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PREDICTION OF HEPATIC CLEARANCE AND AVAILABILITY BY CRYOPRESERVED HUMAN HEPATOCYTES: AN APPLICATION OF SERUM INCUBATION METHOD

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ABSTRACT:

A novel and convenient method was established for the prediction of in vivo metabolic clearance in human liver. The present method applied the in vitro-in vivo extrapolation paradigm previously established in rats to the in vitro data obtained from cryopreserved human hepatocytes. Predicted hepatic availability and clearance were compared with the reported oral bioavailability and plasma clearance in humans for 14 clinically used drugs (naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, timolol, antipyrine, diazepam, quinidine, caffeine, propranolol, diclofenac, and phenacetin). A large interindividual variation was observed in the intrinsic metabolic clearance among separate cryopreserved preparations from different subjects. The prediction generally resulted in a marked underestimation when the biologically based scaling factor (3.1×10^9 cells/kg) was used for the extrapolation of in vitro data (milliliters per minutes per cells) to in vivo value (milliliters per minutes per kilograms). Reasonably good in vitro-in vivo correlations were obtained with empirically calculated scaling factors, 8.5×10^9 (cells/kg) from 10 individual preparations and 10.8×10^9 (cells/kg) from pooled preparation of two selected lots, which were 3- to 4-fold larger than the biologically based scaling factor. These data suggested that the calibration of inherent interindividual variation of metabolic activities among different cryopreserved preparations of human hepatocytes to obtain the empirical scaling factor, which is applicable only to the preparation used, was an essential step for more reliable and quantitative prediction of in vivo metabolic activity in humans.

Hepatic clearance for the metabolism of compounds kinetically consists of two major determinants: intrinsic (metabolic) clearance of the unbound compound and unbound fraction of compound in the blood (or plasma when corrected by the blood-to-plasma partition). Generally, the intrinsic clearance for the unbound compound is measured in vitro by the incubation of isolated hepatocytes or subcellular fractions such as *S*-9 and microsomes in the protein-free medium. The in vitro metabolic parameters thus obtained are extrapolated by using anatomical parameters such as cell numbers and protein content in the intact liver for the prediction of in vivo metabolic activity (Houston and Carlile, 1997; Iwatsubo et al., 1997; Obach, 1999). Separate experiments necessarily are further carried out to measure the unbound fraction in the plasma. Many technical problems including adsorption of compounds to the apparatus during the equilibrium dialysis and ultra-filtration often hamper the accuracy of the evaluated values (Bertilsson et al., 1979; Desoye, 1988). To improve the accuracy and avoid complexity for predicting in vivo metabolic clearance from in vitro experiments, we have recently developed a novel and convenient in vitro method for predicting in vivo metabolic clearance by using freshly isolated rat hepatocytes suspended in rat serum (Shibata et al., 2000). Oral bioavailability and hepatic clearance for 16 widely used compounds were well predicted directly from the in vitro metabolic clearance values obtained from a single incubation without separate evaluation of unbound fraction in the plasma. The purposes of the present study were to 1) determine whether the same methodology was applicable to the prediction of in vivo metabolic activity in humans by using cryopreserved human hepatocytes and 2) establish the in vitro-in vivo scaling-up paradigm to calibrate the interindividual variation of the metabolic activities among cryopreserved preparations from different subjects for more reliable and quantitative prediction in humans.

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Materials and Methods

Naloxone and lidocaine were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. Buspirone, metoprolol, phenacetin, propranolol, quinidine, timolol, and verapamil were obtained from Sigma-Aldrich (St. Louis, MO). Antipyrine, caffeine, diazepam, diclofenac, and imipramine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Blood was collected from three healthy male volunteers aged 25 to 40 years old and allowed to coagulate for 3 h at room temperature. The blood was later centrifuged (15 min, 1800*g*) to obtain serum. The serum was stored at −80°C until use. The pH of human serum was adjusted to 7.4 at 37°C by adding 1N-HCl solution before use. Cryopreserved human hepatocytes (lot numbers 56, 57, 64, 70, 73, 83, 97, 100, 106, and 120) were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Cell viability was assessed using the 0.4% trypan blue exclusion test, and the count of living cells was started 5 min after mixing the pigment. Cell viabilities were between 45 and 60%. Hepatocytes were resuspended in 100% human serum at an ice-cold temperature at the following densities: 1×10^6 cells/ml for naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, and timolol; 2×10^6 cells/ml for quinidine, caffeine, propranolol, diclofenac, and phenacetin; and 5×10^6 cells/ml for antipyrine and diazepam. Suspensions of hepatocytes (370 μl) were pipetted into 1.5-ml tubes, and an aliquot (3.7 μl) of 100 μM compound in a water (or 50% CH_3CN for quinidine and phenacetin) was added to obtain the final concentration of 1 μM (or 50 μM for

antipyrine and 10 μM for caffeine). Each sample (50 μl) was transferred to two 96-well plates with flat bottoms ($n = 3$), each of which was used for the incubation or for the control. Ninety-six well plates were incubated at 37°C with shaking at 150 rpm under 95% O_2/5% CO_2 in the water bath incubator. After the onset of incubation, the plates were placed on ice at the designated time point, and the reaction was terminated by the addition of a 150-μl ice-cold ethanol solution containing the internal standard (no internal standard for antipyrine and caffeine). The sample was centrifuged (10,000g × 10 min), and the amount of compound remaining in the supernatant was measured by HPLC[1]-UV (antipyrine and caffeine) or LC-MS/MS (others) as described below.

