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Short-period Double-dosing for Simultaneous Evaluation of Intestinal Absorption and Hepatic Disposition in a Single Conscious Rat Using Cephalexin as Test Drug

TAKASHI ITO, KIYOSHI YAMAOKA AND TERUMICHI NAKAGAWA

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

Abstract

A new method has been developed for simultaneous evaluation of local absorption from the intestine into the portal system and local disposition through the liver, and for assessment of the bioavailability of a drug in a single conscious rat. The method is based on the difference between plasma concentrations in portal and systemic blood (PS method).

Because cephalexin is known to be absorbed completely from the intestine and not to be eliminated through the liver, it was used as test drug to confirm the validity of the new method. The portal vein and the femoral artery of a rat were simultaneously cannulated and blood samples were obtained from both sites. Two methods of administration, single-dosing and double-dosing, were investigated and the efficacy of double-dosing (DD) was demonstrated. Rats received an intra-arterial (group A) or oral (group B) dose in single-dosing, whereas rats used for double-dosing received an oral dose 3 h after an intra-arterial dose (group C). After administration of cephalexin, the portal and arterial plasma concentrations were determined by HPLC. Groups A and B were monitored for 4 h and group C for 8 h. The portal-blood flow rate was measured by means of an electromagnetic flow-meter. Global and local moments were calculated by trapezoidal integration with extrapolation to infinite time. On the basis of the PS method, the local absorption ratio (F_a) and the mean local absorption time ($\bar{t}_a$) were estimated to be $0{\cdot}975 \pm 0{\cdot}104$ and $2{\cdot}19 \pm 0{\cdot}51$ h, respectively, in group B. By comparing the averaged moments between groups A and B, the extent of bioavailability (F), the mean absorption time (MAT) and the hepatic recovery ratio (F_H) were calculated to be 1·01, 1·92 h and 1·04, respectively. The mean hepatic transit time ($\bar{t}_H$) was negligible. In group C, $F_a = 0{\cdot}936 \pm 0{\cdot}107$, $\bar{t}_H = 1{\cdot}55 \pm 0{\cdot}32$ h, $F = 1{\cdot}08 \pm 0{\cdot}07$, $MAT = 1{\cdot}55 \pm 0{\cdot}40$ h and $F_H = 1{\cdot}17 \pm 0{\cdot}14$ h, the mean values being close to those from groups A and B.

In conclusion, the PS method with short-period double-dosing (PS-DD method) can offer an effective means of evaluating the local absorption kinetics of drugs, because F, MAT and F_H are obtained from a single conscious rat, and consequently the standard deviations of the quantities can be quickly estimated.

When a drug is absorbed from the intestinal tract through the portal system into the systemic circulation, some of the drug transferred from the intestinal lumen is eliminated in the intestine and the liver (Pond & Tozer 1984; Greenblatt 1993). Because low intestinal absorption and high hepatic elimination (possibly pulmonary elimination) result in low drug bioavailability, many studies have attempted to achieve high bioavailability. Some drugs have been intra-portally infused and orally administered to evaluate intestinal absorption, and intra-portally infused and intravenously administered to estimate hepatic elimination (Harris & Riegelman 1969; Cassidy & Houston 1980; Zhong et al 1994). Intraperitoneal administration has also been used in an attempt to avoid intestinal metabolism because drugs given by this route are absorbed directly into the portal system and by-pass the gut wall before arriving at the systemic circulation (Gibaldi & Perrier 1975; Pang & Gillette 1978; Wang et al 1989). The gut and hepatic availability of drugs have been estimated by kinetic analysis after intravenous and oral doses, on the assumption that extrahepatic metabolism is negligible after intravenous administration (Thummel et al 1996). Using inhibitors (ketoconazole and erythromycin) against the intestinal metabolism of cyclosporine, the fraction of the drug absorbed through the gastrointestinal membranes was estimated, assuming that the intestinal metabolism of cyclosporin was completely blocked by the inhibitors (Wu et al 1995). For direct estimation of both intestinal absorption and hepatic metabolism, the intestine and the liver were simultaneously perfused (Pang et al 1985; Soria & Zimmerman 1993), and the portal vein was exchanged with the inferior vena cava (Effeney et al 1982; Lo et al 1982; Souich et al 1995).

