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PHARMACOKINETICS OF STEROIDAL MUSCLE RELAXANTS IN ISOLATED PERFUSED RAT LIVER

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Abstract—Both in humans and animals hepatic elimination is an important factor determining the duration of action of non-depolarizing neuromuscular blocking drugs. To elucidate the hepato-biliary disposition of muscle relaxants the pharmacokinetics of several structurally related but physicochemically distinct steroidal neuromuscular blocking drugs were studied in isolated perfused rat livers. Pharmacokinetics analysis with the DIFFIT computer program enabled the simultaneous fitting of independently measured perfusate disappearance and biliary excretion rate curves using a numerical approach. The hepatic disposition of the steroidal muscle relaxants could be adequately described by a three compartment model with elimination from the peripheral compartment V_2 (biliary excretion) and storage in a deep compartment (V_3) connected to V_2. In addition, for vecuronium only slow ester hydrolysis occurring in V_2 and V_3 was included in the model. The lipophilicity rather than the relative mobility of the muscle relaxants showed a positive relationship with biliary clearance (Cl_{20}) and the initial hepatic uptake (Cl_{12}), indicating that hepato-biliary transport of these organic cations is highly dependent on the hydrophobic character of the compounds. In addition, net hepatic uptake of the steroidal cations was influenced markedly by transport from the liver to perfusate (hepatic efflux). This hepatic efflux (k_{21}) decreased with increasing lipophilicity. In contrast, the extent of intracellular sequestration into deep compartments, indicated by high k_{23}/k_{32} ratios, seemed to be inversely related to the lipophilicity of the muscle relaxants and might explain the observed prolonged hepatic storage of some of these compounds. In combination with data from subfractionation studies the results indicate that the pharmacokinetic analysis of the hepatic disposition of steroidal muscle relaxants may be used to evaluate actual transport phenomena participating in the hepatic disposition of these drugs.

One of the important factors determining the duration of action of peripheral neuromuscular blocking drugs is the disappearance of the muscle relaxant from the plasma. Rapid biotransformation, as with suxamethonium [1], or chemical degradation, as is the case with the Hofmann elimination of atracurium [2], may lead to efficient elimination of the active substance from the plasma. Yet, in general, most peripheral muscle relaxants are excreted unchanged by renal and/or hepatic pathways. The liver is important in the uptake and elimination of mono- and bisquaternary ammonium compounds and influences the effects of many of the neuromuscular blocking agents. For example, the rate of plasma clearance of *d*-tubocurarine in the dog [3] and in humans [4], of pancuronium in the cat [5], and of hexafluronium in the rat and in humans [6] is dependent, to a large extent, upon hepatic uptake. Bencini *et al.* [7] demonstrated in cats that the hepatic removal of vecuronium from the circulation is a major determinant of the duration of action. Moreover, large concentrations of vecuronium were found in a number of liver biopsies of patients as early as 30 min after injection and it was estimated that as much as 80% of the dose of vecuronium might be present in the liver half an hour after injection [8]. Analogous results have been described in animals for several other neuromuscular blocking agents [5, 9]. These data indicated that the uptake of these muscle relaxants may proceed at a faster rate than the excretion to bile, which may result in the accumulation of the drug in the liver.

An important factor in the transport of muscle relaxants from blood to bile appears to be the physicochemical character of the drug, such as relative mobility and lipophilicity [10, 11]. To elucidate the pharmacokinetics of several structurally related but physicochemically distinct steroidal neuromuscular blocking agents in the hepatobiliary transport of muscle relaxants, we studied isolated perfused rat livers. This preparation has been shown to preserve the hepato-biliary transport function for organic cations, anions and uncharged compounds [12]. In this model the liver is the only organ of elimination, thus allowing adequate analysis of the transport phenomena under study.

MATERIALS AND METHODS

Materials. [16β-*N*-methyl-^{3}H]Pancuronium (sp. act. 9.9 Ci/mmol) and [16β-*N*-methyl-^{14}C]Org 6368 (sp. act. 57.3 mCi/mmol) were synthesized by Dr F. Kaspersen from Organon Drug Metabolism Research

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Labs (Oss, The Netherlands). Radiochemical purity exceeded 99% as checked by TLC in two solvent systems. Pancuronium, Org 6368, vecuronium and 3-hydroxyvecuronium were kindly donated by Organon International B.V. (Oss, The Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

Animals. Male Wistar rats weighing 240–270 g and maintained on a standard rat diet and tap water were used in all experiments. The animals were fasted for 16 hr before the experiment.