Antipyrine was measured at 254 nm by the Alliance 2690–2487 HPLC-UV system (Waters Corp., Milford, MA) that was connected to Inertsil ODS-3 4.6 × 250 mm (GL-Sciences, Tokyo, Japan), which acted as an analytical column. The HPLC method involved the isocratic elution with acetonitrile/water (25:75) containing 10 mM ammonium acetate at the flow rate of 1 ml/min. The retention time of antipyrine was 7 min. Caffeine was measured by the UV wavelength of 280 nm at a flow rate of 1 ml/min of acetonitrile/water (10:90) containing 0.1% trifluoroacetic acid. The retention time of caffeine was 16 min. Other compounds were measured by the Alliance HT 2790 HPLC (Waters Corp.), PU-1580 HPLC pump (Jasco, Tokyo, Japan), NANOSPACE SI-2 switching valves (Shiseido, Osaka, Japan), and API-3000 LC-MS/MS detector (PerkinElmerSciex Instruments, Boston, MA) with a turbo ionspray interface. Multiple reaction monitoring of positive-ion mode was used for all analyses. Analytical methods including the selection of ions and parameters for multiple reaction monitoring were automatically obtained for each compound by the application software, Analyst (PerkinElmerSciex Instruments). Mass number of molecular ions and product ions for each compound was identified as follows (molecular > product): naloxone 328.4 > 310.4, buspirone 386.2 > 122.2, verapamil 455.3 > 165.2, lidocaine 235.2 > 86.2, imipramine 281.1 > 86.0, metoprolol 268.3 > 116.0, timolol 317.1 > 261.1, diazepam 285.1 > 193.3, quinidine 325.1 > 307.3, propranolol 260.2 > 116.3, diclophenac 295.9 > 215.2, and phenacetin 180.1 > 138.0. A fast-gradient condition using two switching valves and pumps (3.5 min/cycle) was used for the analysis. Capcell Pak UG-120 4.0 × 10 mm (Shiseido) was used as an analytical column, and the flow rate of 1 ml/min of acetonitrile/water (10:90) containing 10 mM ammonium acetate was the initial condition used. After the injection of a sample (5 μl), the ratio of acetonitrile/water was changed to 90:10 linearly for 1 min and maintained for the next 0.5 min. The column was then washed with acetonitrile/water (90:10) containing 10 mM ammonium acetate at a back flow rate of 1 ml/min. The effluent was split with 0.2 ml/min, and only the effluent from 0.5 to 1.5 min after the injection was introduced into the LC-MS/MS detector. Modified conditions were used for metoprolol, timolol, and phenacetin. In the case of metoprolol and timolol, Symmetry Shield RP18/3.5 μM 2.1 × 10 mm (Waters Corp.) was used as the analytical column. After the injection of a sample, the ratio of acetonitrile/water was changed linearly to 66:34 for 1.4 min, and the effluent from 0.4 to 1.5 min after the injection was introduced into the LC-MS/MS detector. In the case of phenacetin, Inertsil ODS-3 2.1 × 150 mm (GL-Sciences) was used as the analytical column. After the injection of a sample, the ratio of acetonitrile/water was changed linearly to 34:66 for 3 min and then changed linearly to 90:10 for the next 2 min. The effluent from 0.5 to 5 min after the injection was introduced into the LC-MS/MS detector. Diazepam was commonly used as the internal standard. When diazepam was the analyte, quinidine was used as the internal standard.

For standard compounds, the following assumptions were reasonably applied to the prediction, 1) the hepatic metabolism is the major route of elimination, 2) all metabolic enzymes in the cryopreserved preparation of human hepatocytes remain active comparably to in vivo, and 3) the absorption is complete for all standard compounds. The in vitro intrinsic clearance ($CL_{int,\ in\ vitro}$) was calculated from the following equation by using cell density (D), incubation time (T, 120 min for the calculation of $CL_{int,\ in\ vitro}$), and the ratio (R) of unchanged compound concentration at time T to that at time 0 when the unbound drug concentration was much lower than its K_m value (Shibata et al., 2000); $CL_{int,\ in\ vitro} = (-\log_e R)/(D \times T)$. To extrapolate the in vitro clearance to the in vivo value, the empirical scaling factor (average SF_{70+73}) for the optimized cryopreserved preparation pooled from equal volumes of human hepatocytes (lot numbers 70 and 73) was calculated according to the method described under the *Results* section. The value of average SF_{70+73} was 10.8×10^9 cells/kg of body weight and used for the extrapolation as follows: $CL_{H,\ int,\ in\ vitro,\ 70+73} = CL_{int,\ in\ vitro,\ 70+73} \times$ average SF_{70+73} where $CL_{H,\ int,\ in\ vitro,\ 70+73}$ and $CL_{int,\ in\ vitro,\ 70+73}$ represent the in vitro hepatic intrinsic clearance and in vitro intrinsic clearance, respectively, measured in the pooled preparation of lot 70 and 73. We chose the dispersion model as a liver model because a good predictability of hepatic availability (F_H) for high clearance drugs was previously reported (Iwatsubo et al., 1997). The hepatic clearance ($CL_{H,\ predicted,\ 70+73}$) was predicted from the obtained in vitro hepatic intrinsic clearance ($CL_{H,\ int,\ in\ vitro,\ 70+73}$) by using the following equation (eq. 1) with the dispersion model (Iwatsubo et al., 1997);

$$CL_{H,\ predicted,\ 70+73} = Q_H \times R_B \times (1 - 4a/((1 + a)^2 \times \exp[(a - 1)/(2 \times D_N)] - (1 - a)^2 \times \exp[-(a + 1)/(2 \times D_N)])$$

where $R_N = (CL_{H,\ int,\ in\ vitro,\ 70+73})/(Q_H \times R_B)$ and $a = (1 + 4 \times R_N \times D_N)^{0.5}$

F_H was further calculated from $F_H = 1 -$ hepatic extraction ratio (E_H) $= 1 - CL_H/(Q_H \times R_B)$. In these equations, the liver blood flow rate (Q_H) and dispersion number (D_N) for humans were assumed to be 20.7 ml/min/kg (Davies and Morris, 1993) and 0.17 (Roberts and Rowland, 1986), respectively. The blood-to-plasma concentration ratio (R_B) was used as reported or assumed to be unity if the value was not available.