A drug for oral use is usually administered to a conscious patient. Therefore, for prediction of the disposition of a drug in man, it is important to know the value for conscious animals. Recently, a method of portal–systemic concentration difference (PS method) using conscious animals and simultaneous sampling of portal and systemic bloods has been developed for direct determination of the intestinal absorption of drugs into the portal system (Hoffman et al 1995; Tabata et al 1995; Uhing & Kimura 1995a, b). Hoffman et al (1995) attempted to estimate bioavailability in a single rat by administration of consecutive intravenous and intra-jejunal doses with an interval for washing out of the drugs. A possible problem with their method is that the experiment is time-consuming and the physiological conditions (e.g. microbial infection and exhaustion) of the cannulated rat can change after first administration.

The purpose of this work was to develop the PS method with short-period double-dosing (PS-DD method) for effective

Correspondence: K. Yamaoka, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan.

evaluation of intestinal absorption and hepatic elimination, and to assess the bioavailability of a drug in a single conscious rat. The number of rats killed can be reduced and the variances in bioavailability and hepatic first-pass effect can also be estimated.

The PS-DD method is basically designed for evaluating the absorption kinetics of a drug with low bioavailability. However, cephalexin with high bioavailability was selected as a test drug to confirm the validity of the PS-DD method, because cephalexin has no physiological effect on the blood vascular system and is well known to be absorbed completely from the intestine into the portal system and not to be eliminated through the liver in rat and man (Sullivan et al 1969; Nightingale et al 1975; Nakagawa et al 1978).

Materials and Methods

Chemicals

Cephalexin was obtained from Wako Pure Chemical Industries (Osaka, Japan) and heparin from Novo Industries (Denmark). Sodium pentobarbital solution (nembutal for animal injection; Abbott Laboratories, North Chicago, IL) was used to anaesthetize the rats for measurement of portal-blood flow-rate. Other reagents used for the animal experiment and for the assay of cephalexin were of guaranteed reagent grade or HPLC grade.

Measurement of portal-blood flow-rate

Male Wistar rats, 230–260 g (n = 7), were used for measurement of portal-blood flow-rate. Rats were fasted for 16 h and anaesthetized by intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1}). After opening of the abdomen by midline incision, the upper part of portal vein close to liver was dissected to free it from surrounding tissue. A polyethylene catheter (PE-10) filled with saline was inserted into the portal vein from the confluence of the portal vein and the inferior pancreaticoduodenal vein (Waynforth & Flecknell 1992) and was advanced along the portal vein so that the tip of catheter lay in front of the point where the probe was to be attached. The catheter was secured to the mesentery with adhesive. After 5 min a perivascular flow sensor (internal diameter 1·5 mm; Skalar Medical, Delft, the Netherlands) was placed around the portal vein. The signals for the portal-blood flow-rate were measured for 3 min with a compact electromagnetic flowmeter (MDL1401; Skalar Medical). The measurement was performed twice at an interval of 15 min, and the mean value was regarded as the individual portal-blood flow-rate. During the 15-min interval the probe was removed from the portal vein which was placed back into the abdomen. After completion of the second measurement the blood flow rate in the pyloric vein was measured with the perivascular flow-sensor (internal diameter 0·6 mm).