Octanol/Krebs partition coefficient. The relative lipophilicity of the muscle relaxants, expressed as the octanol/Krebs partition coefficient, was determined as described by Neef and Meijer [13].

Isolated perfused liver preparation. A perfusion technique was used as described by Meijer *et al.* [14], with some slight modifications. Rats were anaesthetized with pentobarbital (60 mg/kg, i.p.) and the bile duct was cannulated with PE tubing. The portal vein was cannulated and the liver was perfused for a few seconds to wash away blood. An outflow cannula was inserted in the superior vena cava and the inferior vena cava was ligated just above the renal vein. The liver was excised and placed in the perfusion apparatus. The recirculating medium consisted of Krebs bicarbonate buffer supplemented with 1% bovine serum albumin and was constantly gassed with 95% oxygen and 5% carbon dioxide. The perfusate flow was maintained at 35 mL/min at a hydrostatic pressure of 12 cm of water to assure sufficient oxygen supply. The pH was monitored on line and ranged between 7.35 and 7.45. The temperature was kept at 38°. An infusion of sodium taurocholate (15 μmol/hr) was given to replace bile salts.

Experimental procedure. After a 30 min recovery period following the surgical procedure, 1 mg of one of the respective steroidal muscle relaxants was added to the perfusion medium. A volume of 100 mL of perfusion medium was used in all experiments. Perfusion was performed over a 2 hr period. During the experiments the viability of the liver was checked by measuring bile flow, pH and flow of the recirculating perfusate. Perfusate samples were collected at 2, 4, 6, 8, 10, 15, 20, 30, 40, 60, 80, 100 and 120 min after injection. Bile was collected in fractions at 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min after injection. At the end of the experiment the liver was homogenized in 35 mL of an appropriate buffer and the amount of drug was determined as described below. In the experiments with vecuronium and 3-hydroxyvecuronium the pH of the perfusate and bile samples was brought to 4–5 by titration with 1 M sodium dihydrogen phosphate to prevent hydrolytic degradation of vecuronium and 3-hydroxyvecuronium. Similarly, the livers were homogenized in 1 M sodium dihydrogen phosphate. Subsequently the samples were stored at −20° to await further analysis.

Radiochemical analysis. In the experiments with the radiolabeled muscle relaxants, [^{3}H]pancuronium and [^{14}C]Org 6368, perfusate samples (500 μL) and bile samples were mixed with an appropriate scintillation fluid (AquaLuma 3M, Schaesberg, The Netherlands) and counted in a Beckman LS 1800 liquid scintillation counter. Quenching was estimated by means of an external standard. TLC (solvent system: sodium iodide 3% w/v in isopropanol) revealed that only trace amounts of metabolites of both muscle relaxants were formed during the perfusion experiments. Livers were homogenized in saline and 250 μL samples were submitted to liquid scintillation counting.

HPLC analysis. Vecuronium and 3-hydroxyvecuronium concentrations were assayed by HPLC [15]. This method enables estimation of the concentration of the unchanged compound and the metabolites in the samples. The assay is based on solid-phase extraction, HPLC and post-column ion-pair extraction with the fluorescent anion 9,10-dimethoxyanthracene-2-sulphonate to enable sensitive and selective fluorimetric detection.

Pharmacokinetic analysis of the data. Liver content at various time intervals was calculated by subtracting from the administered dose the amount in the perfusate plus the amount excreted into the bile. The amount of drug recovered in the perfusate, perfusate samples, bile and liver amounted to 95.0 ± 6.8% of the dose and was taken to reflect the mass participating in the kinetic analysis. The perfusate disappearance and biliary excretion rate versus time curves were fitted with the computer program DIFFIT, developed by one of us (F.R.). This program enables simultaneous fitting of perfusate disappearance and biliary excretion rate curves, yielding the best model to explain these independently measured profiles. In contrast with the traditional non-linear curve fitting methods [16, 17], this method is based on defining compartment models using the accompanying differential equations, which indicate rate of input and output of the drug, and subsequent simulation of disappearance and appearance patterns by a numerical approach. For example, the model as depicted in Fig. 4 can be described by the following set of simple equations:

