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Pharmacokinetics of N^4-Octadecyl-1-β-D-Arabinofuranosylcytosine in Plasma and Whole Blood after Intravenous and Oral Administration to Mice

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Abstract

N^4-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC) is a new cytotoxic derivative of cytosine arabinoside with improved cytotoxic activity and stability against deamination. Its pharmacokinetics were studied in mice.

The drug was administered intravenously and orally to ICR mice to assess its pharmacokinetic parameters in plasma and whole blood. The lipophilic drug was administered in small unilamellar liposomes 100–400 nm in diameter. The concentrations of NOAC in plasma and erythrocytes were determined by high-performance liquid chromatography (HPLC). When given orally a rather low amount of the delivered NOAC was absorbed as the unchanged drug, resulting in a bioavailability of 1·1% from the plasma and 12·9% from whole blood. As shown elsewhere, the amount of drug absorbed is sufficient to provide excellent cytotoxic activity in the L1210 leukemia and in human xenograft models after oral administration. The mean residence time of NOAC after intravenous administration was 3·5 h in plasma and 6 h in whole blood giving NOAC a terminal half-life in blood substantially longer than that of cytosine arabinoside. After oral administration the mean residence time was 18 h in plasma and whole blood.

In summary, NOAC has a prolonged half-life after intravenous administration compared with cytosine arabinoside. The distribution of NOAC in blood is highly dependent on its mode of administration.

1-β-D-Arabinofuranosylcytosine (cytosine arabinoside) is one of the most important agents for the treatment of acute myelogenous leukaemia (Keating et al 1982; Plunkett & Gandhi 1993). However, it is rapidly inactivated by deamination to the biologically inactive metabolite 1-β-D-arabinofuranosyluracil (Ho & Frei 1971). This biological instability necessitates intravenous administration of the drug either as a continuous infusion over 5 days at the low dose of 100–200 mg m^{-2} day^{-1} or as a short infusion over 2 h at a high dose of 1–3 g m^{-2} day^{-1} (Momparler 1974; Bolwell et al 1988).

Many different approaches have been tried in attempts to improve the stability and, consequently, the in-vivo cytotoxic activity of cytosine arabinoside. Combination therapy with deaminase inhibitors such as tetrahydrouridine (Kreis et al 1988; Marsh et al 1993) did not significantly improve the cytotoxic efficacy of cytosine arabinoside. Another improvement of the biological stability of cytosine arabinoside can be achieved by chemical modification, mainly at two positions in its molecular structure; steroids (Hong et al 1979) or phospholipids (Matsushita et al 1981) have been attached to the carbon atom at position 5 of the arabinofuranoside acyl chains (Gish et al 1971). However, significantly increased stability was not achieved, which is not unexpected because the amino group remains exposed to deamination. Thus a large number of N^4-derivatives of cytosine arabinoside have been synthesized with the aim of increasing the cytotoxic activity by protection against deamination and alteration of the pharmacokinetic properties (Wempen et al 1968; Kanai & Ichino 1974; Rosowsky et al 1982). Whereas short-chain modifications of cytosine arabinoside at the N^4-amino group generally resulted in weak enhancement of cytotoxicity (Aoshima et al 1976), lipophilic derivatives with long-chain fatty acids showed strong antitumour activity in murine tumour models (Kataoka & Sakurai 1980; Tsuruo et al 1980).

Different N^4-acyl derivatives (Yamada et al 1980; Rubas et al 1986) were reported to be active in murine L1210 leukaemia and B16 melanoma at lower concentrations than cytosine arabinoside (Rubas et al 1986). However, protection was only partially achieved and suggested to be still insufficient in a pilot phase I/II study (Schwendener et al 1989).

Derivatives containing an alkyl chain with 16 or 18 carbon atoms linked to the amino group of cytosine (N^4-hexadecyl- and N^4-octadecyl-1-β-D-arabinofuranosylcytosine, NHAC and NOAC; Schott et al 1994) resulted in the most active compounds in the murine L1210 leukaemia model; they were active at molar concentrations one-sixteenth of those of cytosine arabinoside (Schwendener & Schott 1992) and were strongly cytotoxic when administered as single-dose therapy, suggesting a long-lasting drug effect. The structural formula of NOAC is depicted in Fig. 1.