Animal experiments

The study was performed with male Wistar rats, 186–268 g. After 16 h fasting the abdomen of each rat was opened by midline incision under light ether anaesthesia. The first catheter (PE-10) filled with heparinized (100 int. units mL^{-1}) normal saline was inserted into the portal vein from the confluence of the portal vein and the inferior pancreaticoduodenal vein and was advanced along the portal vein so that the tip of the catheter lay close to the liver. The catheter was secured to the mesentery with adhesive. The free end of the catheter was exteriorized through a small puncture in the abdominal wall. Simultaneously the right femoral artery of each rat was cannulated and the free end of the second cannula containing heparinized (100 int. units mL^{-1}) normal saline was subcutaneously conducted and exteriorized at the back of the leg. Each rat was held in the Bollman cage and was left for 2 h to recover from the light ether anaesthesia. During the experiment water was freely available to every rat. A small volume of portal blood was collected from every rat for measuring the hematocrit (Ht) and centrifuged for 15 min at 3000 rev min^{-1}. Cephalexin dissolved in an isotonic phosphate buffer (pH 7·4) was administered intra-arterially (group A) or orally (group B) at a dose of 25 mg kg^{-1}. In group C, cephalexin (25 mg kg^{-1}) was administered orally 3 h after intra-arterial administration of cephalexin at the same dose. At designated times blood samples (60 μL) were drawn simultaneously from the portal vein and femoral artery of each group. After each sampling, the reduction in blood volume was supplemented with half the volume of heparinized normal saline. After centrifugation for 5 min at 2000 *g* separated plasma samples were analysed on the same day.

The research adhered to the 'Principles of Laboratory Animals Care' (NIH publication #85–23, revised 1985).

Analytical procedure

HPLC (LC-10A; Shimadzu, Kyoto, Japan) was performed with a 250 mm × 4·6 mm i.d. Capcellpak C18 column (Shiseido, Tokyo, Japan). The mobile phase for samples from groups A and B was 100 mM NaH_2PO_4–MeOH (4 : 1, v/v, adjusted to pH 6·2 with NaOH). For more rapid analysis of samples from group C, the mobile phase was H_2O–MeOH (7 : 2, v/v, adjusted to pH 3·05 with acetic acid; Yano et al 1991). The detector wavelength, the flow rate and the column temperature were 264 nm, 1·0 mL min^{-1} and 40°C, respectively. Peak size was measured with a Chromatopac C-R6A (Shimadzu).

To determine cephalexin concentration in the plasma, 30 μL plasma was vortex-mixed vigorously for 30 s with 90 μL methanol then centrifuged at 2000 *g* for 5 min. The supernatant (15 μL) was analysed by HPLC. The detection limit was 0·1 μg mL^{-1}. Calibration lines were freshly prepared by adding cephalexin (five different concentrations in the range 0·5 to 100 μg mL^{-1}) to the plasma. All correlation coefficients were greater than 0·999.

Data analysis

The rate of absorption (dA(t)/dt) from the intestinal tract into the portal system was calculated by use of equation 1:

$$dA(t)/dt = Q_b(C_b^{por}(t) - C_b^{art}(t)) = \bar{Q}_p(C_p^{por}(t) - C_p^{art}(t)) \quad (1)$$

where Q_p and $\bar{Q}_p$ are the blood and effective plasma flow-rates in the portal vein, respectively, $C_b(t)$ and $C_p(t)$ are, respectively, the time-courses of the blood and plasma concentrations of the drug, and the superscripts 'por' and 'art' specify 'portal' and 'artery', respectively. The effective plasma flow rate ($\bar{Q}_p$) was calculated by use of equation 2, taking into consideration the weight of the rat:

$$\bar{Q}_p = Q_b(1 - Ht)(1 + k_b)Wt/250 \quad (2)$$

where Wt is the weight of the rat and Ht is the haematocrit of the portal vein. The partition ratio (k_b) of cephalexin between erythrocytes and plasma is regarded as negligible (Yano et al 1991). The local moments for the absorption rate–time curve are defined by equations 3 and 4:

$$F_a = \int_0^\infty \frac{dA(t)}{dt} dt/D \quad (3)$$

$$\bar{t}_a = \frac{\int_0^\infty t \cdot \frac{dA(t)}{dt} dt}{\int_0^\infty \frac{dA(t)}{dt} dt} \quad (4)$$

where F_a is the absorption ratio of the dose from the intestinal lumen into the portal system and $\bar{t}_a$ is the mean local absorption time from the gastrointestinal tract into the portal system. D is the dose of cephalexin. The bioavailability (F) and the mean absorption time (MAT) of cephalexin were calculated by use of equations 5 and 6, assuming the pulmonary local disposition was negligible.

$$F = \frac{AUC^{art}_{p.o.}}{AUC^{art}_{i.a.}} = F_a \cdot F_H \quad (5)$$

$$MAT = MRT^{art}_{p.o.} - MRT^{art}_{i.a.} = \bar{t}_a + \bar{t}_H \quad (6)$$

where F_H is the hepatic recovery ratio, $\bar{t}_H$ is the mean hepatic transit time (which is predicted to be negligible), superscript 'art' specifies 'arterial blood' and subscripts p.o. and i.a. specify oral and intra-arterial administration, respectively.