$$dA_1/dt = k_{21} \cdot A_2 - k_{12} \cdot A_1$$

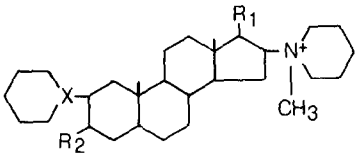
$$dA_2/dt = k_{12} \cdot A_1 - (k_{21} + k_{23} + k_{20} + k_{2m}) \cdot A_2 + k_{32} \cdot A_3$$

$$dA_3/dt = k_{23} \cdot A_2 - (k_{32} + k_{3m}) \cdot A_3$$

$$dA_0/dt = k_{20} \cdot A_2$$

in which A_1 is the amount of drug in the perfusate, A_0 is the amount in the bile and A_2 and A_3 are the amounts of drug in the compartments within the liver. The various rate constants are indicated as k_{12}, k_{21} etc., with k_{20} being the rate constant for excretion to the bile and k_{2m} and k_{3m} the rate constants for the conversion into the 3-hydroxymetabolite.

Consequently the other compartment models to be analysed were described using the same principle by a comparable set of equations. Thus, the procedure offers the possibility to test several multicompartment models without the necessity to derive the complex equations by integration of sets of differential equations as perfomed in the traditional compartment analysis. Instead the fitting procedure occurs via the simple principle of iterative changing of the parameters, such as rate constants



	X	R_1	R_2	MW_{cation}	Octanol/Krebs partition coefficient
Vecuronium	N	$OCOCH_3$	$OCOCH_3$	557	2.56
3-Hydroxyvecuronium	N	$OCOCH_3$	OH	515	0.032
Org 6368	N^+—CH_3	H	$OCOCH_3$	514	0.0145
Pancuronium	N^+—CH_3	$OCOCH_3$	$OCOCH_3$	572	0.0033

Fig. 1. Structural formulas and physicochemical data of the steroidal muscle relaxants involved in this study.

and volume of distribution (V_1). This leads to repeated comparison of the experimental data with the calculated curves using least-squares regression analysis, minimizing $(C_{perf} \cdot V_1 - A_1)^2 + (A_{bile,meas} \cdot A_0)^2$, in which C_{perf} is the measured concentration of drug in the perfusate and $A_{bile,meas}$ is the measured amount of drug excreted into the bile. The combination of rate constants and distribution volume was adapted until a minimal sum of squares was attained using the Simplex procedure for an optimal simultaneous change of parameters [18]. After the fitting procedure the optimal model was discriminated by application of the F-ratio test [16]. Apparent volumes of distribution V_2 and V_3 were estimated from the amounts in the various compartments under simulated steady-state conditions, i.e. $dA_2/dt = 0$ and $dA_3/dt = 0$. Clearances Cl_{12} and Cl_{20} were calculated from $Cl_{12} = k_{12} \cdot V_1$ and $Cl_{20} = k_{20} \cdot V_2$. In the fitting procedure corrections were applied for the loss of drug and circulating volume by sampling of the perfusate. Fitting of experimental versus simulated curves was assumed to be optimal when the change in the sum of squares was less than 0.01%. The experimental data were weighted according to the y^{-2}-method [16].

RESULTS

Although the four muscle relaxants used in this study show marked similarities in their chemical structures (Fig. 1), pronounced differences were observed in the hepato-biliary transport of these structurally related compounds. Figure 2 depicts the hepato-biliary transport of vecuronium in the isolated perfused liver. Vecuronium, the monoquarternary analogue of pancuronium, showed the most efficient hepato-biliary transport of the muscle relaxants studied with an initial clearance of 20.4 ± 4.2 mL/min. Vecuronium was also effectively excreted in bile, both unchanged and as its 3-hydroxy metabolite (in total, 60% of the dose in 2 hr). During the experiment the metabolite also appeared in the perfusate, but only very low concentrations of 3-

