology, University of Salamanca, Avda. Campo Charro s/n, 37007 Salamanca, Spain

Abstract

The kinetic behaviour of drugs in extracellular space is of interest as it influences the drug's access to, and permanence in, those areas of the body upon which drugs exert pharmacological or toxicological actions. A series of experiments was carried out to characterize the vascular, interstitial and cellular spaces of the isolated hindlimb of the rat. Certain specific experimental conditions were met: body weight was under 230 g to avoid fat tissue in the preparations; a perfusion flow rate of $3\,mL\,min^{-1}$; and 3% of bovine albumin in the perfusate supplied at 25°C to the tissues.

The isolation of the hindlimb followed the method described by Ruderman with some modifications to restrict the perfusion to the right hindlimb. Tritiated water, netilmicin and methylene blue were injected separately and efferent fluid samples were collected for 15 min after solute injection. Analysis of the efferent curves was performed to calculate the statistical moments (AUC, area under concentration–time curve; MTT, mean transit time; VTT, variance of mean transit times) and solute distribution volumes, which were subsequently used to estimate the tissue spaces of the isolated hindlimb.

The results revealed that methyl blue and netilmicin can be respectively used as alternatives to radiolabelled indicators of the vascular and extracellular spaces of tissues included in the rat isolated hindlimb.

The isolated hindlimb of the rat is a preparation initially described by Ruderman et al (1977) and recently used in pharmacokinetic studies as an experimental model to determine drug distribution (Wu et al 1993, 1995, 1997; Weiss & Roberts 1996; Weiss et al 1997; Sánchez-Navarro et al 1999). This type of preparation offers a method for determining the kinetic behaviour of drugs in muscle, bone and skin (and fat, if so desired) as a whole component, avoiding interference by kinetic processes occurring simultaneously in the rest of the body. Characterization of the kinetic behaviour in a space which constitutes over 70% of the total body weight is of interest as regards all drugs, since such behaviour strongly determines their access to, and permanence in, other body spaces constituting those areas in which pharmacological or toxicological action is exerted. It is of particular interest in the case of formulations intended for drug targeting. This type of preparation can also be used to study controlled-release preparations designed for subcutaneous or intramuscular administration as well as intrabone implant devices. By analysis of efferent fluid level curves, both the efficiency of the formulation to selectively deliver the drug and the kinetics of systemic drug delivery can be evaluated. The isolated hindlimb is therefore potentially a very useful preparation in the pharmacokinetic field regarding the evaluation of new formulations, since these are essentially designed to obtain controlled-release delivery or drug targeting.

Recently, some authors have studied this preparation under different experimental conditions involving such parameters as flow rate, the composition of the artificial medium, or temperature (Wu et al 1993, 1995). Information about the flow rates to the tissue components has also been documented in the literature (Sánchez-Navarro 1995; Casquero-Dorado & Sánchez-Navarro 1996) and data concerning the volume of the vascular, interstitial and cellular spaces, obtained under different experimental conditions and using different

Correspondence: A. Sánchez-Navarro, Departamento de Farmacia y Tecnología Farmacéutica, Universidad de Salamanca, Avda. Campo Charro s/n, 37007 Salamanca, Spain.
E-Mail: asn@gugu.usal.es

indicators, are also available (Wu et al 1993; Heatherington & Rowland 1994).

The aim of this study was to determine, under certain experimental conditions, the three physiological spaces delimited by the vascular and cellular membranes in the isolated hindlimb of the rat, using non-standard solutes — methylene blue and netilmicin — as vascular and interstitial indicators, respectively.

Material and Methods

Materials

Tritiated water was obtained from Amersham UK and netilmicin was from Schering-Plough. Bovine serum albumin (fraction V; 96–99% pure) and Triton (Triton X-100) were purchased from Sigma Chemical Co., pentobarbital powder was from Abbott Laboratories S.A., heparin (5%) was from Rovi Lab S.A.; scintillation liquid was from Beckman Instruments and trichloroacetic acid (20%) was from Merck. All other chemicals were of analytical grade (Panreac).

