Characterization of the Uptake of Rocuronium and Digoxin in Human Hepatocytes: Carrier Specificity and Comparison with \textit{in Vivo} Data\textsuperscript{1}

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\textbf{ABSTRACT}

Mechanisms of drug transport in the liver have been investigated predominantly in rodents. Most of the \textit{in vitro} drug research in the liver is performed in liver preparations of animals. The results of such experiments frequently are discussed in relation to anticipated metabolic profiles in man, but these extrapolations are often inappropriate because of large interspecies differences in drug metabolism. In the present study, the mechanisms and specificity of the uptake of the organic cation rocuronium and the cardiac glycoside digoxin were investigated in human hepatocytes and were compared with results obtained in rat hepatocytes. The extraction ratio for the intact liver was calculated from the measured uptake rates of the compounds in the human cells \textit{in vitro} and compared with published \textit{in vivo} data. The initial hepatic extraction ratio, calculated from the \textit{in vitro} uptake data for digoxin and rocuronium, very well reflected the initial extraction ratio for distribution in the liver \textit{in vivo} in man. Uptake of 100 \textmu M rocuronium was inhibited by 40 \textmu M K-strophantoside (80\% inhibition), and although not significantly, by 160 \textmu M procainamide ethobromide, whereas no inhibitory effect was found in the presence of 160 \textmu M taurocholic acid. In a previous study in rat hepatocytes, marked inhibition of digoxin uptake by quinine and only minimal inhibition by the diastereomer quinidine was demonstrated, showing clear stereoselectivity in transport inhibition. Unexpectedly, the uptake of digoxin in human hepatocytes was not inhibited significantly by quinidine or quinine, which indicates clear species differences. This is the first study to investigate the uptake mechanisms of organic cations and cardiac glycosides in human hepatocytes in some detail. The results show that uptake characteristics of drugs found in rats can not be extrapolated directly to humans.

Uptake and subsequent elimination of endogenous and exogenous compounds in the liver is one of the main functions of the liver. These processes have been studied widely with \textit{in vitro} techniques. The isolated perfused liver, isolated hepatocytes and plasma membrane vesicles have been used extensively for studies on membrane transport of drugs (Meijer, 1989). Nearly all the research focused on transport phenomena in the liver was performed in rodents and many attempts were made to extrapolate such data to man by scaling techniques (Gronert et al., 1995). However, because of the qualitative interspecies differences, such scaling procedures might lead to false conclusions.

With the expansion of liver transplantation programs, human liver tissue became increasingly available for research purposes. Because of the introduction of the isolation technique for human hepatocytes (Bojar et al., 1976; Groothuis et al., 1995; Guguen-Guillouzo et al., 1982; Iqbal et al., 1991; Rogiers, 1993; Strom et al., 1982), detailed assessment of the uptake mechanism of drugs in humans is now possible.

We previously studied the uptake of three different classes of compounds, i.e., the anionic bile acid taurocholic acid, the uncharged cardiac glycoside ouabain and the cationic muscle relaxants vecuronium and rocuronium. These three classes of drugs are supposed to be transported by separate carriers in the sinusoidal domain of the liver plasma membrane of the rat (Meijer, 1989; Oude Elferink et al., 1995; Steen et al., 1992). The observed uptake rates in the isolated rat and human cells seemed to reasonably reflect the relative hepatic uptake rates found in the intact organism (Sandker et al., 1994). Moreover, a significant correlation between the rate of uptake of the vinca alkaloids in human hepatocytes and the

\textbf{ABBREVIATIONS:} PAEB, procainamide ethobromide; BSA, bovine serum albumin; KHB, Krebs-Henseleit buffer; HBSS, Hanks’ balanced salt solution.

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in vivo plasma clearance of the drugs was found by others (Zhou et al., 1994).