In groups A and B, the area under the curve (AUC) and the mean residence time (MRT) were calculated using the linear trapezoidal method with extrapolation to infinite time. For group C, AUC and MRT of the front portion, arising from the first intra-arterial dose, were calculated in the same way as for groups A and B. The plasma concentration predicted to arise in the artery from the intra-arterial dose was subtracted from the concentration in the rear portion after the second oral dose. AUC and MRT of the time-course after an oral dose were calculated using the subtracted time-course data in the rear portion, regarding the second dosing time as the origin.

All experimental results were expressed as arithmetic means and standard deviations of results from four rats. Statistical analysis was performed by two-way analysis of variance at the 5% significance level.

Results

In this experiment the portal-blood flow-rate was estimated to be 15·3 ± 2·2 mL min^{-1}/250 g (where 250 g is the nominal body weight), close to that reported in the literature (Hadengue et al 1988; Daemen et al 1989). A lower value, 13·3 mL min^{-1}/250 g, was reported in our previous paper (Fujieda et al 1996); this difference is because the portal blood was sampled though the pyloric vein in the previous experiment and consequently the blood flow through the pyloric vein was blocked. The pyloric vein was not occluded in this experiment and the portal blood flow measured included that through the pyloric vein. The blood flow rate in the pyloric vein was evaluated to be 1·30 ± 0·42 mL min^{-1}/250 g (n = 7), approximately 10% of portal blood-flow. It is possible that pentobarbital might affect the portal flow-rate. Hadengue et al (1988) reported that portal-blood flow-rate was not affected by pentobarbital anaesthesia nor portal vein cannulation. Therefore, the obtained flow rate of portal blood is considered to be close to that in the conscious rat.

Fig. 1 shows the time-courses of the portal and arterial plasma concentrations of cephalexin in rats after intra-arterial and oral doses (single-dosing). Each point in the figure represents the mean of results from groups A (n = 4) and B (n = 4). There was no difference between portal and arterial plasma concentrations in group A, whereas the portal plasma concentration was constantly higher than the arterial plasma concentration in group B. It has previously been shown that the concentration difference between portal and arterial bloods