Study design

Fifteen male Wistar rats, $196 \pm 31{\cdot}33$ g mean body weight, were used in the experimental study which was carried out in accordance with the Principles of Laboratory Animal Care (NIH publication, revised 1985). The rats were distributed into three groups of five. Each group received a different solute (methylene blue, netilmicin or tritiated water). All rats underwent the same surgical procedure to isolate and perfuse the hindlimb, after receiving a dose of pentobarbital ($60\,mg\,kg^{-1}$, i.p.) to induce anaesthesia and a dose of heparin (0·5 mL of 5% solution, i.p.) to avoid blood clotting. The surgical procedure was a modification of the Ruderman technique (Ruderman et al 1977), consisting of tying two additional ligatures, one around the contralateral iliac vessels to restrict artificial perfusion to one hindlimb and the other around the lower joint of the perfused limb to exclude the paw from the perfused circuit.

Tissues were perfused immediately at a flow rate of $3\,mL\,min^{-1}$ in a single-pass perfusion mode.

A solution of the following composition was used to prepare the perfusate (mM): 137·0 NaCl, 1·5 KCl, 1·8 $CaCl_2.6H_2O$, 0·4 $NaH_2PO_4.2H_2O$, 1·0 $MgCl_2.6H_2O$ and 12·0 $NaHCO_3$. This buffer solution was freshly prepared and then glucose and bovine albumin were added in sufficient amounts to achieve a final composition of 5·0% and 3·0%, respectively.

The perfusate, at 25°C, was oxygenated with a mixture of 95% O_2 and 5% CO_2 for 5 min before perfusion and throughout the experiments.

After an initial stabilization period of 20 min, 10 μCi of tritiated water, 0·5 mg of methyl blue or 1 mg of netilmicin was injected as a bolus through the inflow cannula. Automatic sample collection of efferent fluid started at the moment of solute administration. The sample collector was programmed for different time-intervals for a period of time of 15 min. After collection, samples were stored at −20°C until the analytical assays.

Complementary experiments were carried out maintaining all the above experimental conditions but in the absence of the hindlimb to evaluate and correct the influence of the catheters on the experimental results.

Analyses

Tritiated water. The concentration of tritiated water in the outflow perfusate samples was determined by liquid scintillation counting (Beckman Instruments). Standard solutions were prepared using a solution with the same composition as the perfusate, to which different amounts of tritiated water were added, to prepare standard solutions in the 0·025–0·5 μCi concentration range. Each efferent fluid sample and standard solution was mixed with a scintillation liquid (Ready Protein, Beckman) at a ratio of 1 : 1. After vortexing for 30 s, the mixture was collected in a scintillation vial and counting was performed. The relationship between the counts min^{-1} and the concentration of tritiated water was established by linear regression analysis and the concentration of tritiated water in each efferent fluid sample was determined from the standard curve.

Netilmicin. Netilmicin levels were determined by a previously described ion-pair HPLC technique (Santos et al 1995), with pre-column derivatization using a fluorescence detector. Briefly, this consisted of the derivatization of netilmicin with a mixture of *o*-phthaldialdehyde, methanol and 2-mercaptoethanol. Before derivatization, netilmicin samples were subjected to the following treatment: 100 μL of trichloroacetic acid was added to 100 μL of sample; after shaking and centrifugation the supernatant was collected and mixed with 100 μL of 0·1 M NaOH. This mixture was then added to 1 mL of aqueous monobasic potassium phosphate (previously adjusted to pH 11) and 2 mL of dichloromethane. The resulting mixture was shaken again, and centrifuged (3 200 rpm) for 5 min. Following this, the supernatant was collected and 1 mL of the

derivatizing agent was added. After shaking for 30 s, 500 mg of anhydrous sodium carbonate was added, followed by shaking once more. Finally, 2-propanol was added to extract the derivatized netilmicin. The chromatographic conditions were as follows: a mobile phase composed of 50%–60% of A (water – acetic acid – 0·1 M heptane sulphonic acid, 80 : 10 : 10, v/v/v) and 40%–50% of B (acetonitrile) set up in a gradient of 10 min, at a flow rate of 2 mL min^{-1}; a reverse-phase column (RP-18) and a fluorescence detector, with an excitation and emission wavelength of 337 nm and 437 nm, respectively.