Because of the scarceness of the human material and the much higher variations in uptake of drugs in human hepatocytes than in rat cells (Sandker et al., 1994), it is impossible to study transport kinetics and competitive patterns as they are studied routinely in animal. For instance, measuring $K_m$ and $V_{max}$ values for each agent with the present techniques is not feasible even within an experimental period of 2 to 3 years. Generally, inhibition of the uptake of substrates in hepatocytes, in principle, would be performed at different substrate concentrations or inhibitor concentrations to investigate if the inhibition is competitive and noncompetitive. However, because of the reasons mentioned above, this is practically not possible with freshly isolated human hepatocytes in a reasonable time span. Therefore, in the present study, the uptake of a limited number of agents was studied in human hepatocytes to be able to compare the data with results of previous studies in rat hepatocytes in our laboratory. This also was done to obtain a first clue on potential interspecies differences. In the present study, we extended the investigations on the mechanisms of drug uptake in human hepatocytes with special reference to the specificity of organic cation carriers. In rat liver, at least two uptake carriers are supposed to be involved in the uptake of organic cations: the type I uptake system for relatively small monovalent organic cations and the type II system for mostly bivalent organic cations with bulky ring structures (Meijer, 1989). In contrast to the uptake of type I agents, the uptake of type II agents can be inhibited largely by cardiac glycosides and bile acids. To determine whether these carrier characteristics are also present in human livers, we studied the influence of several classes of compounds on the uptake of the bulky organic cation rocuronium. We used the cationic PAEB, known as type I model substrate in the rat, K-strophantoside as an uncharged cardiac glycoside also being a potent inhibitor for type II uptake in the rat, as well as the anionic bile acid taurocholic acid that also inhibits the uptake of rocuronium (Steen et al., 1992). In addition, we studied the pharmacokinetic interaction between digoxin and the diastereomers quinidine and quinine. Interaction between these agents has been demonstrated in vivo in animals and humans. Quinidine has inhibited both the renal excretion and biliary clearance of digoxin (Hedman et al., 1990), whereas quinine, the diastereomer of quinidine, only decreased biliary clearance of digoxin (Hedman et al., 1990). In rat hepatocyte experiments (Hedman and Meijer, submitted), a marked inhibition of digoxin uptake by quinine and only minimal inhibition by the diastereomer quinidine was demonstrated. These results indicate that the decrease in clearance and distribution volume of digoxin by quinidine and quinine found in humans can be explained by inhibition of the uptake of digoxin in the liver or uptake in the kidney or both. Therefore, we studied the influence of quinidine and quinine on digoxin uptake in human hepatocytes.

Furthermore, to elucidate if uptake rates of drugs in human hepatocytes adequately predict the hepatic disposition in humans in vivo, we correlated the obtained in vitro data in human hepatocytes with the known hepatic extraction fraction of the particular agents in humans.

**Methods**

**Materials.** The following compounds were obtained from the indicated sources: Collagenase P from Boehringer Mannheim (Mannheim, Germany); BSA from Organon Teknika (Boxtel, The Netherlands); Percoll from Pharmacia AB (Uppsala, Sweden); [3H]Digoxin (12a-labeled; specific activity, 16.1 Ci/mmol) from Du Pont NEN Research Products (Boston, MA); Quinine hydrochloride and quinidine hydrochloride monohydrate from Sigma Chemical Co (St. Louis, MO). The University of Wisconsin organ preservation solution (UW) was from Du Pont Critical Care (Waukegah, IL). [16-f-N-methyl-3H]Rocuronium and unlabeled rocuronium were kind gifts from Organon International BV (Oss, The Netherlands). Sodium taurocholate was from Fluka (Buchs, Switzerland). PAEB was kindly donated by E.K. Squibb & Sons Inc. (Princeton, NJ). K-strophantoside was purchased from Roth (Karlsruhe, Germany). All other chemicals were of analytical grade and were obtained from commercial sources.

