The Use of Human Hepatocytes to Select Compounds Based on Their Expected Hepatic Extraction Ratios in Humans

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Purpose. The present investigation retrospectively evaluates the use of human hepatocytes to classify compounds into low, intermediate or high hepatic extraction ratio in man.

Methods. A simple approach was used to correlate the *in vivo* hepatic extraction ratio of a number of compounds in man (literature and in-house data) with the corresponding *in vitro* clearance which was determined in human hepatocytes. The present approach assumes that, for compounds eliminated mainly through liver metabolism, intrinsic clearance is the major determinant for their *in vivo* hepatic extraction ratio and subsequently their bioavailability in man. The test compounds were selected to represent a broad range of extraction ratios and a variety of metabolic pathways.

Results. The present data show that *in vitro* clearances in human hepatocytes are predictive for the hepatic extraction ratios *in vivo* in man. Most of the test compounds (n = 19) were successfully classified based upon human hepatocyte data into low, intermediate or high hepatic extraction compounds, i.e. compounds with potential for high, intermediate or low bioavailabilities in humans.

Conclusions. The present approach, validated so far with 19 test compounds, appears to be a valuable tool to screen for compounds with respect to liver first-pass metabolism at an early phase of drug discovery.

KEY WORDS: human hepatocytes; extraction ratio; pharmacokinetics; clearance; *in vitro* models.

INTRODUCTION

Drugs are most frequently administered orally. The majority of these are intended to act systemically and, therefore, absorption is a prerequisite for activity. A number of steps can limit the systemic availability of an oral drug. In particular, its removal as it passes through the liver is one of the most critical steps that can dramatically reduce the systemic availability. Thus, knowing whether or not a compound is subject to a high oral first-pass effect is an important piece of information in the drug development process, and this can be particularly relevant to the selection of development candidates in pharmaceutical research.

A strategy to screen for liver first-pass metabolism at the discovery stage using hepatocytes has been established in our laboratories. Since any new drug candidate can be metabolized by a variety of enzymes, hepatocytes are the model of choice because of their predictive value for the *in vivo* situation with respect to drug metabolism (1) due to a broad spectrum of enzyme activities. Furthermore, *rat* hepatocytes have already been shown to allow for the classification of drugs into low and high extraction groups (2), by providing very accurate estimates of the *in vivo* clearances for a variety of compounds (3,4). However, there are only few studies where the *in vivo* metabolic clearance of a drug in *human* was directly predicted from *in vitro* data (5).

The goal of the present investigation was to identify threshold values of *in vitro* clearances in human hepatocytes allowing compounds to be classified into low, intermediate or high hepatic extraction ratio compounds in man. Therefore, for a number of test compounds, a correlation was established between *in vitro* intrinsic clearances in human hepatocytes and the corresponding *in vivo* hepatic extraction ratios. This approach is then expected to be used for screening purposes with respect to liver first-pass metabolism in order to identify compounds with potential for high or intermediate bioavailabilities in man, and to exclude those compounds with a potentially low bioavailability in man. The test compounds were selected to represent a broad range of extraction ratios and a variety of metabolic pathways in order to be representative of future compounds to be screened.

MATERIALS AND METHODS

In Vitro Experiments in Human Hepatocytes in Primary Culture

Human liver specimens were collected from Hautepierre Hospital (Strasbourg, France) from hepatic surgical resection in accordance with the guidelines of the local Ethical Committees. The population used in this study was mainly represented by cancer patients (hepatic metastasis of colorectal cancers) and was rather heterogeneous as far as age (20 to 78 years) and comedications are concerned. However, each single liver preparation was characterized with respect to its metabolic competence by using at least one test compound (midazolam). The liver samples were collected in cold Eurocolins and processed within 1.5–2 h to prepare hepatocytes. The isolation of hepatocytes involved a two-step perfusion (6,7): at first with a Ca^{2+}-free buffer containing EGTA and then with a buffer containing Ca^{2+} and collagenase (200 I.U./mL). The cell viability, assessed by erythrosin B exclusion, was greater than 80%. Human hepatocytes were seeded without Percoll purification, in collagen coated dishes at the density of 1.5×10^6 viable cells per 1 mL of culture medium. After 16–18 h attachment, the different compounds were incubated at one concentration for up to 72 h in 1 mL of culture medium containing 10% fetal calf serum. At each sampling time, the extracellular medium was collected into Eppendorf vials. The intracellular medium was obtained by scraping the cell monolayer with 1 mL acetonitrile-H_2O or methanol-H_2O (50:50, v/v). The unchanged parent compounds were analysed in either the extracellular medium or in both the intra + extracellular medium, by specific HPLC assays. For all test compounds, a preliminary experiment was performed with analysis of the respective unchanged compounds in both the extra- and intracellular medium. In further experiments, the analysis was restricted to the extracellular medium when

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the intracellular concentrations represented less than 10% of the corresponding extracellular concentrations. Otherwise, unchanged compounds were analysed in a mixture of the extra- and intracellular medium.