Methylene blue. Methylene blue was determined by a UV-spectrophotometric technique at a wavelength of 620 nm. Standard curves were prepared using a fraction of the efferent perfusate obtained during the stabilization period, immediately before solute injection, to avoid the possible interference by colour due to blood remaining in the fluid samples. Two standard curves (0·5–5 $\mu g\,mL^{-1}$ and 0·1–1 $\mu g\,mL^{-1}$) were prepared to guarantee a linear relationship between absorbance and solute concentration. Samples were diluted when necessary to obtain a final volume of 0·5mL, the minimum necessary for measurement in the spectrophotometer.

Kinetic analysis. The stochastic approach was applied to the outflow solute concentration curves. The statistical moments were calculated according to equations 1–3 (Yamaoka et al 1978):

$$AUC = \int_0^\infty C(t)\,dt \quad (1)$$

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

$$VTT = \frac{\int_0^\infty (t - MTT)^2\,C(t)\,dt}{\int_0^\infty C(t)\,dt} \quad (3)$$

where C(t) is the solute concentration determined in the outflow perfusate samples, AUC is the area under the concentration–time curve, MTT is the mean transit time and VTT is the variance of mean transit times.

The mean transit time values, calculated from the outflow perfusate curves, were corrected by subtraction of the corresponding statistical moment value established from the experimental data obtained from the experiments carried out in the absence of organ. These corrections avoid the influence of the injection and collection devices in the final results. All moment values were calculated numerically using the trapezoidal and log-trapezoidal rules, with extrapolation of the final log-linear phase to infinite time.

The relative dispersion of the transit time (CV^2) and the distribution volume of each solute (Vw = water volume, Vn = netilmicin volume and Vmb = methyl blue volume) were calculated by equations 4 and 5 (Weiss 1982).

$$CV^2 = VTT/MTT^2 \quad (4)$$

$$V = Q \times MTT \quad (5)$$

where Q is the perfusate flow rate (Q = 3 mL min^{-1}).

Assuming that tritiated water distributes in the total water, netilmicin in the extracellular water and methylene blue in the vascular water of the preparation, the physiological spaces (Vv = vascular space, Vi = interstitial space and Vc = cellular space) were estimated from the volumes calculated for each solute according to the following expressions: Vv = Vmb, Vi = Vn − Vmb and Vc = Vw − Vn.

Experimental efferent levels were normalized by the dose of the injected solute to enable comparison of curve profiles corresponding to the different tissue space indicators.

Results

Table 1 shows the statistical moments calculated from the raw experimental data of each injected solute, together with the corresponding slope of the final monoexponential phase of the curve and the distribution volume.

The MTT, and hence the distribution volume, increased as the solute accessed wider tissue spaces, values of 1·0 ± 0·3 mL being reached for methylene blue, 6·47 ± 2·32 mL for netilmicin and 14·9 ± 2·35 ml for tritiated water. By contrast, the relative dispersion of transit times increased for the solutes with lower distribution volumes, affording values of 8·55 ± 4·19, 5·66 ± 0·8 and 1·86 ± 0·3 for methylene blue, netilmicin and tritiated water, respectively.

Figures 1, 2 and 3 show the normalised mean outflow curves (concentration/dose) of tritiated water, netilmicin and methylene blue, respectively, after administration as a bolus injection in the rat isolated hindlimb. Figure 4 shows typical outflow curves of each indicator obtained from three different experiments carried out under identical experimental conditions.

Discussion

The tritiated water used in this study as an indicator of total tissue water has classically been considered

Table 1. Statistical moments, distribution volumes and slope of the final phase of the curves calculated from experimental data of each solute injected into rat isolated hindlimb.