**Liver material.** Human liver tissue was obtained from livers procured from multiorgan donors (Tx-livers) or from patients after partial hepatectomy because of metastases of colorectal carcinoma (PH-livers). Consent from the legal authorities and from the families concerned was obtained for the explantation of organs for transplantation purposes. The donor livers were obtained from six donors (five males and one female aged from 3 to 32 years). They were reduced to perform reduced size or split liver transplantation. The donor liver was perfused with cold UW organ storage solution in situ before explantation. The livers were stored in cold UW solution on ice until reduction of the liver. The reduction or splitting of the donor organ was performed while it was immersed in UW with ice slush. The liver tissue remaining after bipartition was stored again in cold UW solution until the start of the isolation procedure. Total cold preservation time varied from 12 to 18 hr. The research protocols were approved by the medical ethics committee of our institution. In the case of the PH-livers, consent from the patients ($n = 3$, one man and two women aged from 46 to 64 years) concerned was obtained for the use of liver tissue for research purposes. The research protocols were approved by the medical ethics committee of our institution. The technique of partial hepatectomy was performed as described earlier (Brouwers et al., 1997). After partial hepatectomy, a wedge from the resected liver lobe was cut at a distance of at least 5 cm from the metastasis. Warm ischemia time in PH-livers, defined as the time after clamping of the branches of the hepatic artery and portal vein to the part to be resected, varied from 60 to 85 min. Directly after excision of the piece of tissue, the biopsy wedge was perfused with cold UW and was transported to the laboratory where the isolation procedure was started within 30 min.

**Preparation of human hepatocytes.** Human hepatocytes were isolated by a modification of the method described earlier by Groothuis et al. (1995); the biopsy wedge was cannulated with two to four cannulas as described in detail by Groothuis et al. (1995). The cannulas were filled with ice-cold modified HBSS without Ca$^{2+}$ (containing 112 mM NaCl, 5.4 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES), 10 mM glucose and 25 mM NaHCO$_3$, pH 7.42). The biopcy wedge was placed in a cabinet at 37°C and was perfused for a 10-min single pass with modified HBSS without Ca$^{2+}$ [saturated with 95% O$_2$/5% CO$_2$ (carbogen)], 5 mM of [ethylene bis (oxyslhenilenitri)l] tetraacetic acid (EGTA) and with a pH of 7.42. The flow rate was adjusted to a flow of 30 ml/cannula/min. Thereafter, the liver tissue was perfused during a 2-min single pass with modified HBSS with 5 mM Ca$^{2+}$ [saturated with carbogen]. This buffer was supplemented with 0.05% (w/v) collagenase, and 250 ml of this collagenase buffer solution was perfused in a recirculating mode for 30 min. After the recirculating period, the wedge was put in ice-cold modified KHB (Sandker et al., 1992) supplemented with 1% (w/v) BSA, saturated with carbogen and with a pH of 7.42. The liver capsule was cut and liver tissue was dissociated with the aid of a forceps and subsequently filtered through 250-
100- and 50-μm nylon filters. The obtained cell suspension was pooled and centrifuged at 50 × g for 4 min and washed three times with ice-cold KHB. Nonviable cells were removed by Percoll density centrifugation (Groot Huins et al., 1995). The viability of the final cell suspension, as assessed by trypan blue exclusion (final concentration, 0.2%), was 95 ± 2%.

Uptake and inhibition experiments. The uptake experiments were performed as described previously by Sandker et al. (1994) with 1.5 × 10⁶ cells/ml. The uptake rate of 50 nM digoxin was determined during 3 min in the absence and presence of 50 μM quinidine or 50 μM quinine, and the inhibitors were added 2 min before the addition of digoxin in line with experiments for rat hepatocytes (Hedman and Mejier, submitted). The uptake of 100 μM rocuronium was determined during 3 min in absence and presence of 160 μM taurocholic acid, 160 μM PAEB or 40 μM K-strophansoside, which were added 0.5 min before the start of the experiment. The concentrations were used in line with the experiments performed in rat hepatocytes (Steen et al., 1992). In one experiment, the uptake rate of digoxin was determined at 2.5 nM, the therapeutic concentration in man. In this experiment, the human hepatocytes concentration had to be increased to 15 × 10⁶ cells/ml because of the low specific activity of the [³H]digoxin.