In Vivo Data

The *in vivo* blood clearances from in house data or literature values were used for this evaluation. These were determined after intravenous administration, except for mofarotene where only oral data were available. When binding to erythrocytes was not known (caffeine, theophylline and diltiazem), the blood/plasma partition coefficient was assumed to be unity. Hepatic extraction ratios (E_h) were calculated from the corresponding blood clearances according to the following equation: $E_h = CL/LBF$, where CL represents the *in vivo* blood clearances (mL/min/kg) and LBF the liver blood flow (20 mL/min/kg) in humans.

Data Analysis

In vitro clearances. For each test compound, the intrinsic clearance in human hepatocytes, CL_{int}, was calculated from the ratio of the initial amount of the test compound added to the incubation medium and the corresponding area under the concentration versus time curve (AUC). AUC was calculated using the linear trapezoidal rule assuming linear changes between adjacent concentrations, and extrapolated to time infinity by adding C_t/β to the AUC (C_t is the predicted concentration at the last sampling time and β, the slope of the terminal phase of the log concentration-time curve, determined by linear regression of the last few data points).

In vitro-in vivo correlations. The equation for a well stirred model (8) was used to describe the relationship between the *in vitro* intrinsic clearances in human hepatocytes (CL_{int}; μL/min/10^6 cells) and the corresponding *in vivo* hepatic extraction ratios (E_h):

$$E_h = \frac{SF \times CL_{int}}{LBF + (SF \times CL_{int})},$$

where LBF and SF, respectively, are the liver blood flow in humans (20 mL/min/kg) and the *in vitro* to *in vivo* scaling factor. The parameter of this model (SF) was estimated by non-linear iterative least squares regression, using Excel®. A scaling factor of 8.9 was obtained by using this approach. This figure cannot be compared directly to the number of hepatocytes per kg body weight since it represents an hybrid parameter accounting also for *in vitro/in vivo* protein binding, membrane permeability, etc.

The curve fitted according to the well stirred model was used to calculate the predicted *in vivo* hepatic extraction ratios from the corresponding *in vitro* clearances.

RESULTS AND DISCUSSION

In the present study, a simple model to investigate whether the *in vitro* clearance in hepatocytes (calculated from bound + unbound concentrations in the incubation medium) allows compounds to be classified according to their *in vivo* extraction ratio was evaluated. The model assumes that there may be some degree of similarity between the binding in hepatocytes and plasma or the difference which may exist will not lead to a change in the extraction category (low, intermediate, high) of the compounds.

The relationship between the *in vitro* clearances in human hepatocytes and the *in vivo* hepatic extraction ratios in humans for the different test compounds investigated is depicted in Fig.

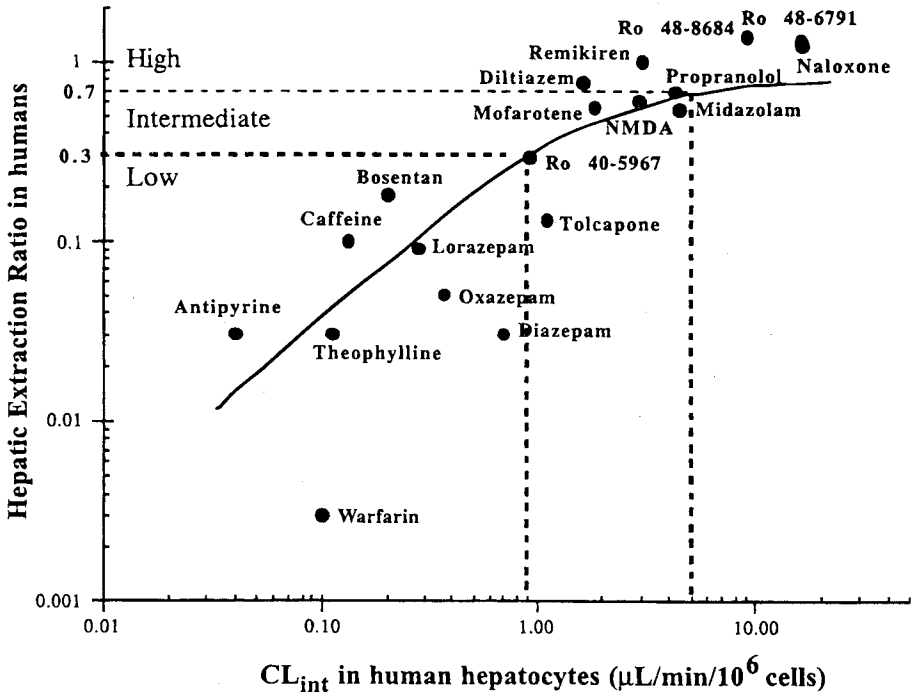

Fig. 1. Relationship between *in vivo* hepatic extraction ratios in humans and *in vitro* intrinsic clearances (CL_{int}) in human hepatocytes.