Indicator	AUC_0^∞ (μCi min mL^{-1} or μg min mL^{-1})	MTT (min)	VTT	CV^2	Vd (mL)	Slope (min^{-1})
Tritiated Water	1·91 ± 0·61	4·96 ± 0·75	46·41 ± 15·89	1·86 ± 0·26	14·87 ± 2·35	0·10 ± 0·01
Netilmicin	198·50 ± 128·38	2·16 ± 0·77	27·01 ± 13·96	5·66 ± 0·85	6·47 ± 2·32	0·14 ± 0·02
Methylene Blue	47·08 ± 5·45	0·33 ± 0·10	1·04 ± 0·69	8·55 ± 4·19	1·00 ± 0·30	0·51 ± 0·03

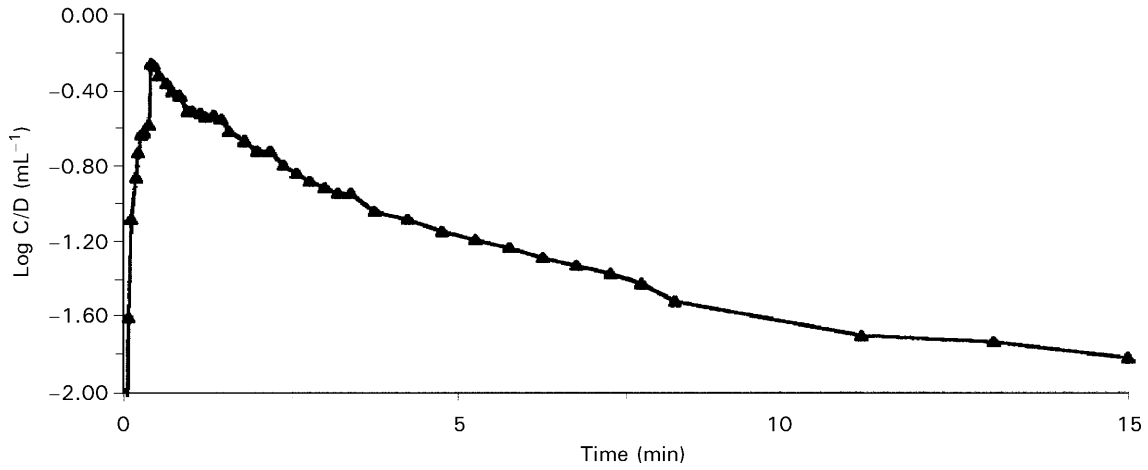

Figure 1. Mean outflow concentration–time profile normalized by the dose of tritiated water in the isolated hindlimb of the rat after bolus administration of 10 μCi.

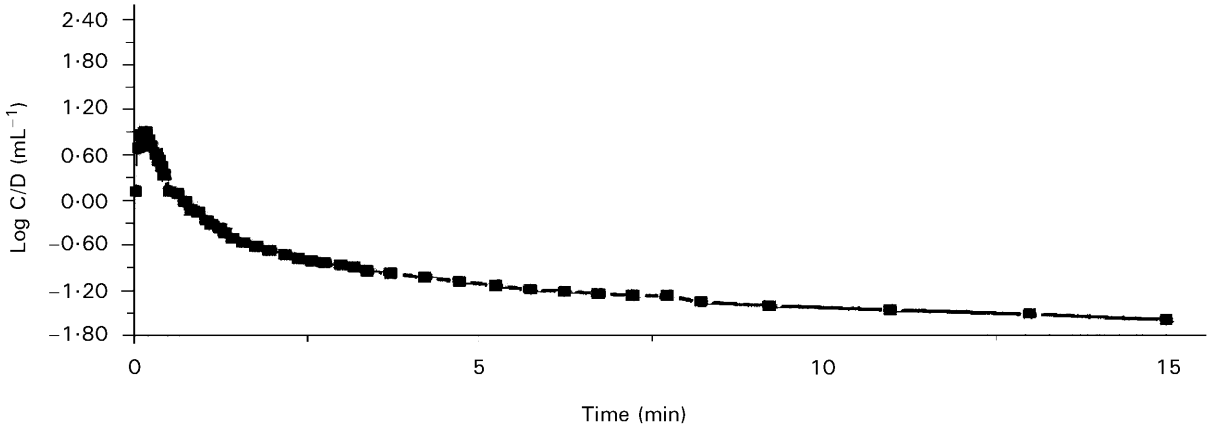

Figure 2. Mean outflow concentration–time profile normalized by the dose of netilmicin in the isolated hindlimb of the rat after bolus administration of 1 mg.