By in-vitro experiments on human tumour cell lines it was demonstrated that the cytotoxic effect of NHAC and NOAC is to a large extent independent of the formation of cytosine arabinoside and its phosphorylated metabolites (Horber et al 1995b). The exact mechanism of action is currently under investigation. Because of their resistance to cytidine deaminase, NHAC and NOAC also have a curative effect in the L1210 mouse leukaemia model and in human xenograft

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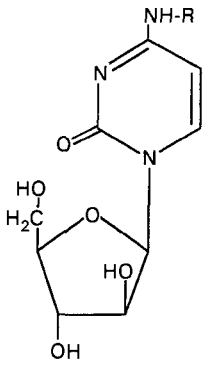

FIG. 1. Structural formulae of 1-β-D-arabinofuranosylcytosine (R = H) and NOAC (N^4-octadecyl-1-β-D-arabinofuranosylcytosine; R = $C_{18}H_{37}$).

models when administered orally (Schwendener et al 1996). In-vitro studies describing the interactions of NHAC with blood components showed that the drug binds rapidly and with high efficiency to erythrocytes, leukocytes and plasma proteins (Horber et al 1995a). In an earlier study with NOAC, in-vitro interactions of NOAC with human blood were described (Koller-Lucae et al 1997). Initially the drug is distributed in erythrocyte membranes; this is followed by re-distribution into plasma proteins and preferentially into the lipoproteins.

In the study described in this paper, the pharmacokinetic properties of NOAC in plasma and erythrocytes were analysed after intravenous and oral application in mice.

Materials and Methods

Lipids and chemicals

Soy phosphatidylcholine was obtained from L. Meyer (Hamburg, Germany). Cholesterol, recrystallized from methanol, and analytical grade buffer salts were from Fluka AG (Buchs, Switzerland). DL-α-Tocopherol and all HPLC-grade solvents were from Merck (Darmstadt, Germany). The synthesis of N^4-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC) has been described elsewhere (Schott et al 1994; Schwendener & Schott 1992).

Preparation of the NOAC formulations

Unilamellar liposomes were prepared by filter extrusion of multilamellar liposome suspensions using a Lipex extruder (Lipex Biomembranes, Vancouver, Canada) as described by Schwendener et al (1996). They were composed of soy phosphatidylcholine (40 mg mL^{-1}), cholesterol (4 mg mL^{-1}) and DL-α-tocopherol (0·2 mg mL^{-1}) in the molar ratio 1:0.2:0.01. NOAC (5 mg mL^{-1}) was added to the lipids in 1:1 (v/v) methanol–dichloromethane.

After evaporation of the organic solvents at 40°C multilamellar liposomes were obtained by addition of phosphate buffer (67 mM, pH 7·4). After extrusion through 0·4-μm Nucleopore membranes (Costar, Sterico, Dietikon, Switzerland), the liposomes were concentrated in an Amicon ultrafiltration cell (Amicon, Lexington, MA; Diaflo YM membranes, Mr = 100 000 cut-off) to furnish drug concentrations of 9·5 mg mL^{-1} (liposomal formulation A) and 4·7 mg mL^{-1} (liposomal formulation B) for oral and intravenous administration experiments, respectively. The preparation A used for intravenous application was also extruded through 0·1-μm membranes; this did not change the binding of NOAC to the liposomes.