Results

Tables 1 and 2 summarize the pharmacokinetic profiles in humans and the results of extrapolations from the in vitro data for the standard compounds tested in the present study. These compounds were chosen to represent a wide range of oral bioavailability (2–96%) and plasma clearance (0.3–28.3 ml/min/kg). These standard compounds are reported to have complete absorption, negligible urinary excretion (<20% of dose), and the major route of elimination by hepatic metabolism. Therefore, it was reasonably assumed that the in vivo plasma clearance ($CL_{P,\ in\ vivo}$) and oral bioavailability ($F_{PO,\ in\ vivo}$) are equal to the hepatic metabolic clearance (CL_H) and F_H, respectively. In vivo values for hepatic intrinsic clearance ($CL_{H,\ int,\ in\ vivo}$) of standard compounds were calculated from $F_{PO,\ in\ vivo}$ by the dispersion model using the iterative calculation method (Goal Seek method in Microsoft Excel). To calculate empirical scaling factor from the comparison of in vivo and in vitro hepatic intrinsic clearance, $CL_{int,\ in\ vitro}$ values were evaluated in the cryopreserved preparations of human hepatocytes obtained from 10 different subjects. An approximately 3- to 5-fold variation in the $CL_{int,\ in\ vitro}$ was observed

[1] Abbreviations used are: HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; $CL_{int,\ in\ vitro}$, in vitro intrinsic clearance observed when test compounds were metabolized by human hepatocytes suspended in human serum; D, cell density of hepatocytes suspended in serum; T, incubation time; R, ratio of intact drug concentration after incubation to that at time 0; SF_{70+73}, scaling factor calculated from $CL_{int,\ in\ vitro,\ 70+73}/CL_{H,\ int,\ in\ vivo}$ for the pooled hepatocyte preparation of lot 70 and 73; $CL_{H,\ int,\ in\ vitro,\ 70+73}$, hepatic intrinsic clearance calculated from in vitro data using the pooled hepatocyte preparation of lot 70 and 73; $CL_{int,\ in\ vitro,\ 70+73}$, in vitro intrinsic clearance observed when test compounds were metabolized in the pooled hepatocyte preparation of lot 70 and 73 suspended in human serum; F_H, hepatic availability; $CL_{H,\ predicted,\ 70+73}$, predicted hepatic clearance from $CL_{int,\ in\ vitro,\ 70+73}$ and average SF_{70+73}; Q_H, hepatic blood flow rate; R_B, blood-to-plasma concentration ratio; D_N, dispersion number; $CL_{P,\ in\ vivo}$, in vivo plasma clearance; $F_{PO,\ in\ vivo}$, oral bioavailability in humans; $CL_{H,\ int,\ in\ vivo}$, hepatic intrinsic clearance calculated from $F_{PO,\ in\ vivo}$ by the dispersion model (using the Goal Seek method attached to Microsoft Excel); SF_{mean}, mean of scaling factor calculated from $CL_{int,\ in\ vitro,\ mean}/CL_{H,\ int,\ in\ vivo}$ for 10 individual lots; average SF_{mean}, average value of SF_{mean} for seven standard compounds; $F_{H,\ predicted,\ 70+73}$, predicted hepatic availability from $CL_{int,\ in\ vitro,\ 70+73}$ and average SF_{70+73}; SF_{biol}, biologically based scaling factor of hepatocellularity (3.1×10^9 cells/kg); average SF_{70+73}, average value of SF_{70+73} for seven standard compounds.

TABLE 1

In vitro-in vivo correlation of seven compounds using 10 individually prepared cryopreserved human hepatocytes and key parameters for in vivo prediction

All these values were quoted from the literature as follows. Naloxone [Asali and Brown (1984); Holford (1998)]; buspirone [Gammans et al. (1986)]; verapamil [Gross et al. (1988); McAllister and Kirsten (1982); Obach (1999)]; lidocaine; [Wing et al. (1984), Remmel et al. (1991)]; imipramine [the pharmacokinetics were mean value of the report of Nagy and Johansson (1975) and Ciraulo et al. (1988)]; metoprolol [Johansson et al. (1974); Regardh et al. (1974); Regardh et al. (1981)]; timolol [Wilson et al. (1982), [Holford (1998].