for this purpose, although netilmicin and methylene blue have not been previously reported as possible indicators of the extracellular and vascular tissue spaces. The choice of netilmicin as a possible indicator of the extracellular space of the hindlimb is based on the well-known distribution characteristics of this aminoglycoside, whose capacity to access cells has been reported to be restricted to the kidney, the inner ear structures and erythrocytes (Banza et al 1975; Lanao et al 1991; Santos et al 1997). The choice of methylene blue was empirically based on the chemical analogy between this product and Evans blue, widely used for the determination of vascular spaces.

Comparison of our results with those obtained by other authors, using the standard tissue space indicators, reveals some interesting analogies and differences. Table 2 shows our data together with other previously reported vascular, extracellular and total water volumes for the isolated hindlimb of

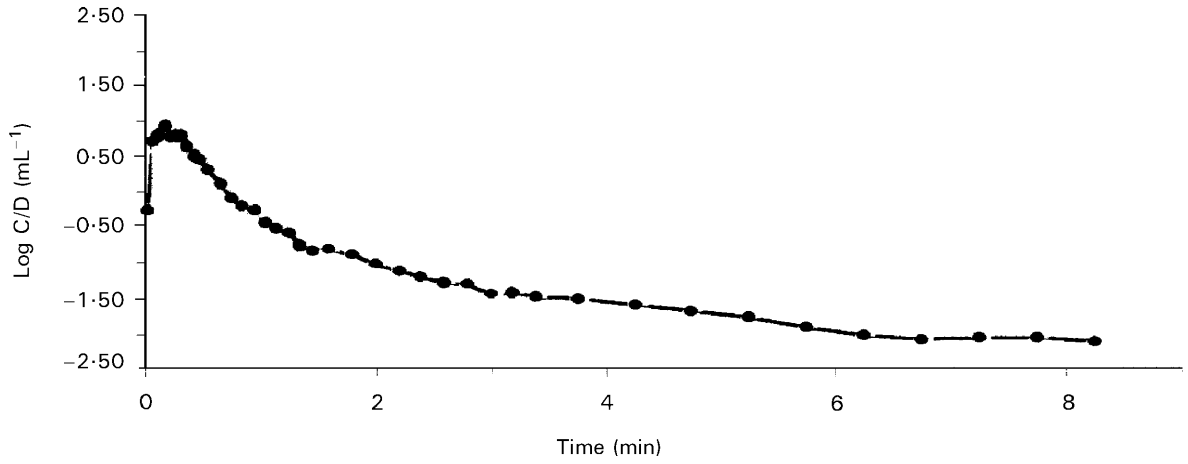

Figure 3. Mean outflow concentration–time profile normalized by the dose of methylene blue in the isolated hindlimb of the rat after bolus administration of 0·5 mg.