Table 1. *In Vivo* Hepatic Extraction Ratios in Man and *In Vitro* Intrinsic Clearances in Human Hepatocytes for 19 Test Compounds Investigated

DRUG (concentrations incubated, μM)	*In vitro* CL_{int} (human hepatocytes) (μL/min/10^6 cells)	E_h observed (calculated from hepatic blood CL)	E_h predicted from *in vitro* data in human hepatocytes	References
antipyrine	0.04	0.03 (I)	0.02 (I)	(11)
(100 μM)	(0.02–0.06)	(0.02–0.04)	(0.01–0.03)	
bosentan	0.22	0.18 (I–II)	0.09 (I)	[a]
(2–20 μM)	(0.20–0.25)	(0.12–0.33)	(0.08–0.10)	
caffeine	0.13	0.10 (I)	0.05 (I)	(12)
(25 μM)	(0.05–0.27)	±0.01	(0.02–0.11)	
diazepam	0.69	0.03 (I)	0.24 (I)	(13)
(2–20 μM)	(0.36–0.83)		(0.14–0.27)	
diltiazem	1.62	0.76 (II–III)	0.42 (II–III)	(14)
(0.5–10 μM)	(1.07–4.25)	(0.52–0.95)	(0.32–0.66)	
lorazepam	0.28	0.09 (I)	0.11 (I)	(15)
(2–20 μM)	(0.15–0.64)		(0.06–0.22)	
midazolam	4.49	0.54 (I–III)	0.67 (II–III)	(16–18)
(5 μM)	(1.50–8.80)	(0.29–0.77)	(0.40–0.80)	
mofarotene	1.83	0.55 (II–III)	0.45 (II)	[a]
(20 μM)	(1.58–2.08)	(0.37–1.00[b])	(0.41–0.48)	
naloxone[§]	16.5	1.00[b] (III)	0.88 (III)	(19)
(0.1–10 μM)	(14.9–18.7)		(0.87–0.89)	
NMDA	2.93	0.60 (II–III)	0.57 (II–III)	[a]
(10 μM)	(1.49–5.42)	(0.30–1.00)	(0.40–0.71)	
oxazepam	0.37	0.05 (I)	0.14 (I–II)	(20)
(0.5–10 μM)	(0.22–0.98)		(0.09–0.31)	
propranolol	4.24	0.67 (II–III)	0.65 (II–III)	(21)
(0.5–10 μM)	(1.87–7.10)	(0.49–0.91)	(0.46–0.76)	
remikiren	3.05	0.98 (III)	0.58 (II)	(22)
(0.1–10 μM)	(2.70–3.59)		(0.55–0.62)	
Ro 40-5967	0.92	0.29 (I–II)	0.29 (I–II)	[a]
(0.5–20 μM)	(0.37–1.81)	(0.18–0.47)	(0.14–0.45)	
Ro 48-6791	16.2	1.00[b] (II–III)	0.88 (III)	[a]
(5 μM)	(7.07–18.3)	(0.66–1.00[b])	(0.76–0.89)	
Ro 48-8684	9.03	1.00[b] (III)	0.80 (III)	[a]
(5 μM)	(4.77–14.9)	(0.92–1.00[b])	(0.68–0.87)	
theophylline	0.11	0.03 (I)	0.05 (I)	(23)
(25 μM)	(0.05–0.20)	±0.003	(0.02–0.08)	
tolcapone	1.10	0.13 (I)	0.33 (I–II)	[a]
(10 μM)	(0.67–1.40)	(0.08–0.19)	(0.23–0.38)	
warfarin[§]	0.10	0.003 (I)	0.04 (I)	(24)
(1–20 μM)	(0.10–0.11)	±0.001	(0.04–0.05)	

Note: median and range or standard deviation for n = 3–4 batches, except[§]: 2 batches. Remark: The observed and predicted extraction ratios were used to classify compounds into low, intermediate or high hepatic extraction. It was not the purpose of this work to compare directly the absolute predicted and observed values reported in this table.

[a] Data on file, F. Hoffmann La Roche Ltd.

[b] Arbitrarily set to 1.00, since the reported *in vivo* clearance in man was larger than the hepatic blood flow. (I) Low extraction ratio ($E_h < 0.3$); (II) intermediate extraction ratio ($0.3 < E_h < 0.7$); (III) high extraction ratio ($E_h > 0.7$).