No.	compound	f_u	R_B	$CL_{P,\ in\ vivo}$	$F_{PO,\ in\ vivo}$	$CL_{H,\ int,\ in\ vivo}$	$CL_{int,\ in\ vitro,\ mean}$	SF_{mean}	$CL_{H,\ predicted,\ mean}$	$F_{H,\ predicted,\ mean}$	$CL_{H,\ predicted,\ biol}$	$F_{H,\ predicted,\ biol}$
				ml/min/kg		*ml/min/kg*	*ml/min/10^9 cells*	*10^9 cells/kg*	*ml/min/kg*		*ml/min/kg*	
1	Naloxone	0.559	1.22*	24.8	0.02	154.9	34.7 ± 8.2	4.5	25.3 ± 0.1	0.00 ± 0.00	23.8 ± 0.7	0.06 ± 0.03
2	Buspirone	0.050	0.81*	28.3 ± 10.3	0.04 ± 0.04	79.1	12.1 ± 6.9	6.5	16.2 ± 0.6	0.04 ± 0.03	13.2 ± 1.9	0.22 ± 0.11
3	Verapamil	0.100	0.77	11.8 ± 5.0	0.20 ± 0.12	31.0	7.3 ± 3.6	4.3	14.5 ± 0.9	0.09 ± 0.05	10.6 ± 1.9	0.34 ± 0.12
4	Lidocaine	0.296	0.84	12.5 ± 1.5	0.24 ± 0.05	29.8	2.3 ± 0.7	12.9	10.6 ± 1.9	0.39 ± 0.11	5.5 ± 1.4	0.68 ± 0.08
5	Imipramine	0.185	1.08	11.8 ± 8.1	0.42 ± 0.08	21.8	1.5 ± 0.6	14.5	8.6 ± 2.7	0.61 ± 0.12	4.0 ± 1.4	0.82 ± 0.06
6	Metoprolol	0.883	1.13	10.8 ± 1.5	0.50 ± 0.11	17.8	2.3 ± 0.8	7.8	12.0 ± 2.5	0.49 ± 0.10	5.8 ± 1.6	0.75 ± 0.07
7	Timolol	0.400	0.84*	7.7 ± 1.2	0.61 ± 0.06	9.1	1.0 ± 0.6	9.0	6.0 ± 2.8	0.65 ± 0.16	2.7 ± 1.4	0.84 ± 0.08
							average SF_{mean}	8.5 ± 4.0				

f_u, unbound fraction in plasma; R_B, blood-to-plasma concentration ratio (reported); $CL_{P,\ in\ vivo}$, plasma clearance in humans, (reported); $F_{PO,\ in\ vivo}$, oral bioavailability in humans (reported); $CL_{H,\ int,\ in\ vivo}$, hepatic intrinsic clearance values calculated from $F_{PO,\ in\ vivo}$ by th dispersion model (using the Goal Seek method attached to Microsoft Excel); $CL_{int,\ in\ vitro,\ mean}$, in vitro intrinsic clearance observed when test compounds were metabolized by 10 individual lots of human hepatocytes suspended in human serum; SF_{mean}, mean of scaling factor calculated from $CL_{int,\ in\ vitro,\ mean}$/ $CL_{H,\ int,\ in\ vivo}$ for 10 individual lots; $CL_{H,\ predicted,\ mean}$, predicted hepatic clearance from $CL_{int,\ in\ vitro,\ mean}$ and average SF_{mean} (8.5×10^9 cells/kg) as a scaling factor; $F_{H,\ predicted,\ mean}$, predicted hepatic availability from $CL_{int,\ in\ vitro,\ mean}$ and average SF_{mean} (8.5×10^9 cells/kg) as a scaling factor; $CL_{H,\ predicted,\ biol}$, predicted hepatic clearance from $CL_{int,\ in\ vitro,\ mean}$ and biologically based scaling factor of hepatocellularity ($SF_{biol} = 3.1 \times 10^9$ cells/kg); $F_{H,\ predicted,\ biol}$, predicted hepatic availability from $CL_{int,\ in\ vitro,\ mean}$ and biologically based scaling factor of hepatocellularity ($SF_{biol} = 3.1 \times 10^9$ cells/kg).

* In house data.

TABLE 2

Profiles of 14 compounds tested with the pooled preparation of human hepatocytes lot 70 and 73 and the key parameters for the in vivo prediction

All these values were quoted from the literature as follows. Naloxone [Asali and Brown (1984); Holford (1998)]; buspirone [Gammans et al. (1986)]; verapamil [Gross et al. (1988); McAllister and Kirsten (1982); Obach (1999)]; lidocaine [Remmel et al. (1991); Wing et al. (1984)]; imipramine [the pharmacokinetics were mean value of the report of Ciraulo et al. (1988) and Nagy and Johansson (1975)]; metoprolol [Johansson et al. (1974); Regardh et al. (1974); Regardh et al. (1981)]; timolol [Wilson et al. (1982), Holford (1998)]; antipyrine [Elfstrom and Lindgren (1978); Vesell et al. (1975)]; diazepam [Divoll et al. (1983); Greenblatt et al. (1980); Maguire et al. (1980)]; quinidine [Guentert et al. (1979); Hardy and Schentag (1988); Hughes et al. (1975)]; caffeine [Blanchard (1982); Newton et al. (1981)]; propranolol [Walle et al. (1989); Wilson et al. (1982)]; diclofenac [Chan et al. (1987); Obach (1999); Willis et al. (1980)]; phenacetin [Raaflaub and Dubach (1975); Vesell et al. (1975)].