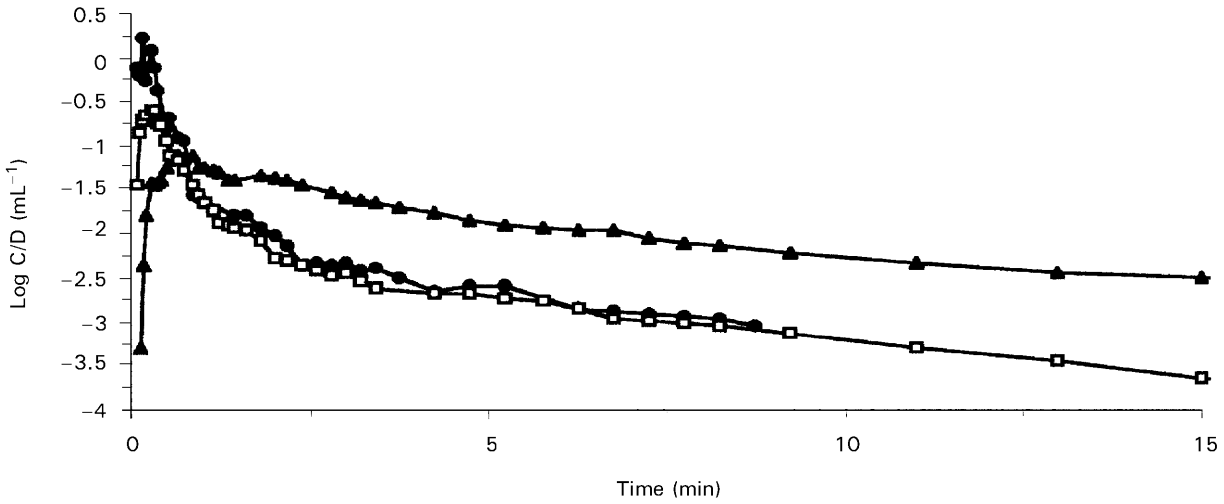

Figure 4. Typical outflow curves of methylene blue (●), netilmicin (□) and tritiated water (▲) from different experiments carried out under identical experimental conditions.

the rat (Wu et al 1993; Heatherington & Rowland 1994) using Evans blue or red blood cells (for the vascular space), sucrose and tritiated water. The weight of the rats and the sampling time corresponding to each study are also shown in Table 2, since they may strongly influence the final characteristics of the experimental preparation (rat weight) or calculation of the statistical moments (sampling time). The volume obtained for methylene blue in our study (1·00 ± 0·30 mL) agrees with the value obtained by Heatherington & Rowland (1994) (1·03 ± 0·26 mL) for red blood cells, although the latter value is lower than the volume obtained by Wu et al (1993) using Evans blue (1·48 ± 0·16 mL). This difference might be due to variations in the body weight of the rats used in the studies (332·8 ± 19·4 g vs 215·0 ± 90 g or 196·46 ± 31·33 g). On comparing the volume of

Table 2. Volumes of indicators obtained in different studies in the isolated hindlimb of the rat.

Indicator	Volume (mL)	Rat weight (g)	Sampling time (min)
Evans blue[a]	1·48 ± 0·16	332·8 ± 19·4	2
Red blood cells[b]	1·03 ± 0·26	215 ± 90	5
Methylene blue[c]	1·00 ± 0·30	196·46 ± 31·33	15
Sucrose[a]	5·24 ± 1·18	332·8 ± 19·9	2
Sucrose[b]	6·62 ± 0·53	215 ± 90	5
Netilmicin[c]	6·47 ± 2·32	196·46 ± 31·33	15
Tritiated water[a]	9·24 ± 4·84	332·8 ± 19·9	2
Tritiated water[b]	10·73 ± 1·01	215 ± 90	5
Tritiated water[c]	14·87 ± 2·35	196·46 ± 31·33	15

[a]Wu et al (1993); [b]Heatherington & Rowland (1994); [c]our results.

netilmicin determined in our study ($6{\cdot}47 \pm 2{\cdot}32$ mL) with the sucrose volumes ($6{\cdot}62 \pm 0{\cdot}53$ mL or $5{\cdot}24 \pm 1{\cdot}18$ mL) reported by the other authors, very similar results were seen in all three cases, although the results from Wu et al (1993) again differ from the other two.

With tritiated water, the results showed the widest variation, even though the same product was used in all three studies. The volume of tritiated water obtained under our experimental conditions was appreciably higher ($14{\cdot}87 \pm 2{\cdot}35$ mL) than the values reported by the other authors (Wu et al 1993; Heatherington & Rowland 1994) ($9{\cdot}24 \pm 4{\cdot}84$ or $10{\cdot}73 \pm 1{\cdot}01$mL). The differences could not be attributed to the body weight of the rats since the lowest volume obtained for tritiated water corresponds to the study carried out using the rats with the highest mean weight. A review of the experimental conditions used in the studies of Wu et al (1993) and Heatherington & Rowland (1994) for data comparison reveals that in both studies the total sampling time (2 and 5 min) is much shorter than the period of time followed in our experiments (15 min). In our opinion, the final monoexponential phase of the efferent curve may not be accurately characterized using data collected over a short period of time.