Pharmacokinetic analysis in mice

Animals. Experiments were performed on female ICR-mice, 22·7 ± 1·5 g. The animals were housed in air-conditioned rooms (25°C) on a 12-h/12-h light/dark schedule. Tap water and a commercial, pelleted maintenance diet were freely available. Before oral treatment the mice were fasted overnight. Irrespective of body weight, either 200 μL liposomal formulation B was injected intravenously into the tail vein or 600 μL liposomal formulation A was administered orally by use of a stomach catheter; this corresponded to a dose of 2·8 and 1·9 mg NOAC per mouse for oral and intravenous application, respectively. Three mice were used for each mode of administration and for each measurement time point (ethical approval No. 96/94, Veterinary Department of the Canton Zurich). The mice were killed by heart puncture under diethyl ether anaesthesia and blood was removed into heparin-coated Vacutainer tubes (Becton Dickinson, Meylan Cedex, France). To minimize deamination of NOAC, a solution of tetrahydrouridine in phosphate-buffered saline (10 mg mL^{-1}; 30 μL) was added to the sampling tubes before acquisition of the blood. Plasma and blood cells were immediately separated by centrifugation (2800 *g* for 10 min) and the erythrocytes were washed once with tetrahydrouridine solution. The total amounts of plasma and washed erythrocytes in the samples were determined by weighing and the erythrocytes were suspended in 1 mL tetrahydrouridine solution. All samples were immediately frozen at −30°C until analysis.

Sample preparation and drug analysis. The methods used for sample preparation and analysis of NOAC by HPLC have been reported previously (Rentsch et al 1995). Briefly, plasma (standards, controls or samples; 1 mL), internal standard (6·25 μg NHAC mL^{-1}; methanol 100 μL) and urea solution (10 M; 1 mL) were incubated overnight at constant temperature (25°C) and 100 rev min^{-1} on a horizontal shaker. Solid-phase extraction (SPE) was performed using BondElut C18 cartridges (Varian, Sunnyvale, CA). The cartridges were prewashed with distilled water (10 mL), methanol (10 mL) and distilled water (10 mL) and the entire volume of the incubated sample was applied to the cartridge, followed by elution with distilled water (1 mL) and 33% methanol (0·5 mL). Finally, NHAC and NOAC were eluted with methanol (3 mL). The methanol was removed by rotary evaporation (Rotavapor, Büchi, Flawil, Switzerland) and the residue dissolved in mobile phase (150 μL), as described below.

Erythrocytes (standards, controls or samples) were suspended in tetrahydrouridine-solution (1·0 mL) and internal standard (6·25 μg NHAC mL^{-1}; methanol 100 μL), phosphate-buffered saline (pH 7·4; 1 mL) and butanol (1 mL) were added and the mixture was sonicated for 2 h (Branson, 90W, Bransonic, Shelton, CT) and then centrifuged at 4800 *g* for 10 min. The supernatant was purified by SPE as described for plasma. Methanol (3 mL) was added to the erythrocyte pellet and the mixture sonicated for 30 min. The samples were then centrifuged at 4800 *g* for 10 min and the methanol supernatant was immediately pipetted into a clean vial. This procedure was repeated once. The methanolic extracts of NOAC were com-

bined and the solvent evaporated. For HPLC analysis the samples were dissolved in 200 μL mobile phase.

The HPLC system consisted of a 9010 pump, a 9100 autosampler with 92-μL loop, a 9050 UV-Vis-detector operated at 275 nm (Varian, Sunnyvale, CA), and a Croco-cil column-heater (Cluzean-info-labo SA, Saint-Foy-la-Grande, France). Two identical columns (Nucleosil C18, 5-μm particle size, 120–Å pore size, 250 × 4 mm i.d.) were connected in series, and protected with a guard column (11 × 4 mm) packed with the same material. The first column was kept at room temperature, the second at 45°C. The mobile phase consisted of a 10:1 mixture of methanol and 0·16 M ammonium formate buffer (pH 2·7). The mobile phase flow rate was 0·9 mL min^{-1}.

Pharmacokinetic analysis. The same dose of the appropriate formulation of NOAC was administered intravenously (1·9 mg) or orally (2·8 mg) to all mice, irrespective of body weight. For direct comparison of the pharmacokinetic parameters all measured concentrations of NOAC were corrected to a dose of 100 μg (g body weight)$^{-1}$. Pharmacokinetic analysis was performed for plasma and whole blood. Whole blood NOAC concentrations were determined relative to the haematocrit using plasma and erythrocyte data. The area under the curve of drug concentration as a function of time (AUC) and the area under the moment curve (AUMC) were determined with the trapezoidal rule for infinite time. Parameters were calculated as follows:

$$\text{Mean resistance time: } MRT = AUMC(\infty)/AUC(\infty) \quad (1)$$

$$\text{Intravenous: } MRT = MRT_{i.v.} \quad (2)$$

$$\text{Oral: } MRT_{p.o.} = MRT_{i.v.} + MAT \quad (3)$$

where MAT is the mean absorption time.