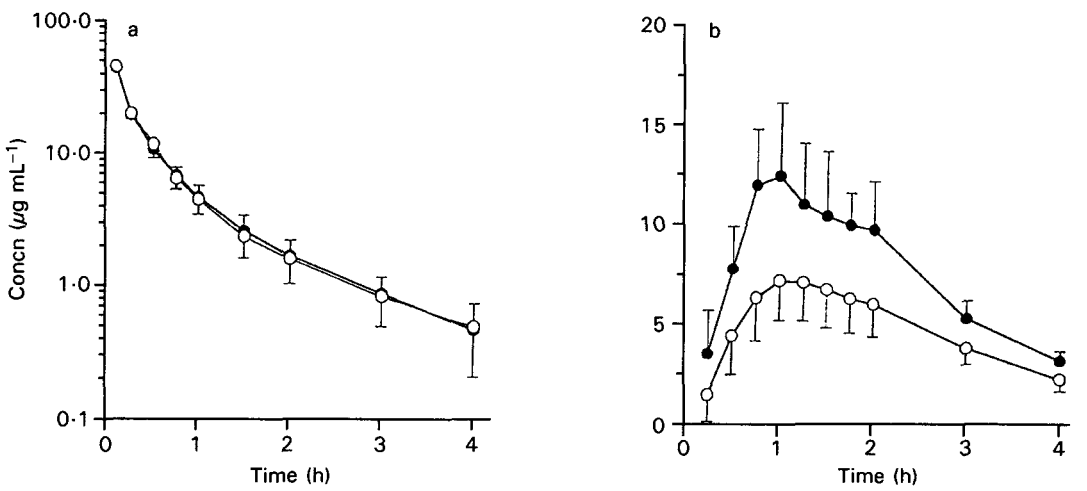

FIG. 1. a. Mean portal (●) and arterial (○) plasma concentration time-courses of cephalexin after intra-arterial (group A) administration (25 mg kg^{-1}); b. mean portal (●) and arterial (○) plasma concentration time-courses of cephalexin after oral (group B) administration (25 mg kg^{-1}). Each point represents the mean and s.d. (n = 4).

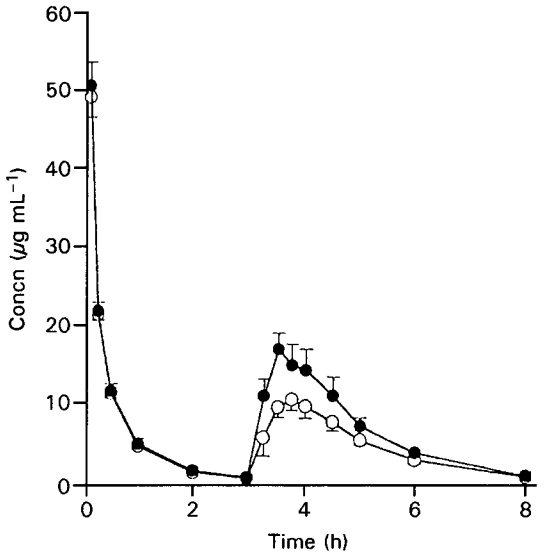

FIG. 2. Mean portal (●) and arterial (○) plasma concentration time-courses of cephalexin after intra-arterial administration then oral administration (group C; 25 mg kg^{-1}). Each point represents the mean and s.d. (n = 4).

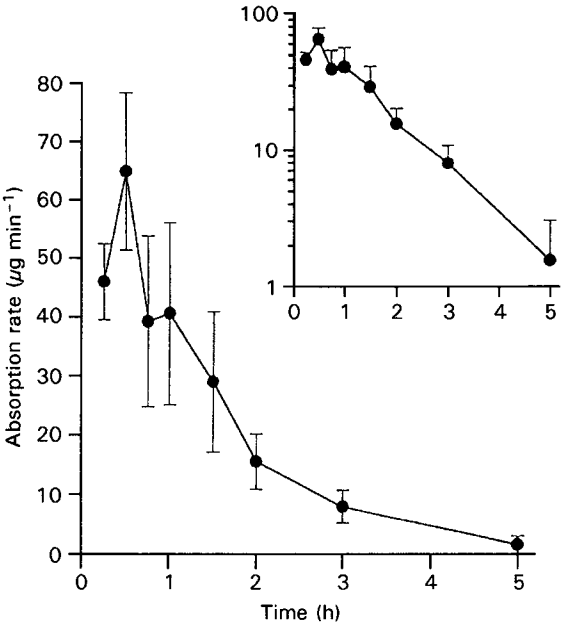

FIG. 3. Predicted time-course of rate of absorption of cephalexin into the portal system. Each point represents the mean and s.d.

was not appreciable after intravenous administration, even in the presence of large hepatic elimination (Tabata et al 1995; Fujieda et al 1996). Therefore, this portal-arterial concentration difference in group B was a result not of hepatic elimination but of absorption of cephalexin from the intestinal tract into the portal system.