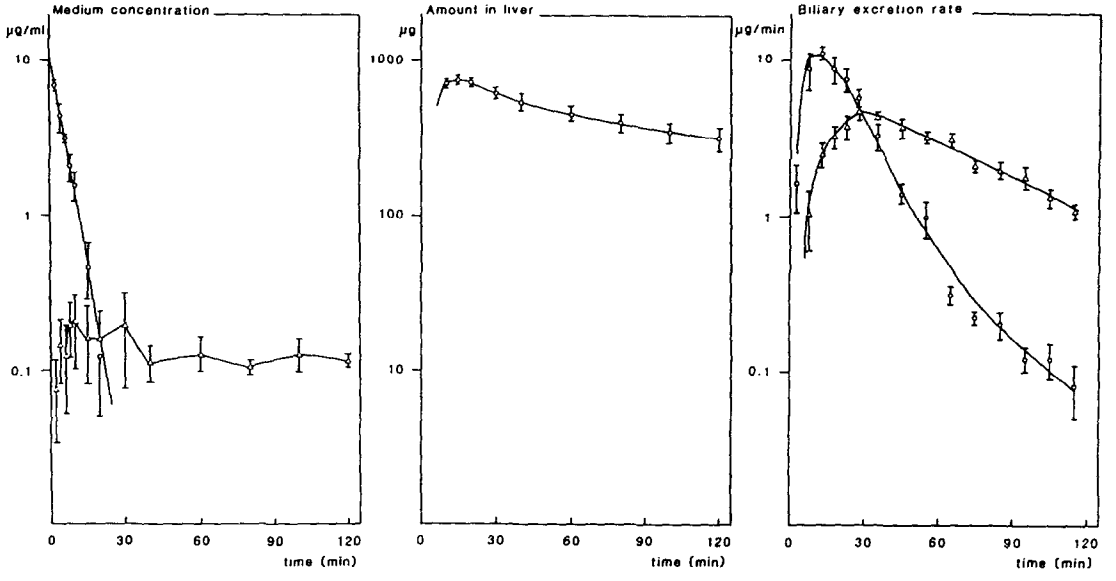


Fig. 2. Kinetics of vecuronium bromide in the isolated perfused rat liver after a bolus injection of 1 mg. Concentration in perfusate and biliary excretion rate of vecuronium (circles) and its metabolite 3-hydroxyvecuronium (triangles) are indicated. The amount in the liver represents the total amount of vecuronium plus its metabolite. Data are the means ± SEM of four experiments. For unchanged vecuronium the depicted curves are obtained after pharmacokinetic analysis of the mean values.