An additional observation supporting our finding of a higher volume with tritiated water refers to the calculated interstitial and cellular spaces, which must be estimated from water volumes. According to our data, the interstitial and cellular water volumes of the isolated hindlimb had values of 36% and 56%, respectively, of the total water volume; these percentages are in good agreement with the physiological values of these spaces reported for muscle, bone and skin (Kawai et al 1994). In contrast, the lower values of tritiated water afforded estimations of 52·4% for the interstitial volume and 43·3% for the cellular volume by Heatherington & Rowland (1994) and respective values of 40·7% and 38·3% by Wu et al (1993). These values are much higher than those established for the interstitial space and hence much lower than those accepted for the cellular spaces of the tissues included in the hindlimb. The differences in sampling times would explain these facts.

Figures 1–3 show the normalized mean outflow curves and Figure 4 includes typical outflow curves of each solute; the vascular indicator (methylene blue) reached the highest peak earliest, followed by the extracellular marker (netilmicin) and finally tritiated water. The log-linear phase was reached earlier by the vascular marker and had a slope value of $0{\cdot}56\ \text{min}^{-1}$, significantly higher ($P = 0{\cdot}004$) than the values obtained for netilmicin ($0{\cdot}14\ \text{min}^{-1}$) and tritiated water ($0{\cdot}13\ \text{min}^{-1}$).

Besides MTT (used to calculate distribution volumes), the relative dispersion of transit times (CV^2) has been estimated. This parameter provides information about the distribution process itself (Weiss & Pang 1992) leading to high values when distribution is not instantaneous and homogeneous, in other words when the tissue does not behave as a well-stirred space. The unexpected high values of CV^2 obtained for the three indicators may be attributed to the structural characteristics of the isolated hindlimb – a complex preparation with a heterogeneous capillary network with solute trapping zones, leading to dispersion numbers much higher than those found in other body tissues such as the liver (Oliver et al 1997). The extremely high value of $CV^2 = 8{\cdot}55$ for methylene blue may also be due to the limitations of the spectrophotometric technique used to determine the outflow levels of this solute; the possible interference of blood remaining in the fluid samples might be responsible.

Considering the potential usefulness of the isolated hindlimb of the rat in the pharmacokinetic field, an exhaustive study of this preparation would be of great interest for establishing the factors affecting its characteristics and hence the optimum experimental conditions for obtaining the most reliable results. Wu et al (1993), who carried out a systematic study of the isolated hindlimb of the rat, highlighted its importance and proved the influence of the flow rate on vascular resistance, oxygen consumption and tissue volumes. The formation of tissue oedema is also dependent on the flow rate together with albumin content of the perfusate and the neurological status of the limb.

In the light of our results netilmicin distributes in the extracellular space of the isolated hindlimb; this finding confirms the theoretical hypothesis that the aminoglycosides distribution is restricted to the extracellular water, except for very particular body spaces. Accordingly, it may be proposed that methylene blue and netilmicin can be used as alternative indicators of the vascular and extracellular spaces, respectively, for the tissues included in the hindlimb. Netilmicin offers an excellent alternative to labelled products used as extracellular markers, since it can be accurately quantified by an HPLC technique; other aminoglycosides are probably also suitable for the determination of the extracellular volume of these tissues, since all show very similar distribution characteristics. With methylene blue, although the vascular volume estimated for the isolated hindlimb is in good agreement with reported values, the observed dispersion (CV^2) is much higher than expected, which can be partially attributed to the

UV-spectrophotometric technique used to quantify outflow levels. The results of our experiments suggest that quantification of methylene blue in the efferent perfusate by the spectrophotometric technique may lead to some analytical errors and consequently a more specific analytical technique, such as HPLC, would be more reliable.

Our results point to the importance of considering some experimental conditions such as the weight of the rat and the length of sampling time; a body weight of under 250 g avoids the presence of fat tissue, reducing qualitative and quantitative inter-experiment tissue variability related to differences in fat-tissue proportions. An extended period of sampling time is recommended for the isolated hindlimb to avoid errors in the estimation of the slope of the final phase of the outflow curve, which might lead to underestimation of the corresponding indicator volume, especially for tritiated water.