Table 1 lists the AUC and MRT of the time-courses of the portal and arterial plasma concentrations after intra-arterial and oral administration. In group A there was no difference between AUC and MRT values in portal and arterial blood at a 5% level of significance on two-way analysis of variance, suggesting no intestinal re-absorption after intra-arterial administration (Tabata et al 1995). In group B, AUC in the portal vein was significantly greater than that in the artery and MRT was significantly smaller. F_a and $\bar{t}_a$ were estimated to be $0{\cdot}975 \pm 0{\cdot}104$ and $2{\cdot}19 \pm 0{\cdot}51$ h, respectively, using the time-curve of the rate of absorption obtained from the portal–arterial plasma concentration difference. The bioavailability (F), defined by equation 5, was calculated to be 1·01 by comparing the arterial AUC values in groups A and B; consequently F_H ($= F/F_a$) was estimated to be 1·04. The mean absorption time (MAT) defined by equation 6 is calculated to be 1·92 h, which was close to the value of $\bar{t}_a$ ($\approx$ MAT). This result demonstrates that the mean hepatic transit time ($\bar{t}_H$) is negligible on this experimental scale.

Fig. 2 presents the time-courses of portal and arterial plasma concentrations of cephalexin in group C (double-dosing). Each point in the figure represents the mean (n = 4). Although there was no difference between portal and arterial plasma concentrations from 0 to 3 h, the portal concentration was always higher than the arterial concentration after 3 h, the time of the subsequent oral dose. Fig. 3 shows the absorption rate-time curve in group C, with the time of oral dosing regarded as the origin. The inset shows the semi-logarithmic plots of Fig. 3, which demonstrate that cephalexin is absorbed into the portal system roughly according to a mono-exponential process.

Table 2 lists the AUC and MRT values of the time-courses of the portal and arterial plasma concentrations for group C. AUC and MRT values for both arterial and portal concentrations in the front portion of group C were not significantly different from those of group A. AUC values and MRT values of both arterial and portal concentrations in the rear portion were not significantly different from those of group B. It is suggested from this comparison that the PS-DD method can provide reasonable moment values. In the rear portion of group C, the AUC for portal blood was significantly greater than that for arterial blood whereas MRT for portal blood was significantly lower than that for arterial blood. The difference between the plasma concentrations for arterial and portal blood in the rear portion was attributed to the absorption of cephalexin from the intestinal lumen into the portal system, as mentioned above. Table 3 lists the local absorption parameters calculated from the global moments in Table 2. F and F_a were $1{\cdot}08 \pm 0{\cdot}07$ and $0{\cdot}936 \pm 0{\cdot}107$, respectively, and consequently F_H was estimated to be $1{\cdot}17 \pm 0{\cdot}14$. MAT ($1{\cdot}55 \pm 0{\cdot}40$ h) was close to $\bar{t}_a$ ($1{\cdot}55 \pm 0{\cdot}32$ h).

Discussion

The bioavailability of a drug is characterized by its extent and time which are specified by F ($= F_a \times F_H$) and MAT ($\approx \bar{t}_a$), respectively. Because the bioavailability is affected by absorption from the intestine into the portal system and the elimination by the liver, the contributions of the intestine and the liver to the bioavailability should be estimated separately. For single-dosing F and F_a of cephalexin were calculated to be close to 100% for groups A and B, and F_H was estimated to be 100%. For double-dosing, F and F_a were calculated to be close to 100% for group C, and F_H was estimated to be 100%. MAT and $\bar{t}_a$ were found to be the same for single- and double-dosing, suggesting that transport of cephalexin from the intestinal tract into portal system is the rate-limiting step and $\bar{t}_H$ is too small to

Table 1. Moment characteristics of cephalexin after intra-arterial (group A) and oral (group B) administration (25 mg kg^{-1}).

Characteristic	Rat no. 1	2	3	4	Mean (s.d.)
Group A					
Haematocrit	0·431	0·470	0·435	0·429	0·441 (0·017)
Area under the plasma concentration–time curve (μg h mL^{-1})					
Portal	24·9	24·5	17·9	24·4	22·9 (3·4)
Artery	25·6	25·1	17·5	24·7	23·2 (3·8)
Mean residence time (h)					
Portal	0·842	0·856	0·512	0·986	0·799 (0·202)
Artery	0·842	0·994	0·471	0·958	0·816 (0·239)
Group B					
Haematocrit	0·485	0·476	0·495	0·473	0·485 (0·011)
Area under the plasma concentration–time curve (μg h mL^{-1})					
Portal	44·5	36·3	30·1	32·1	35·7 (6·4)
Artery	31·7	23·2	17·7	21·3	23·5 (6·0)
Mean residence time (h)					
Portal	2·24	2·51	2·80	2·34	2·47 (0·25)
Artery	2·67	2·88	2·89	2·52	2·74 (0·18)
Local absorption ratio F_a	1·05	1·06	0·954	0·837	0·975 (0·104)
Mean local absorption time $\bar{t}_a$(h)	1·68	2·19	2·88	2·00	2·19 (0·51)