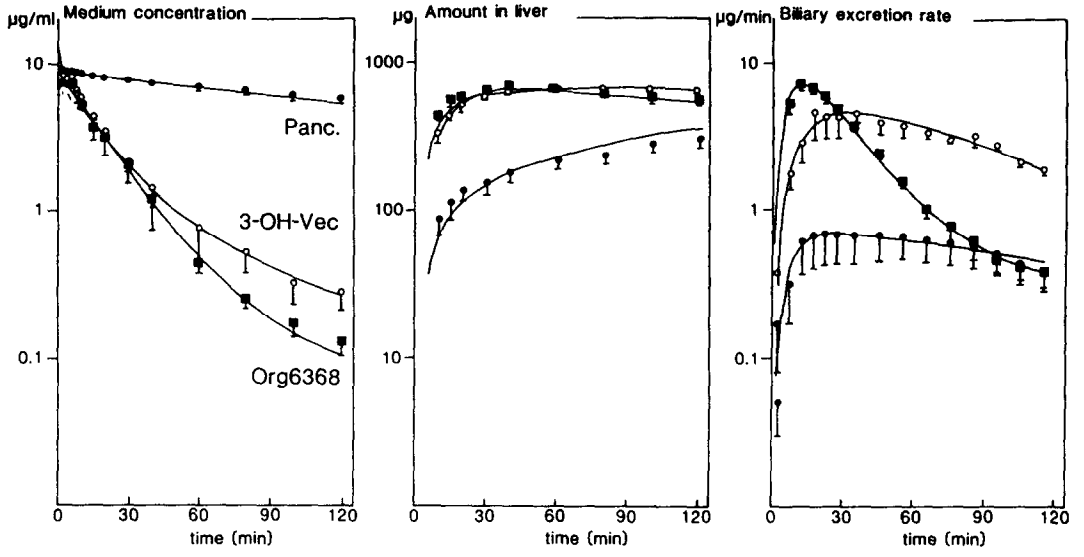


Fig. 3. Kinetics of steroidal muscle relaxants in the isolated perfused rat liver after a bolus injection of 1 mg pancuronium bromide (closed circles), 1 mg Org 6368 (closed squares) and 1 mg 3-hydroxyvecuronium bromide (open circles), respectively. Concentration in perfusate, amount in the liver and biliary excretion rate are indicated. Data are the means ± SEM of three experiments. The depicted curves are obtained after pharmacokinetic analysis of the mean values.

hydroxyvecuronium were measured in the perfusion medium.

Figure 3 shows the hepato-biliary transport of the metabolite 3-hydroxyvecuronium and of the two bisquaternary compounds Org 6368 and pancuronium. The results clearly indicate that the metabolite, which has marked neuromuscular blocking potency itself (*ca.* 65% as compared to vecuronium [19]) was transported from plasma to bile less efficiently than was vecuronium. Furthermore, Fig. 3 shows the modest net transport of pancuronium from plasma to bile. After 2 hr more than 50% of the administered dose was still present in the perfusate, whereas only 6.7 ± 3.8% of the dose was excreted into bile. Interestingly, the liver content versus time curve rose continuously during the experiment, suggesting that a proportion of the liver content was not readily available for biliary excretion. Such a storage phenomenon appeared to be even more pronounced in the case of Org 6368. Whereas at the end of the experiment the liver contained about 70% of the administered dose, the biliary excretion rate had declined to about 0.4 µg/min, indicating that only a minor proportion of the liver content was directly available for biliary excretion. In contrast to pancuronium. Org 6368 exhibited effective hepatic uptake: within 60 min more than 95% of the dose was taken up by the liver.

Pharmacokinetic analysis of the data was performed by curve fitting according to several two, three and four compartment models. Application of the *F*-test indicated that the three compartment model as depicted in Fig. 4 gave significantly better results than the other three and two compartment models tested. No significant improvement was obtained by applying four compartment models. Table 1 summarizes the kinetic parameters for the hepato-biliary transport of the various muscle relaxants as derived from the pharmacokinetic analysis. The vecuronium curves were analysed under the assumption that the metabolite does not affect the transport of vecuronium.

DISCUSSION

Marked differences are observed in the overall hepato-biliary transport of the four structurally related steroidal muscle relaxants. Although, strictly speaking, vecuronium and 3-hydroxyvecuronium are monoquaternary ammonium compounds, it should be noted that these agents are present in bivalent form, since the tertiary amine group is nearly completely protonated at physiological pH. These bivalent cations all have a molecular weight of 500–600 and thus satisfy the conditions for effective hepato-biliary transport, as proposed by Hughes *et al.* [10].

The observed transport differences between the compounds despite their comparable molecular weights indicate that molecular weight is not valid

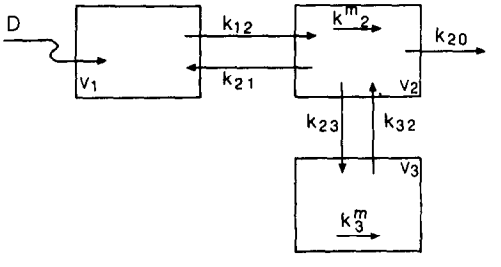


Fig. 4. Compartment model for the hepato-biliary transport of steroidal muscle relaxants.