Total clearance

$$\text{Intravenous: } CL_{tot} = \text{dose}/AUC(\infty) \quad (4)$$

$$\text{Oral: } CL_{tot} = F \times \text{dose}/AUC(\infty) \quad (5)$$

$$\text{Steady-state volume of distribution: } V_{SS} = MRT \times CL_{tot} \quad (6)$$

The initial concentration (C_0) was extrapolated from the intravenous data.

The bioavailability (BA) was calculated using the equation:

$$BA = AUC_{p.o.(\infty)}/AUC_{i.v.(\infty)} \quad (7)$$

To estimate the absorption of NOAC after oral administration, the fraction absorbed (f_a) was calculated according to the Wagner–Nelson equation (Wagner & Nelson 1963):

$$f_a = (A_T/V)/(A_{max}/V) \quad (8)$$

where A_T is the amount of drug which reaches the central compartment at time T, V is the volume of the central compartment and A_{max}/V is the asymptotic value of A_T/V. A_T/V was calculated using the equation:

$$A_T/V = C_T + k_{10} \times AUC_{(0\rightarrow T)} \quad (9)$$

where C_T represents the concentration at time T and k_{10} is the final elimination constant, estimated from the last points of measurement.

The f_a value enables calculation of the absorption constant k_{01} and the respective absorption half-life ($t_{\frac{1}{2}(01)}$).

To estimate the in-vivo binding of NOAC to erythrocytes, the erythrocyte/plasma partition coefficients ($P_{e/p}$) from the intravenous and oral data were calculated using (Roos & Hinderling 1981):

$$P_{e/p} = C_e/C_p \quad (10)$$

where C_e and C_p represent the concentrations of NOAC in the erythrocytes and plasma, respectively.

Results

Liposomal NOAC was either injected as an intravenous bolus or administered orally to mice as described above. The concentrations of NOAC in plasma and erythrocytes were determined by HPLC and the concentration of NOAC in whole blood was calculated relative to the hematocrit. All measured or calculated concentrations of NOAC were corrected to a dose of 100 μg g^{-1}. The corresponding profiles of concentration against time are shown in Fig. 2. Over the whole period of observation the concentrations of NOAC in plasma and whole blood were 10- to 100-fold higher after intravenous administration than after oral administration. The maximum concentration of orally administered NOAC was reached after 4 h in plasma and after 8 h in whole blood. These peak concentrations remained constant over a period of approximately 20 h. Because of the administration of a single dose, steady state can not be reached.

The pharmacokinetic parameters, summarized in Table 1, were calculated from these data with a non-compartmental model. In plasma the area under the curve (AUC) was 90-fold greater after intravenous administration than after oral treatment. In contrast, the mean residence time (MRT) was 5-fold lower after intravenous injection of NOAC than after the oral application, resulting in a mean absorption time (MAT) of 14 h. For the intravenous treatment, the calculated values of total clearance from plasma indicate slow elimination of NOAC (cf Table 1). The steady-state volumes of distribution give evidence that NOAC is accumulated in a deep compartment.

The results of the analysis of the pharmacokinetic data calculated for whole blood are different from those obtained in plasma, especially when considering the AUC, which was only

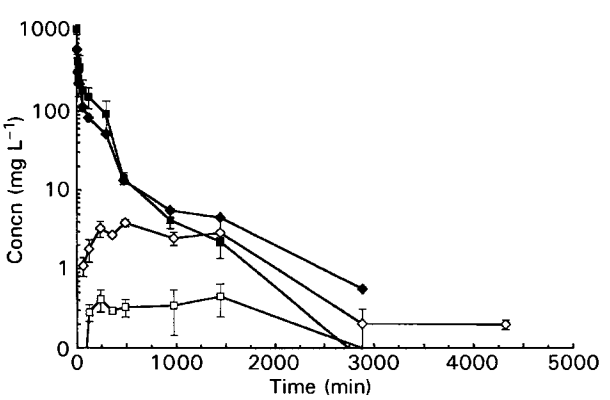

FIG. 2. The concentration of NOAC (mg L^{-1}) in plasma (■, □) and whole blood (◆, ◇) as a function of time (min) after intravenous (■, ◆) and oral (□, ◇) administration of the drug. The vertical bars represent the standard deviations of the mean (n = 3). The administered doses were normalized to 100 μg NOAC (g body weight)$^{-1}$.