Calculation of the extraction ratio for the whole liver. The extraction ratio for the whole liver (E_{calc}) was calculated from the initial uptake rates (V_i) in human hepatocytes, as described previously by Sandker et al. (1994). Initial intrinsic clearance by the cells was calculated from the ratio of the initial uptake velocity (mean of the initial uptake rate of each individual liver) and the substrate concentration. An intrinsic clearance (CL_i) for the whole liver could be calculated from these data, assuming 100 × 10⁶ cells/g liver (Olinga et al., 1993) and a liver weight of 1.5 kg. Based on the well-stirred liver perfusion model, the initial extraction ratio was estimated according to the equation E = CL_i/(CL_i + Q_H), in which E is the initial extraction ratio and Q_H is the plasma flow through the liver (750 ml/min in humans) (Blom et al., 1982; Sandker et al., 1994). The initial hepatic clearance (CL_H, initial) in vivo was estimated from the published pharmacokinetic data of the rocuronium (Wierda and Proost, 1995) and of digoxin (Hedman et al., 1992). The pharmacokinetic analysis was performed with a PK/PD program, as published previously by Proost et al. (1996). The CL_H, initial for digoxin in vivo in man was calculated from the in vivo plasma concentration decay of digoxin, which could be described successfully as a two-compartment model with elimination from the peripheral as well as the central compartment. Assuming that the urinary excretion was proportional to the plasma concentration (central compartment) and that the biliary excretion was proportional to the concentration in the peripheral compartment, the relative contribution of central and peripheral elimination was calculated from the fraction of the dose excreted unchanged in urine and bile, respectively. The CL_H, initial was estimated from the rate constant k_{12} and the initial distribution V_i (CL_H, initial = k_{12}V_i). From studies performed in man, it is known that a large part of the administered dose is distributed to skeletal muscles. Therefore, it was assumed that only 10% of the initial clearance represents the transport to the liver (Doherty et al., 1967).

For rocuronium, the CL_H, initial in man in vivo was calculated from the in vivo plasma concentration decay of rocuronium in patients with use of a three-compartment model. Based on the published data (Wierda and Proost, 1995), the third compartment was considered to be the liver and the fraction of the dose excreted by the liver was taken to be 70%. The rate constant of hepatic uptake k_{12} and the initial volume of distribution V_i were used to estimate the CL_H, initial. The E_{in vivo} was calculated from the CL_H, initial divided by the plasma flow Q_H.

Statistics. Results were compared by use of the Student’s t test. A P value <.05 was considered significant.

Results

It was found in a pilot experiment that the uptake of digoxin at 37°C in human hepatocytes was at least linear up to 2 min. Digoxin uptake in human hepatocytes showed a clear temperature dependence (fig. 1). The uptake rate at 4°C was only 5% of the velocity found at 37°C, the uptake of digoxin was linear during 2 min. The uptake of 50 nM digoxin was not inhibited in the presence of 50 μM quinidine or quinine (fig. 2). The CL_H, initial calculated in humans in vivo was 1.4 ml/min/kg and the E_{in vivo} calculated was 0.13 (table 1). The extraction ratio calculated from the initial uptake rate of digoxin (50 nM) (determined up to 2 min) was slightly higher (0.33) than the value that was calculated with in vivo data from human subjects, 0.13 (Hedman et al., 1992).

Only one initial uptake experiment in human hepatocytes was performed with a concentration of 2.5 nM digoxin, the therapeutic concentration in humans, because of the high amounts of hepatocytes needed (low specific activity of the labeled digoxin). With 2.5 nM digoxin, a CL_i of 1.12 × 10⁻³ ml/min/10⁶ was found and the extraction ratio that was calculated (E_{calc}) was 0.18, close to the E_{in vivo} (1).