Table 1. Pharmacokinetic microparameters for the hepato-biliary transport of steroidal muscle relaxants in isolated perfused rat liver

	Vecuronium (N = 4)		3-Hydroxyvecuronium (N = 3)		Org 6368 (N = 3)		Pancuronium (N = 3)	
	Mean	± SEM	Mean	± SEM	Mean	± SEM	Mean	± SEM
k_{12} (min^{-1})	0.212	0.018	0.0716	0.0218	0.0783	0.0054	0.0140	0.0017
k_{21} (min^{-1})	1.35×10^{-7}	6.99×10^{-8}	0.00540	0.00337	0.0503	0.0203	0.0895	0.0171
k_{23} (min^{-1})	0.0428	0.0057	0.00486	0.00086	0.0865	0.0113	0.0362	0.0111
k_{32} (min^{-1})	0.00619	0.00228	0.000420	0.000038	0.00206	0.00061	8.42×10^{-12}	2.55×10^{-12}
k_{20} (min^{-1})	0.0238	0.0046	0.00736	0.00123	0.0318	0.0067	0.00940	0.00425
k_{2^m} (min^{-1})	0.0212	0.0047	—	—	—	—	—	—
k_{3^m} (min^{-1})	0.0253	0.0028	—	—	—	—	—	—
V_1 (mL)	95.5	5.6	99.2	3.3	93.3	3.3	100.0	1.1
V_2 (mL)	976	271	576	77	104	23	14.7	1.7
V_3 (mL)	9493	3409	6935	1569	4861	1231	1.24×10^{11}	0.79×10^{11}
Lagtime (min)	2.36	0.22	2.08	0.32	2.87	0.03	2.20	0.08

For each muscle relaxant studied mean values ± SEM of the calculated microparameters are presented.

Table 2. Summary of pharmacokinetic data on the hepato-biliary transport of steroidal muscle relaxants in isolated perfused liver

	$P_{Oct/Krebs}$	Log $P_{Oct/Krebs}$	Cl_{12} (mL/min)	Cl_{20} (mL/min)	k_{12} (min^{-1})	k_{21} (min^{-1})	k_{12}/k_{21}	k_{23}/k_{32}	k_{20} (min^{-1})	V_2 (mL)	V_3 (mL)	Cytosol/ perfusate concn ratio	Bile/ cytosol concn ratio	Particles/ cytosol concn ratio
Vecuronium	2.56	0.408	20.2	23.2	0.212	1.35×10^{-7}	1.57×10^{6}	6.9	0.0238	976	9493	15.8	27.7	3.0
3-Hydroxyvecuronium	0.032	−1.49	7.10	4.24	0.0716	0.00540	13.3	11.6	0.00736	576	6935	ND	ND	ND
Org 6368	0.0145	−1.84	7.31	3.31	0.0783	0.0503	1.56	42.0	0.0318	104	4861	1.57	31.2	9.8
Pancuronium	0.0033	−2.48	1.40	0.138	0.0140	0.0895	0.156	4.3×10^{9}	0.00940	14.7	1.24×10^{11}	0.52	5.7	4.2*

Calculated parameters are based on the mean values derived from the pharmacokinetic analysis. Data on concentration gradients are taken from Ref. 27.
* Determined under non-steady-state conditions in the liver.
ND, not determined.

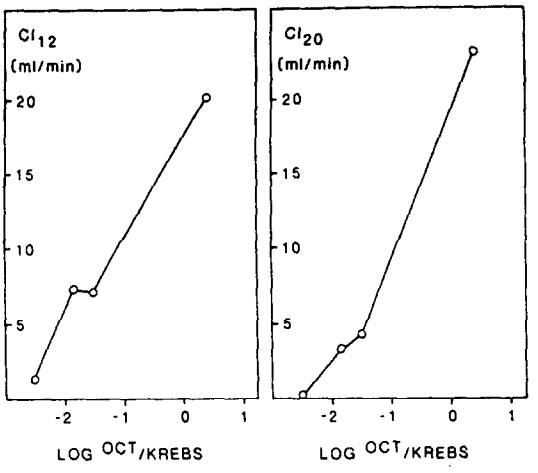


Fig. 5. Left panel: relation between the lipophilicity of the muscle relaxants and the initial hepatic uptake (Cl_{12}) in isolated perfused liver. Right panel: relation between the lipophilicity of the muscle relaxants and the biliary clearance (Cl_{20}) in isolated perfused liver.

as a primary parameter to predict the effective hepato-biliary transport of the steroidal muscle relaxants. The lipophilicity of the compounds seems a better parameter in relation to the overall efficacy of hepato-biliary transport [11]: the relatively lipophilic muscle relaxant vecuronium is most efficiently transported from perfusate into bile, whereas the hydrophilic compounds are transported to a lesser degree.