1 and the corresponding values are reported in Table 1. Oral bioavailabilities were not correlated with the the *in vitro* clearance in human hepatocytes, since a low bioavailability can be due to causes other than first-pass hepatic metabolism.

The data were used to identify threshold values of intrinsic clearances in human hepatocytes that allow the test compounds to be classified into low, intermediate or high hepatic extraction compounds, i.e. with potential for high, intermediate or low bioavailabilities in human. The relationships shown in Figure 1 indicates that compounds with intrinsic clearances ≤0.9 μL/min/10^6cells in human hepatocytes are "low extraction compounds" ($E_h < 0.3$) and thus have potentially high (>70%) oral bioavailabilities in humans. Compounds with *in vitro* clearances in human hepatocytes between 0.9 and 5 μL/min/10^6cells are "intermediate extraction compounds" ($0.3 < E_h < 0.7$). While those with *in vitro* clearances above 5 μL/min/10^6cells are "high extraction compounds" ($E_h > 0.7$) with low (<30%) expected oral bioavailabilities in man. Based on these threshold values, the results obtained with human hepatocytes assigned most of the compounds to their correct extraction category.

Remikiren, a high extraction compound, would have been misclassified based on human hepatocyte data into the intermediate extraction group. Some biliary excretion of unchanged compound in man, as observed in rats, may explain the discrepancy between the *in vivo* and *in vitro* data.

Up to 10 fold overestimations of the observed extraction ratios in man were observed for a number of low extraction and highly bound compounds, namely warfarin, diazepam,

oxazepam and tolcapone. Since the extraction ratios of these compounds are sensitive to the extent of binding, these large overestimations, may be related to a lower binding in the hepatocyte medium than in plasma. For compounds like antipyrine, caffeine and theophylline which are only poorly bound to plasma proteins, the predicted extraction ratio is much closer to the observed value, in comparison to the aforementioned low extraction and highly bound compounds. Surprisingly, a good agreement between the predicted and observed extraction ratios was observed for bosentan and lorazepam which are also low extraction and highly bound compounds. This apparent discrepancy observed between the compounds in this category could be related to differences in the relative binding in plasma and hepatocytes and/or in the relative stabilities of the enzymes in hepatocyte cultures. However, it is worth mentioning that this discrepancy did not compromize the validity of the present approach because most of the compounds, except tolcapone, were still classified into their correct extraction ratio category.

Ideally, the parameter intrinsic clearance, which is a pure measure of enzyme activity towards a compound, should be used for the extrapolation of *in vitro* data to the *in vivo* situation (2). This requires to take into account protein binding in plasma and hepatocytes, the use of an adequate liver model to derive intrinsic clearance from hepatic clearance, and the determination of a physiologically based scaling factor to scale the *in vitro* clearance to the *in vivo* situation. Further investigations (e.g. determination of the binding to hepatocytes, permeability, stability of liver enzymes, cofactor depletion. . .) are now ongoing in our laboratories in order to generate the data necessary for such *in vitro—in vivo* scaling based on intrinsic clearance.

Intersubject variability in drug metabolism is considered to be an important issue in obtaining valuable information from human *in vitro* liver preparations (9). In the present study, up to a 4-fold variation in extraction ratios when determined from 3–4 liver samples was observed; this *in vitro* variability compares favourably with the *in vivo* situation for the different compounds investigated. The variability observed in such experiments is likely to be due to the intrinsic nature of the liver samples (content and activity of the constitutive enzymes) rather than to technical factors related to the preparation/culturing of the human hepatocytes. Thus, the standard conditions used to prepare and culture hepatocytes as well as the characterization of each liver preparation with respect to its metabolic competences have certainly contributed to the acceptable variability observed in the present study.

At the drug discovery stage, the enzymes involved in the metabolism of a compound are largely unknown. Therefore, the drugs evaluated so far were selected to represent a variety of metabolic reactions including cytochrome P450 isoenzymes, such as CYP 3A4 for midazolam (10), CYP 2D6 for propranolol (10), CYP 1A2 for antipyrine, caffeine and theophylline (10) and CYP 2C9 for Ro 48-6791 and Ro 48-8684 (in house data). Furthermore, oxazepam, naloxone and tolcapone were metabolized exclusively by phase II reactions (glucuronidation, sulfation, methylation, etc.). Despite the variety of metabolic pathways, the range of protein binding and clearance, most of the test drugs were classified into the correct category based on *in vitro* data in human hepatocytes.

In conclusion: the present study demonstrates for the first time that, *in vitro* clearances in human hepatocytes can be used to classify compounds into low, intermediate or high hepatic extraction compounds, i.e. with potential for high, intermediate or low bioavailabilities in humans. The approach, validated so far with 19 test compounds, will be evaluated with additional compounds. It appears to be a valuable tool to screen for compounds, with respect to liver first-pass metabolism, at an early phase of drug discovery.

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