It was found in a pilot experiment that the uptake of rocuronium at 37°C in human hepatocytes was at least linear up to 2 min. The uptake of rocuronium at 37°C and 4°C is depicted in figure 3. The uptake rate at 4°C was only 35% of that measured at 37°C, which also indicates a clear temperature dependence of the uptake. The uptake of rocuronium was linear up to 3 min. The influence of K-strophansoside, PAEB and taurocholic acid on the initial uptake rate of rocuronium is shown in figure 4. The uptake of 100 μM rocuronium was not inhibited by 160 μM taurocholic acid in human hepatocytes. In contrast, the initial uptake rate of 100 μM rocuronium was inhibited by a 40 μM concentration of the cardiac glycoside K-strophansoside: a reduction of about 80% (P < .05) was found. The uptake seemed also to be inhibited by 160 μM type I organic cation PAEB, but because of the large interindividual variation the difference was not statistically significant (P = .067) on a 5% level.

The intrinsic clearance of rocuronium was calculated from the mean initial uptake rate found in human hepatocytes and the calculated extraction ratio (table 1). The CL_H, initial in humans in vivo was calculated to be 4.4 ml/min/kg, which resulted in an E_{in vivo} of 0.41 (table 1).

![Fig. 1. Uptake of 50 nM digoxin in human hepatocytes (○) at 37°C and (●) at 4°C. Data are the mean of four separate experiments ± S.E.](image-url)
Discussion

Human hepatocytes isolated from Tx-livers and PH-livers were used without distinction in this study. In a preceding study we showed that hepatocytes from both sources have similar viability (ATP content, trypan blue exclusion, metabolic capacity and transport capacity) (Olinga et al., 1998a). Digoxin uptake in human hepatocytes was temperature-dependent, which indicates a carrier-mediated transport for this compound in humans. The extraction ratio for the whole liver \( (E_{\text{calc}}) \), calculated from the in vitro values found with the cell experiments, reasonably reflected the extraction as found in humans in vivo, taking into account the various assumptions used for these calculations (number of cells/g liver, plasma flow, liver weight, well-stirred liver model) and the high interindividual variations in humans. This indicates that the hepatic uptake rate of digoxin is reflected quantitatively in the uptake as measured in isolated human hepatocytes. However, quinidine and quinine were not able to inhibit the uptake of digoxin. This is in contrast to the result obtained in rat hepatocytes, in which a clear stereoselective inhibition was found (Hedman and Mejier, submitted). The observed results in human and rat hepatocytes indicate marked species differences in the hepatic uptake mechanisms of digoxin. The hypothesis that the inhibition of the biliary excretion of digoxin by quinidine and quinine found in vivo was caused by inhibition of hepatic uptake was not confirmed in this study. Other explanations need to be found.

It could be hypothesized that human hepatocytes are more vulnerable to the collagenase digestion (protease activity) than rat hepatocytes. However, in pilot experiments with human and rat liver slices, no inhibition of quinidine and quinine on the uptake of digoxin in human liver slices was found either (Olinga, unpublished data), whereas, in rat liver slices, similar stereoselective uptake was found as in rat hepatocytes (Olinga et al., 1998b). Moreover, the digoxin uptake rates reported from in vivo studies correspond quite well with the present hepatocyte uptake studies (table 1). This indicates that the transport function of the human hepatocytes is well retained.

The present results indicate that the well-known pharmacokinetic interaction between cardiac glycosides and quinoline-like compounds as found in man in vivo does not seem to be related to the uptake process in the liver. Inhibition of the excretion of digoxin by quinidine and quinine at the biliary level may account for the decrease in biliary clearance. Interaction at the level of metabolism does not seem to be likely because quinidine and quinine are metabolized mainly by a cytochrome P450-dependent reaction [probably by the CYP3A family (Wanwimolruk et al., 1995)], whereas the metabolic conversion of digoxin is not cytochrome P450 mediated (Lacarelle et al., 1991).