Interesting differences in transport rate were observed between the three hydrophilic muscle relaxants. The two bisquaternary agents Org 6368 and pancuronium have lipophilicities in the same order of magnitude, but Org 6368 appears to be handled by the liver much more efficiently than is pancuronium. The data in Table 2 indicate that this may be due to both a more efficient uptake (Cl_{12}) and biliary excretion (Cl_{20}) combined with a more rapid sequestration into deep pharmacokinetic compartments (presumably cellular organelles).

Interestingly, in the experiments with vecuronium the metabolite 3-hydroxyvecuronium gradually appears in the perfusion medium, due mainly to hydrolysis in the liver and transport of 3-hydroxyvecuronium from the liver into the perfusate. Control experiments indicate that only a small part of the 3-hydroxyvecuronium in the perfusion medium arises from direct ester hydrolysis in the perfusion medium of the 3-*O*-acetyl group of vecuronium, although this may contribute significantly in the initial 15 min of the experiment where the concentration of vecuronium in the perfusate is still high and a concentration peak of the 3-hydroxy-metabolite is observed. In addition, a slight contamination of the vecuronium through hydrolysis in the administered material could play a role in this respect.

The different kinetic constants for the hepato-biliary transport of the muscle relaxants, as revealed by pharmacokinetic analysis, point to remarkable differences in hepato-biliary transport and hepatic storage. The limited net hepatic uptake of pancuronium is partially due to a low k_{12} value, but also appears to be caused by a large "backflux" (k_{21}) of the drug to the perfusate. The k_{21} value increases with decreasing lipophilicity of the muscle relaxants. Cellular extrusion systems for cationic anti-cancer drugs have been described for various cell types, including hepatocytes [20]. However, immuno-histological studies indicate that these systems are predominantly localized at the canalicular membrane and that transport is positively correlated with lipophilicity.

With respect to the biliary clearance Cl_{20} of these steroidal cations a clear relationship with lipophilicity was observed (Fig. 5). Similarly, the initial hepatic uptake, expressed as Cl_{12}, increased with increasing lipophilicity. These data clearly indicate that the hepato-biliary transport of these steroidal muscle relaxants at both sinusoidal and canalicular level is highly dependent on the hydrophobic character of the compounds.

The kinetic analysis of the hepato-biliary transport of steroidal muscle relaxants revealed a deep distribution compartment. The affinity of the muscle relaxants for this intracellular distribution compartment, expressed as the k_{23}/k_{32} value, is inversely related with lipophilicity. For pancuronium an exceptionally small k_{32} value was calculated, suggesting almost irreversible sequestration of this agent in this compartment. It implies that once the compound has penetrated the hepatocyte, the tendency to be accumulated in the deep compartment is very high. In the hepato-biliary transport of *d*-tubocurarine an intracellular deep compartment was also inferred [12]. In analogy with *d*-tubocurarine and metocurine, the lysosomes are a likely candidate for this deep compartment [21, 22]. However, data on subcellular distribution of the steroidal cations revealed that the mitochondrial fraction is the major storage compartment at the dose employed in the present study [23]. In line with these results, autoradiographic studies in mice demonstrated that prolonged accumulation of [^{14}C]pancuronium predominantly occurred in the liver [24]. Additional studies will be necessary to elucidate the molecular prerequisites for these intrahepatic storage mechanisms.

Although the calculated distribution volumes are only apparent entities, the decrease in V_2 in the order $V_{2\text{Vecuronium}} > V_{2\text{Org6368}} > V_{2\text{Pancuronium}}$ correlates rather well with the decrease in the cytosol/plasma concentration ratios found in the subcellular distribution studies [23]. This may imply that the apparent distribution volume V_2, as determined in the pharmacokinetic analysis, represents the hepatocyte cytoplasm as estimated in subfractionation studies. Similarly, the k_{20} values obtained from the kinetic analysis showed an excellent relation with the bile/cytosol concentration ratios as estimated in the subfractionation studies [23]. These data suggest that the pharmacokinetic analysis of the hepatic disposition of steroidal muscle relaxants in the intact organ, as described in this study, may be used to evaluate the membrane transport processes involved in the hepato-biliary transport of these drugs.

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