No.	compound	f_u	R_B	$CL_{P,\ in\ vivo}$	$F_{PO,\ in\ vivo}$	$CL_{H,\ int,\ in\ vivo}$	D	R	$CL_{int,\ in\ vitro,\ 70+73}$	SF_{70+73}	$CL_{H,\ predicted,\ 70+73}$	$F_{H,\ predicted,\ 70+73}$
				ml/min/kg		*ml/min/kg*	*10^6 cells/ml*	*after 2-h*	*ml/min/10^9 cells*	*10^9 cells/kg*	*ml/min/kg*	
1	Naloxone	0.559	1.22*	24.8	0.02	154.9	1.0	0.07 ± 0.01	18.31 ± 0.69	8.5	25.2 ± 0.0	0.01 ± 0.00
2	Buspirone	0.050	0.81*	28.3 ± 10.3	0.04 ± 0.04	79.1	1.0	0.33 ± 0.01	7.66 ± 0.26	10.3	16.5 ± 0.0	0.02 ± 0.00
3	Verapamil	0.100	0.77	11.8 ± 5.0	0.20 ± 0.12	31.0	1.0	0.51 ± 0.01	4.65 ± 0.13	6.7	14.9 ± 0.1	0.07 ± 0.00
4	Lidocaine	0.296	0.84	12.5 ± 1.5	0.24 ± 0.05	29.8	1.0	0.70 ± 0.03	2.43 ± 0.26	12.3	13.4 ± 0.6	0.23 ± 0.03
5	Imipramine	0.185	1.08	11.8 ± 8.1	0.42 ± 0.08	21.8	1.0	0.77 ± 0.01	1.78 ± 0.06	12.3	13.3 ± 0.3	0.40 ± 0.01
6	Metoprolol	0.883	1.13	10.8 ± 1.5	0.50 ± 0.11	17.8	1.0	0.81 ± 0.05	1.47 ± 0.39	12.1	12.1 ± 2.0	0.49 ± 0.09
7	Timolol	0.400	0.84*	7.7 ± 1.2	0.61 ± 0.06	9.1	1.0	0.91 ± 0.03	0.69 ± 0.23	13.2	6.5 ± 1.7	0.62 ± 0.10
8	Antipyrine	0.970		0.7 ± 0.1	0.96 ± 0.06	0.8	5.0	0.98 ± 0.01	0.03 ± 0.02		0.4 ± 0.2	0.98 ± 0.01
9	Diazepam	0.013	0.71	0.3 ± 0.1	0.94 ± 0.20	0.9	5.0	0.96 ± 0.02	0.05 ± 0.03		0.7 ± 0.4	0.95 ± 0.03
10	Quinidine	0.146	0.92	4.9 ± 1.6	0.70 ± 0.17	7.1	2.0	0.84 ± 0.04	0.61 ± 0.14		6.1 ± 1.2	0.68 ± 0.06
11	Caffeine	0.650		1.0 ± 0.4	0.92 ± 0.04	1.7	2.0	0.96 ± 0.01	0.13 ± 0.03		1.6 ± 0.4	0.92 ± 0.02
12	Propranolol	0.123	0.89*	17.3 ± 2.0	0.32 ± 0.04	24.4	2.0	0.52 ± 0.01	2.28 ± 0.07		13.6 ± 0.2	0.26 ± 0.01
13	Diclofenac	0.003	0.55	4.1 ± 0.8	0.58 ± 0.14	6.7	2.0	0.80 ± 0.04	0.77 ± 0.17		6.2 ± 0.8	0.46 ± 0.07
14	Phenacetin	0.600	1.01*	19.6 ± 4.5	0.02 ± 0.03	127.5	2.0	0.01 ± 0.00	14.72 ± 0.29		20.8 ± 0.0	0.01 ± 0.00
									average SF_{70+73}	10.8 ± 2.4		

f_u, unbound fraction in plasma; R_B, blood-to-plasma concentration ratio (reported); $CL_{P,\ in\ vivo}$, plasma clearance in humans (reported); $F_{PO,\ in\ vivo}$, oral bioavailability in humans (reported); $CL_{H,\ int,\ in\ vivo}$, hepatic intrinsic clearance values calculated from $F_{PO,\ in\ vivo}$ by the dispersion model (using the Goal Seek method attached to Microsoft Excel); D, cell density of hepatocytes suspended in serum; R, the ratio of unchanged compound concertration at 2-hr incubation to that at time 0; $CL_{int,\ in\ vitro,70+73}$, in vitro intrinsic clearance observed when test compounds were metabolized in the pooled preparation of Lot 70 and 73 suspended in human serum; SF_{70+73}, scaling factor calculated from $CL_{int,\ in\ vitro,\ 70+73}$ / $CL_{H,\ int,\ in\ vivo}$ for pooled preparation of Lot 70 and 73; $CL_{H,\ predicted,\ 70+73}$, predicted hepatic clearance from $CL_{int,\ in\ vitro,\ 70+73}$ and average SF_{70+73} (10.8×10^9 cells/kg) as a scaling factor; $F_{H,\ predicted,\ 70+73}$, predicted hepatic availability from $CL_{int,\ in\ vitro,\ 70+73}$ and average SF_{70+73} (10.8×10^9 cells/kg) as a scaling factor.

* In house data.

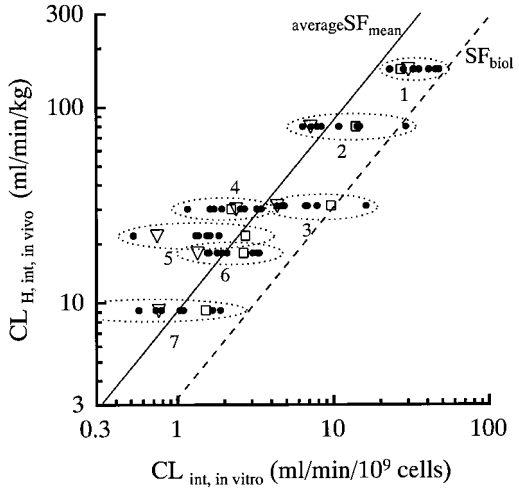

FIG. 1. *$CL_{H, int, in vivo}$ versus $CL_{int, in vitro}$ in 10 cryopreserved preparations of human hepatocytes.*

Each symbol represents the mean of three determinations. Data obtained from lot 70 and 73 are shown by open triangles and squares, respectively, whereas those in the individual preparations are shown by closed circles. Solid and broken line represent the predictions by the empirical scaling factor (average SF_{mean} in Table 1) and biologically based scaling factor (SF_{biol}, see Text), respectively, from 10 cryopreserved preparation.