The uptake of rocuronium in human hepatocytes was temperature-dependent, and this is one factor that indicates that carrier-mediated transport is involved. Comparison of the initial hepatic extraction ratio of rocuronium, as established from the present in vitro data with the in vivo extraction found in patients (Wierda and Proost, 1995), shows that human hepatocytes can predict the in vivo situation rather well. Yet, the uptake rate of rocuronium was highly variable between hepatocytes from individual livers and, on the basis of these results, large interindividual differences in distribution to the liver are anticipated. The absence of the inhibition

| TABLE 1 |
| Initial clearance Cl (in \( 10^{-3} \) ml/min/10^6) and hepatic extraction \( E_{\text{calc}} \) and \( E_{\text{in vivo}} \) for rocuronium and digoxin |
| Cl | \( E_{\text{calc}} \) | \( E_{\text{in vivo}} \) |
| Rocuronium | 1.29 | 0.21 | 0.41 |
| Digoxin |
| 50 nM | 2.40 | 0.33 | |
| 2.5 nM | 1.12 | 0.18 | 0.13 |
of the uptake of rocuronium by taurocholic acid clearly indicates that at the concentration range chosen, taurocholic acid is not taken up by the same carrier system. This seems to contrast with the data reported in the rat (Steen et al., 1992). However, bile acids may only significantly inhibit uptake of organic cations at a relatively high concentration level of these cationic agents. In such a concentration range, a multispecific carrier system, which also accommodates bile acids, largely contributes to the overall uptake rate of the type II cations in the rat. It cannot be excluded that, in human cells, inhibition of rocuronium uptake by taurocholic acid will be found at a higher rocuronium concentration.

K-strophantoside showed a significant inhibition of the initial uptake rate of rocuronium. The effect of K-strophantoside may be explained by assuming that the organic cation carrier has a considerable higher affinity for K-strophantoside than for taurocholic acid. Earlier studies in rat hepatocytes showed that in contrast to bile acids, K-strophantoside inhibits type II cation uptake in rat cells over the entire concentration range investigated (Steen et al., 1992). This indicates similar specificity, in this respect, in humans and rats. However, it could be argued that, because human Na+/K+ ATPase is much more sensitive for cardiac glycoside than rat ATPase (Abeywardena et al., 1984), the Na+/K+ ATPase in the human hepatocytes was inhibited by K-strophantoside and that, thereby, the uptake of rocuronium was inhibited indirectly via the perturbation of Na+ and/or K+ gradients. However, this is not very likely because perturbation of Na+ and/or K+ gradients does not influence hepatic uptake rate of organic cations (Oude Elferink et al., 1995).

With PAEB, a type I organic cation in the rat, an inhibition of the uptake of rocuronium was observed, although no statistical significance was reached at the P = .05 level. This trend may suggest that the classification in type I and II carriers for organic cations is not entirely valid in humans or that overlapping substrate specificity exists. This is confirmed by the results of (Gorboulev et al., 1997) who recently cloned a polypeptide from human liver that mediated transmembrane transport of organic cations in frog oocytes. They observed vectorial transport and mutual inhibition of uptake of type I and II compounds in cell lines transfected with this human cation transporter. Further experiments are needed, therefore, to elucidate the specificity of organic cation transporters in human hepatocytes.

In conclusion, this is the first study with freshly isolated human hepatocytes that investigates mechanisms and specificity of carrier-mediated transport of organic cations and cardiac glycosides in human hepatocytes. It is shown that human hepatocytes exhibit transport activity that resembles quantitatively the uptake rates of drugs in the human liver in vivo. Furthermore, this study confirms the hypothesis that extrapolation of data on drug transport from rat to man is hazardous.

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References


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