Table 1. Pharmacokinetic data of NOAC in plasma and whole blood after intravenous and oral administration.

	Intravenous administration		Oral administration	
Parameter	Plasma	Whole Blood	Plasma	Whole Blood
Number of animals for each data point	3	3	3	3
Initial concentration (mg L^{-1})	3839	707	0	0
Area under the drug concentration–time curve extrapolated to infinity (mg h L^{-1})	1218 (5·0)*	837 (4·8)	13·4 (0)	108 (0·43)
Mean residence time (min)	211	366	1062	1100
Mean absorption time (min)	0	0	851	734
Total clearance(mL min^{-1})	0·031	0·045	0·031	0·045
Volume of distribution at steady state (mL)	6·56	16·5	33·0	49·7

The parameters were calculated by use of the equations given in the text. *Percent extrapolated.

8-fold higher after intravenous treatment than after oral administration. The MRT was 3-fold lower after intravenous than after oral application. The MAT was determined to be 12 h. After oral administration the MRT values in plasma and whole blood were similar, in contrast with the values obtained after intravenous bolus injection where the MRT in plasma was approximately 60% of the MRT value in whole blood. These differences and their implications in relation to the distribution of NOAC will be discussed below.

The bioavailability of NOAC after oral administration was calculated from the AUC. A bioavailability of 1·1% was obtained from the plasma data; the respective value for whole blood was 12·9%.

To assess the absorption half-life of NOAC after oral administration the fraction absorbed was calculated and was indicative of first-order absorption kinetics for plasma and whole blood. This means that the rate of absorption is proportional to the concentration of NOAC at the site of absorption. The absorption constants and the respective half-lives were determined by plotting $(1 - f_a)$ against time after administration of the drug. For plasma the absorption half-life was 10 h; for whole blood it was 9 h. Although the plots of concentration against time were described by a three-compartment model, the Wagner–Nelson equation (Wagner & Nelson 1963) could be applied because k_{10} was a good estimate of the linear elimination process as compared with compartmental modelling (data not shown).

The in-vivo binding of NOAC to erythrocytes was estimated by calculating the erythrocyte/plasma partition coefficient for any time point of measurement. Steady state was not reached after intravenous administration of the drug and therefore the $P_{e/p}$ value could not be determined. After oral application of NOAC, an erythrocyte/plasma partition coefficient of 13 was calculated, reflecting a 13-fold accumulation of NOAC in the erythrocytes compared with the plasma.

Discussion

The main finding in this study was that when NOAC was given orally a large amount was absorbed unchanged. This is in sharp contrast with cytosine arabinoside; although this drug is susceptible to enzymatic deamination the lipophilic derivative NOAC is highly resistant against deamination and therefore is not degraded in the gastrointestinal tract (Horber et al 1995b). Despite the rather low bioavailability, the amount of NOAC absorbed after oral treatment was sufficient to furnish excellent oral antitumour activity, as has already been reported (Schwendener et al 1996). In an earlier study it was shown that the in-vitro distribution of NOAC in blood is independent of whether it is administered as a liposomal formulation or solubilized in DMSO. This indicates that NOAC is very rapidly released from the liposomes (Koller-Lucae et al 1997).

Analysis of the pharmacokinetic data for NOAC has been divided into three parts. Firstly, the results of plasma analysis will be discussed, secondly the whole blood data will be examined and lastly the differences in the distribution of NOAC after intravenous and oral administration will be compared.