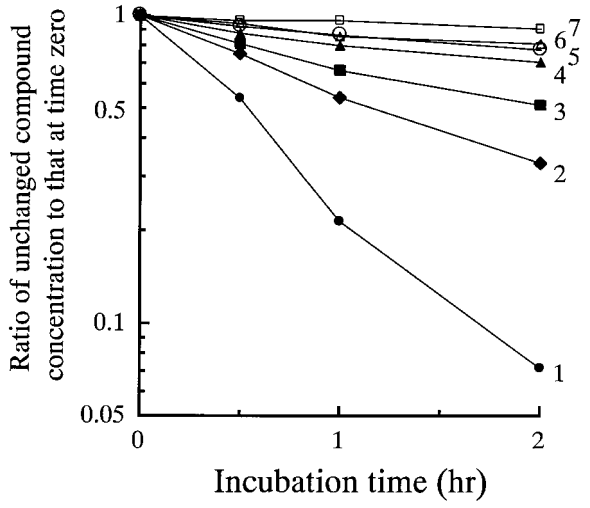

FIG. 2. *Disappearance curves of seven standard compounds in the pooled human hepatocytes preparation (lot 70 and 73) suspended in serum.*

Each symbol represents the mean of three determinations. The numbers correspond to the compound numbers listed in Table 1.

among 10 cryopreserved preparations of human hepatocytes. The prediction of $CL_{H, int, in vivo}$ resulted in a marked underestimation when the biologically based scaling factor [3.1×10^9 cells/kg, calculated from the assumption that each gram of human liver contains 120×10^6 cells/g liver (Iwatsubo et al., 1997), and an average human has 1800 g of liver (Davies and Morris, 1993)] was used to extrapolate $CL_{int, in vitro}$ to $CL_{H, int, in vivo}$ (Fig. 1). Mean values for the empirical scaling factor (SF_{mean}) were calculated by averaging the ratio of $CL_{H, int, in vivo}$ to the corresponding in vitro values ($CL_{int, in vitro}$) for each standard compound (Table 1). The average SF_{mean} value among seven standard compounds was found to be 8.5×10^9 cells/kg, which was approximately 3 times larger than that of biologically based value (3.1×10^9 cells/kg). Reasonably accurate predictions were achieved (Table 1; Fig. 1) when the scaling factors, thus empirically obtained, were used for the extrapolation.

The averaged results from 10 or more preparations of human hepatocytes appeared to provide more reliable predictions for the human liver metabolism, whereas it was less convenient and cost-effective. It was found that the pooled preparation of two lots (lot 70 and 73) achieved the same extent of predictability for all seven standard compounds as the averaged results from 10 individual preparations (Tables 1 and 2). Pooled preparation demonstrated that the metabolic activity was constant during a 2-h incubation time period for standard compounds (Fig. 2). The in vitro- in vivo-correlation study was further extended to another seven compounds by using pooled cryopreserved preparation of human hepatocytes (Table 2). The predictions of $CL_{H, predicted, 70+73}$ and $F_{H, predicted, 70+73}$ were carried out with the average value of scaling factor (average SF_{70+73}, 10.8×10^9 cells/kg) for total 14 compounds, which was obtained empirically as described for 7 standard compounds in the pooled hepatocyte preparation of lot 70 and 73. Reasonably good correlations were obtained for both oral bioavailability (Fig. 3, panel A) and hepatic clearance (Fig. 3, panel B). These data demonstrated that the in vitro metabolic clearance obtained in the pooled preparation from cryopreserved human hepatocytes reasonably well predicted in vivo hepatic clearance and availability with the aid of empirical scaling factor.

Discussion

Isolated hepatocytes have been recognized as more in vivo relevant in vitro systems than the subcellular fractions such as liver *S*-9 and microsomes for the prediction of in vivo metabolism. Although the freshly isolated human hepatocytes appeared to be one of the best preparations for the prediction of in vivo metabolism in humans (Lavé et al., 1999), the cryopreserved human hepatocytes instead became more prevalent and widely used for the routine analysis (Li, 2001). Cryopreserved human hepatocytes have been reported to quantitatively retain most of the phase I metabolic activities observed in the fresh liver, whereas some phase II metabolic activities to certain substrates were lower in the cryopreserved preparation than the intact human liver (Li et al., 1999; Steinberg et al., 1999; Hengstler et al., 2000; Rialland et al., 2000). In addition, consistent with the fact that each drug metabolizing enzyme activity in the human liver is known to individually vary between subjects, an approximately 3- to 5-fold variation was found in the in vitro metabolic clearance for the standard compounds among preparations from different human subjects (Table 1; Fig. 1). The interindividual variation in the metabolic capacity in the liver appears to reflect the observed large variation in the clearance in humans.