Acknowledgements

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

References

Banza, M., Brown, R., Gibaldi, M., Weinstein, L. (1975) Predictability of blood levels of gentamicin in man. J. Infect. Dis. 132: 165–174

Casquero-Dorado, A. C., Sánchez-Navarro, A. (1996) The isolated hindlimb preparation as a drug distribution model. Meth. Find. Exp. Clin. Pharmacol. 18: 453–458

Heatherington, A. C., Rowland, M. (1994) Estimation of reference spaces in the perfused rat hindlimb. Eur. J. Pharm. Sci. 2: 261–270

Kawai, R., Lemaire, M., Steimer J. L., Bruelisauer A., Niederberger W., Rowland M. (1994) Physiologically based pharmacokinetic study on a cyclosporin derivative, SDZ IMM 125. J. Pharmacokin. Biopharm. 22: 327–365

Lanao, J. M., López, A., Domínguez-Gil, A., Martin, J., Gomez, J. R., Tabernero, M. (1991) Distribution kinetics of netilmicin in human blister fluid: effect of renal impairment. Biopharm. Drug Dispos. 12: 149–162

Oliver, R. E., Heatherington, A. C., Jones, A. F., Rowland, M. (1997) A physiologically based pharmacokinetic model incorporating dispersion principles to describe solute distribution in the perfused rat hindlimb preparation. J. Pharmacokin. Biopharm. 25: 389–412

Ruderman, N. B., Goodman, M. N., Berger, M., Hagg, S. (1977) Effect of starvation on muscle glucose metabolism: studies with the isolated perfused rat hindquarter. Biochem. J. 36: 171–176

Sánchez-Navarro, A. (1995) A theoretical approach to the estimation of tissue flows using tritiated water as indicator. J. Pharm. Sci. 84: 643–646

Sánchez-Navarro, A., Casquero-Dorado, A. C., Weiss, M. (1999) Distribution and binding kinetics of ciprofloxacin and ofloxacin in the isolated hindlimb of the rat. Pharm. Res. 16: 587–591

Santos, M., Garcia, E., López, S. G., Lanao, J. M., Domínguez-Gil, A. (1995) Determination of netilmicin in plasma by HPLC. Pharm. Biomed. Analysis 13: 1059–1062

Santos, M., Arevalo, M., Colino, C. I., López, F. G., Sánchez-Navarro, A. S., Domínguez-Gil, A., Lanao, J. M. (1997) Study on pharmacokinetic and nephrotoxicity of netilmicin in rabbit using a new dosage regimen. Meth. Find. Exp. Clin. Pharmacol. 19: 53–59

Weiss, M. (1982) Moments of physiological transit time distribution and the time course of drug disposition in the body. J. Math. Biol. 15: 305–318

Weiss, M., Pang, K. S. (1992) The dynamics of drug distribution. I. Role of the second and third curve moment. J. Pharmacokin. Biopharm. 20: 253–278

Weiss, M., Roberts, M. S. (1996) Tissue distribution kinetics as determinant of transit time dispersion of drugs in organs: application of a stochastic model to the rat hindlimb. J. Pharm. Sci. 24: 173–198

Weiss, M., Koester, A., Wu, Y. W., Roberts, M. S. (1997) Distribution kinetics of diazepam, lidocaine and antipyrine in the isolated perfused rat hindlimb. Pharm. Res. 14: 1640–1643

Wu, Z. Y., Rivory, L. P., Roberts, M. S. (1993) 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. Pharmacokinet. Biopharm. 21: 653–688

Wu, Z. Y., Cross, S. E., Roberts, M. S. (1995) Influence of physicochemical parameters and perfusate flow rate on the distribution of solutes on the isolated perfused rat hindlimb determined by the impulse-response technique. J. Pharm. Sci. 84: 1020–1027

Wu, Z. Y., Smithers, B. M., Roberts, M. S. (1997) Tissue and perfusate pharmacokinetics of melphalan in the isolated perfused rat hindlimb. J. Pharmacol. Exp. Ther. 282: 1131–1138

Yamaoka, K., Nakagawa, T., Uno, T. (1978) Statistical moments in pharmacokinetics. J. Pharmacokinet. Biopharm. 6: 547–558