Plasma

The intravenous pharmacokinetic data for NOAC in plasma were significantly different from those for cytosine arabinoside. With NOAC a mean residence time (MRT) of 3·5 h was obtained in mice in this study, equivalent to 27 h in man (Dedrick et al 1970). In man the equivalent value for cytosine arabinoside was reported to be approximately 3 h (DeAngelis et al 1992), i.e. one-ninth the corresponding value for NOAC. After oral administration of the drug the MRT in plasma was 5-fold higher than for intravenous bolus injection, but the AUC was 90-fold lower. Possibly the concentrations of NOAC in plasma were below the limit of detection of the HPLC assay 48 h after oral administration of NOAC. It has previously been reported (Rentsch et al 1995) that the detection limit of the HPLC method is 15 $\mu g\ L^{-1}$ if 1 mL plasma is used. The amount of plasma available from each mouse was approximately 0·35 mL, which increases the limit of detection to 50 $\mu g\ L^{-1}$. After oral treatment a very long MRT value was measured, reflecting a low but long-persisting NOAC concentration in plasma owing to the slow absorption process.

The bioavailability (BA) of NOAC determined in plasma was 1·1%, a rather low proportion of the drug administered. Because little is known about the metabolism of NOAC the putative metabolites could not be included in the calculation of the BA. This might be an explanation of the low BA value determined in plasma. Studies on the cellular pharmacology of the C_{16}-analogue of NOAC revealed that only small amounts of these lipophilic cytosine arabinoside derivatives are metabolized to ara-CTP, the cytotoxically active metabolite of cytosine arabinoside (Horber et al 1995b, c). Therefore, it must be considered that possible differences in the rate of metabolism of NOAC after intravenous or oral administration might have a significant influence on the BA value calculated. The absorption half-life of NOAC for oral application was calcu-

lated to be 10 h, but even 1 h after oral administration measurable concentrations of NOAC were found in the plasma.

Whole blood

Analysis of the pharmacokinetic data of NOAC in whole blood led to an interpretation different from that inferred from the plasma concentration results. Because of the design of the study steady state cannot be reached during the whole observation period, resulting in ongoing distribution processes of NOAC in blood. Therefore, the whole blood data provide a better description of the pharmacokinetic behaviour. The AUC of whole blood after intravenous administration was lower than that for plasma, whereas the MRT was considerably higher. After oral administration the AUC was 8-fold higher than for plasma, but the MRT was similar to that calculated for plasma. Accordingly, the extent of the accumulation of NOAC in erythrocytes is strongly dependent on its mode of administration. As will be discussed later, intravenous and oral administration of NOAC result in a different distribution pattern.

Consideration of the whole blood concentrations of NOAC led to calculation of a BA of 12·9% after oral administration; this was significantly higher than that calculated from plasma concentrations. This can be explained by the rapid uptake of NOAC by erythrocytes after oral administration and by the large accumulation of NOAC in the red blood cells. Again, possible differences in the metabolism of NOAC after intravenous and oral administration have to be considered. For the reasons mentioned above the BA obtained for whole blood represents the in-vivo situation most realistically. From analysis of whole blood, the absorption half-life calculated for oral administration was 9 h. At the first measurement time point (1 h after administration) the concentration of NOAC in whole blood was already considerable, indicating a short lag time for the slow absorption process.

Distribution of NOAC in blood

The distribution of a drug between plasma and erythrocytes can be estimated by calculation of the erythrocyte/plasma partition coefficient. Analysis of the $P_{e/p}$ values calculated for each measurement time point indicates a slow process of accumulation of NOAC in the erythrocytes. However, even 2 h after oral administration of the drug the distribution of the drug between the erythrocytes and the plasma had already reached a steady state.

The interpretation of these results requires the analysis of the distribution of NOAC in blood; this is depicted hypothetically in Fig. 3. Immediately after intravenous bolus injection of NOAC, all the drug is still associated with the liposome membranes. As reported by Horber et al (1995a), on incubation of NHAC with plasma the drug is rapidly released from the liposomes and binds with high affinity to plasma proteins, mainly lipoproteins, and erythrocytes. This mode of administration leads to different distribution processes (liposome-bound drug compared with free drug, free drug compared with protein-bound drug, free drug compared with cell-bound drug), each again representing a combination of different equilibria.