The empirical scaling factors for the in vitro-to-in vivo extrapolation (8.5×10^9 cells/kg from 10 individual preparations in Table 1; 10.8×10^9 cells/kg from pooled preparation in Table 2) were approximately 3 to 4 times larger than the anatomically calculated value (3.1×10^9 cells/kg). In addition, the variation of scaling factor obtained from 10 individual preparations (SF_{mean} in Table 1) between different compounds was much larger than that obtained from the pooled-cryopreserved preparation of human hepatocytes (SF_{70+73} in Table 2). These data suggested that the empirical scaling factor applicable only to the preparation used in the prediction was critically important for more reliable and rational predictions, which might compensate the inherent interindividual variation and/or loss of metabolic activities among different cryopreserved preparations.

In summary, the present study demonstrates that the direct evaluation of metabolic clearance in cryopreserved human hepatocytes in the presence of human serum was a convenient and useful tool for the prediction of hepatic clearance and availability. The calibration paradigm described in this report minimized the interindividual variation

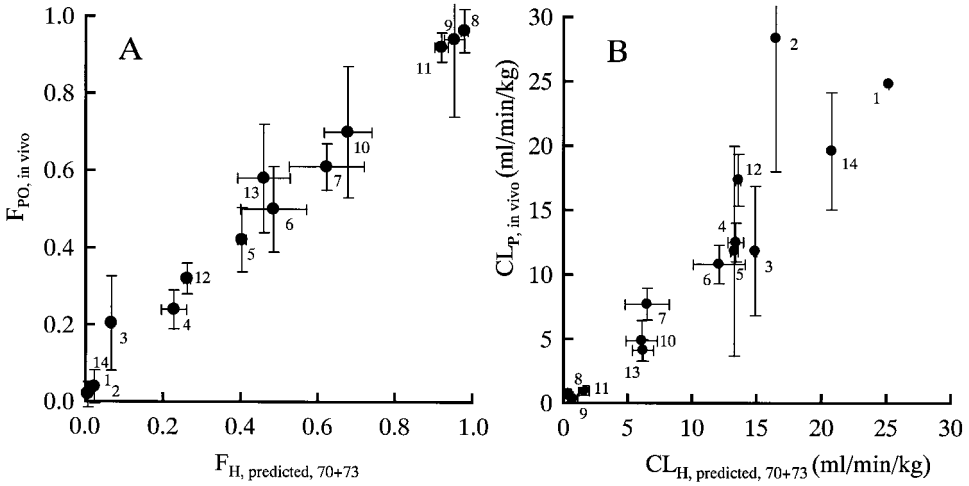

FIG. 3. *Prediction of oral bioavailability (A) and hepatic clearance (B) using the cryopreserved preparation of human hepatocytes pooled from two separate lots.*

The hepatocyte suspension was prepared by combining equal volumes of cryopreserved preparations of human hepatocytes from lot 70 and 73. Each symbol represents the mean ± S.D. of three determinations of predicted values from cryopreserved preparations (*x*-axis) and those of observed values taken from the literature (*y*-axis). Numbers correspond to the compound numbers listed in Table 2. Predictions ($F_{PO,\ predicted,\ 70+73}$ and $CL_{H,\ predicted,\ 70+73}$) were carried out as described under the *Materials and Methods* section by using the average of empirical scaling factor (average SF_{70+73}) of 10.8×10^9 cells/kg (Table 2).

of metabolic activities among different subjects and improved the predictability of the in vitro data for the in vivo metabolic clearance with the aid of empirical scaling factor. The present method could be helpful at the early discovery stage to identify more promising candidates for further development that have lower hepatic clearance and higher oral bioavailability in humans.

References

Asali LA and Brown KF (1984) Naloxone protein binding in adult and foetal plasma. *Eur J Clin Pharmacol* **27:**459–463.

Bertilsson L, Braithwaite R, Tybring G, Garle M, and Borga O (1979) Techniques for plasma protein binding of demethylchlorimipramine. *Clin Pharmacol Ther* **26:**265–271.

Blanchard J (1982) Protein binding of caffeine in young and elderly males. *J Pharm Sci* **71:**1415–1418.

Chan KK, Vyas KH, and Brandt KD (1987) In vitro protein binding of diclofenac sodium in plasma and synovial fluid. *J Pharm Sci* **76:**105–108.

Ciraulo DA, Barnhill JG, and Jaffe JH (1988) Clinical pharmacokinetics of imipramine and desipramine in alcoholics and normal volunteers. *Clin Pharmacol Ther* **43:**509–518.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res (NY)* **10:**1093–1095.

Desoye G (1988) Error analysis in equilibrium dialysis: evaluation of adsorption phenomena. *J Biochem Biophys Methods* **17:**3–16.

Divoll M, Greenblatt DJ, Ochs HR, and Shader RI (1983) Absolute bioavailability of oral and intramuscular diazepam: effects of age and sex. *Anesth Analg* **62:**1–8.

Elfstrom J and Lindgren S (1978) Influence of bed rest on the pharmacokinetics of phenazone. *Eur J Clin Pharmacol* **13:**379–383.

Gammans RE, Mayol RF, and LaBudde JA (1986) Metabolism and disposition of buspirone. *Am J Med* **80:**41–51.

Greenblatt DJ, Allen MD, Harmatz JS, and Shader RI (1980) Diazepam disposition determinants. *Clin Pharmacol Ther* **27:**301–312.

Gross AS, Heuer B, and Eichelbaum M (1988) Stereoselective protein binding of verapamil enantiomers. *Biochem Pharmacol*. **37:**4623–4627.

Guentert TW, Holford NH, Coates PE, Upton RA, and Riegelman S (1979) Quinidine pharmacokinetics in man: choice of a disposition model and absolute bioavailability studies. *J Pharmacokinet Biopharm* **7:**315–330.