Table 2. Moment characteristics of cephalexin after intra-arterial administration then oral administration (group C; 25 mg kg^{-1}).

Characteristic	Rat no. 1	2	3	4	Mean (s.d.)
Group A					
Haematocrit	0·400	0·411	0·407	0·434	0·413 (0·013)
Intra-arterial					
Area under the plasma concentration–time curve (μg h mL^{-1})					
Portal	24·4	23·3	25·6	26·5	24·9 (1·4)
Artery	24·8	22·8	22·8	25·1	23·9 (1·3)
Mean residence time (h)					
Portal	0·759	0·732	0·675	0·631	0·699 (0·057)
Artery	0·921	0·558	0·522	0·652	0·663 (0·180)
Oral					
Area under the plasma concentration–time curve (μg h mL^{-1})					
Portal	35·4	38·4	35·9	36·4	36·5 (1·1)
Artery	25·8	25·6	26·5	25·0	25·7 (0·5)
Mean residence time (h)					
Portal	2·15	1·57	2·46	1·75	1·98 (0·35)
Artery	2·37	1·69	2·73	2·06	2·21 (0·38)

AUC values after oral administration are corrected as described in data analysis.

Table 3. Local absorption parameters of cephalexin after intra-arterial administration then oral administration (group C; 25 mg kg^{-1}).

Parameter	Rat no. 1	2	3	4	Mean (s.d.)
Bioavailability F	1·04	1·12	1·16	1·00	1·08 (0·07)
Local absorption ratio F_a	0·837	1·11	0·856	0·944	0·936 (0·107)
Hepatic recovery ratio F_H	1·24	1·01	1·36	1·06	1·17 (0·14)
Mean absorption time (h)	1·45	1·13	2·21	1·40	1·55 (0·40)
Mean local absorption time $\bar{t}_a$ (h)	1·63	1·34	2·03	1·19	1·55 (0·32)

be estimated on this experimental scale. Thus, the contributions of the intestine and the liver to the bioavailability were estimated separately in a conscious rat. Double-dosing is superior to single-dosing, because double-dosing requires fewer animals, and all local absorption parameters including F, MAT and F_H were obtained from a single rat. For double-dosing the second dosing time is important for correct estimation of local absorption parameters. In this experiment the β phase on the cephalexin time-course was observed after approximately 30 min and the second dosing time (3 h) was approximately four times the MRT of intra-arterial administration. The time of second dosing could be set earlier. Cephalexin with high bioavailability ($F = 100\%$) was selected to confirm the validity of the PS-DD method. However, the PS-DD method presented here should be used for evaluation of intestinal local absorption and hepatic local disposition of drugs with low bioavailability. The PS-DD method is inappropriate for drugs which are eliminated and absorbed very slowly, because all experiments must be finished within one day at most. The method is also inappropriate for drugs for which a large volume of blood is required to achieve the assay sensitivity requested, and for drugs whose first intra-systemic administration considerably changes their global disposition after the second oral dose.

Concerning blood volume, the number of sampling points can be reduced by curve-fitting techniques. If the concentrations in portal and arterial blood were not appreciably different after intra-arterial (and intravenous) administration, portal blood sampling in the front portion can be omitted to reduce the total volume of blood sampled.

In conclusion, the PS-DD method can furnish reliable local and global kinetic parameters and it is possible to estimate bioavailability (F) and hepatic recovery ratio (F_H) in a single conscious rat, in addition to local absorption ratio (F_a). Therefore, analysis of variance can be used for statistical testing of changes in F and F_H with a change in animal experimental conditions.

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