When liposomes are administered orally, they are degraded during uptake from the intestinal mucosa (Kaufmann-Kolle et al 1994) and the drug is released from its carrier. By analogy with the known mechanisms of absorption of lipids (Thomson et al 1993) we hypothesize that NOAC most probably interacts with bile acids for its intestinal absorption and is afterwards integrated into chylomicrons. This enables immediate direct

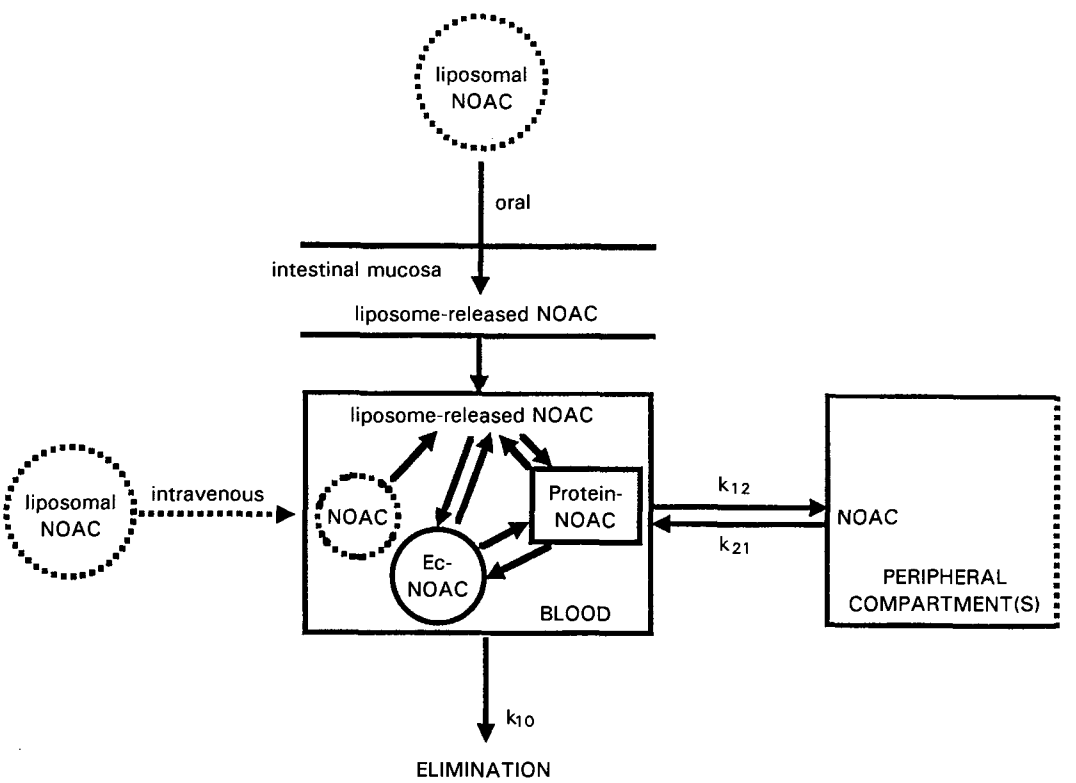

FIG. 3. Hypothetical scheme of the distribution of NOAC in blood after oral (p.o.) and intravenous (i.v.) administration. Blood represents the central compartment and liver and other tissues the peripheral compartment(s).

contact of the absorbed NOAC with the erythrocytes in the portal vein and therefore results in a distribution pattern after a single dose which is completely different from that after intravenous application. Identical MRT values determined in plasma and whole blood after oral administration indicate free exchange of NOAC between the different blood compartments.

In conclusion, liposomal NOAC is absorbed to a considerable extent after oral administration. Analysis of the pharmacokinetic data reveals a half-life after intravenous administration considerably longer than that of cytosine arabinoside and a large accumulation of the drug in the erythrocytes. The distribution of NOAC in blood is highly dependent on its mode of administration. Studies on the kinetics of the in-vitro distribution of NOAC in blood components were reported elsewhere (Koller-Lucae et al 1997).

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