Hardy BG and Schentag JJ (1988) Lack of effect of cimetidine on the metabolism of quinidine: effect on renal clearance. *Inter J Clin Pharmacol Ther Toxicol* **26:**388–391.

Hengstler JG, Utesch D, Steinberg P, Platt KL, Diener B, Ringel M, Swales N, Fischer T, Biefang K, Gerl M, et al. (2000) Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction. *Drug Metab Rev* **32:**81–118.

Holford N (1998) *Clinical Pharmacokinetics: Drug Data Handbook*, 3rd ed, ADIS Press Limited Auckland, New Zealand.

Houston JB and Carlile DJ (1997) Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. *Drug Metab Rev* **29:**891–922.

Hughes IE, Ilett KF, and Jellett LB (1975) The distribution of quinidine in human blood. *Br J Clin Pharmacol* **2:**521–525.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, and Sugiyama Y (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* **73:**147–171.

Johansson KA, Appelgren C, Borg KO, and Elofsson R (1974) Binding of two adrenergic beta-receptor antagonists, alprenolol and H 93–26, to human serum proteins. *Acta Pharm Suec* **11:**333–346.

Lavé T, Coassolo P, and Reigner B (1999) Prediction of hepatic metabolic clearance based on interspecies allometric scaling techniques and in vitro-in vivo correlations. *Clin Pharmacokinet* **36:**211–231.

Li AP (2001) Screening for human ADME/Tox drug properties in drug discovery. *Drug Discov Today* **6:**357–366.

Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, and Silber PM (1999) Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability and drug-drug interaction potential. *Chem-Biol Interact* **121:**17–35.

Maguire KP, Burrows GD, Norman TR, and Scoggins BA (1980) Blood/plasma distribution ratios of psychotropic drugs. *Clin Chem* **26:**1624–1625.

McAllister RG Jr and Kirsten EB (1982) The pharmacology of verapamil. IV. Kinetic and dynamic effects after single intravenous and oral doses. *Clin Pharmacol Ther*. **31:**418–426.

Nagy A and Johansson R (1975) Plasma levels of imipramine and desipramine in man after different routes of administration. *Naunyn-Schmiedeberg's Arch Pharmacol* **290:**145–160.

Newton R, Broughton LJ, Lind MJ, Morrison PJ, Rogers HJ, and Bradbrook ID (1981) Plasma and salivary pharmacokinetics of caffeine in man. *Eur J Clin Pharmacol*. **21:**45–52.

Obach RS (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos* **27:**1350–1359.

Raaflaub J and Dubach UC (1975) On the pharmacokinetics of phenacetin in man. *Eur J Clin Pharmacol*. **8:**261–265.

Regardh CG, Borg KO, Johansson R, Johnsson G, and Palmer L (1974) Pharmacokinetic studies on the selective beta1-receptor antagonist metoprolol in man. *J Pharmacokinet Biopharm* **2:**347–364.

Regardh CG, Jordo L, Ervik M, Lundborg P, Olsson R, and Ronn O (1981) Pharmacokinetics of metoprolol in patients with hepatic cirrhosis. *Clin Pharmacokinet* **6:**375–388.

Remmel RP, Copa AK, and Angaran DM (1991) The effects of hemodilution, pH and protamine on lidocaine plasma protein binding and red blood-cell uptake in vitro. *Pharm Res* **8:**127–130.

Rialland L, Guyomard C, Scotte M, Chesne C, and Guillouzo A (2000) Viability and drug metabolism capacity of alginate-entrapped hepatocytes after cryopreservation. *Cell Biol Toxicol* **16:**105–116.

Roberts MS and Rowland M (1986) Correlation between in-vitro microsomal enzyme activity and whole organ hepatic elimination kinetics: analysis with a dispersion model. *J Pharm Pharmacol* **38:**177–181.

Shibata Y, Takahashi H, and Ishii Y (2000) A convenient in vitro screening method for predicting in vivo drug metabolic clearance using isolated hepatocytes suspended in serum. *Drug Metab Dispos* **28:**1518–1523.

Steinberg P, Fischer T, Kiulies S, Biefang K, Platt KL, Oesch F, Bottger T, Bulitta C, Kempf P, and Hengstler J (1999) Drug metabolizing capacity of cryopreserved human, rat and mouse liver parenchymal cells in suspension. *Drug Metab Dispos* **27:**1415–1422.

Vesell ES, Passananti GT, Glenwright PA, and Dvorchik BH (1975) Studies on the disposition of antipyrine, aminopyrine, and phenacetin using plasma, saliva and urine. *Clin Pharmacol Ther* **18:**259–272.

Walle T, Walle UK, Cowart TD, and Conradi EC (1989) Pathway-selective sex differences in the metabolic clearance of propranolol in human subjects. *Clin Pharmacol Ther* **46:**257–263.

Willis JV, Kendall MJ, and Jack DB (1980) A study of the effect of aspirin on the pharmacokinetics of oral and intravenous diclofenac sodium. *Eur J Clin Pharmacol* **18:**415–418.

Wilson TW, Firor WB, Johnson GE, Holmes GI, Tsianco MC, Huber PB, and Davies RO (1982) Timolol and propranolol: bioavailability, plasma concentrations and beta blockade. *Clin Pharmacol Ther* **32:**676–685.

Wing LM, Miners JO, Birkett DJ, Foenander T, Lillywhite K, and Wanwimolruk S (1984) Lidocaine disposition–sex differences and effects of cimetidine. *Clin Pharmacol Ther* **35:** 695–701.