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 preparations from different subjects. The prediction generally resulted in a marked underestimation when the biologically based scaling factor (3.1 × 10³ 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 × 10⁶ (cells/kg) from individual preparations and 10.8 × 10⁶ (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 from different subjects for more reliable and quantitative prediction in vivo metabolic activity in humans.

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 × 10⁶ cells/ml for naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, and timolol; 2 × 10⁶ cells/ml for quinidine, caffeine, propranolol, diclofenac, and phenacetin; and 5 × 10⁶ 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% CH₃CN 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% O₂/5% CO₂ 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-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 (PerkinElmerSciei Instruments, Boston, MA) with a turbo ion spray interface. Multiple reaction monitoring of positive-ion mode was used for all analyses. Methodological studies including the selection of ions and parameters for multiple reaction monitoring were automatically obtained for each compound by the application software, Analyst (PerkinElmerSciei 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 262.2 > 116.3, diclofenac 295.9 > 215.2, and phenacetin 180.1 > 138.0. A fast-gradient condition using two switching valves and pumps (3.5 ml/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 pressure of 1 ml/min. The effluent was split with 0.2 ml/min, and 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 with acetonitrile/water (90:10) containing 10 mM ammonium acetate was the initial condition used. 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 (CLint, in vitro) was calculated from the following equation by using cell density (D), incubation time (T, 120 min for the calculation of CLint, in vitro), and the ratio (R) of unchanged compound concentration at time T to that at time 0 when the undrung drug concentration was much lower than its Km value (Shibata et al., 2000); CLint, in vitro = (\(-\log_{10} R/(D \times T)\)). To extrapolate the in vitro clearance to the in vivo value, the empirical scaling factor (average SF10–73) 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 SF70, 73 = 10.8 × 10³ cells/kg of body weight and used for the extrapolation as follows: CLint, in vitro, 70 + 73 = CLint, in vitro, 70 + 73 × average SF70, 73. The in vitro hepatic intrinsic clearance and in vivo 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 (Fp) for high clearance drugs was previously reported (Iwatsubo et al., 1997). The hepatic clearance (CLH, p, in vivo) was predicted from the obtained in vitro hepatic intrinsic clearance (CLH, int, in vitro, 70 + 73) by using the following equation (eq. 1) with the dispersion model (Iwatsubo et al., 1997); CLH, predicted, 70 + 73 = Qh × Rb × (1 - 4a/(1 + a²)) × exp[a · (1/2 × Dp)]

\[-(a - 1/4) × \exp\left[-(a + 1)/(2 × Dp)\right]\]

where Rb = (CLH, int, in vitro, 70 + 73)/(Qh × Rb) and a = (1 + 4 × Rb × Dp)⁻⁰.⁵

Fp was further calculated from Fp = 1 – hepatic extraction ratio (Ep) = 1 - CLH/(Qh × Rb). In these equations, the liver blood flow rate (Qh) and dispersion number (Dp) 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 (Rb) 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 (CLH, in vivo) and oral bioavailability (Fpo, in vivo) are equal to the hepatic metabolic clearance (CLH) and Fp, respectively. In vivo values for hepatic intrinsic clearance (CLH, int, in vivo) of standard compounds were calculated from Fpo, 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, CLint, 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 CLint, in vitro was observed.
### 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 [Gammians et al. (1986)]; verapamil [Ginos et al. (1986); 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 Caradaj (1988)]; metoprolol [Johansson et al. (1974); Regardh et al. (1974); Regardh et al. (1981)]; timolol [Wilson et al. (1982); Holford (1998)].

<table>
<thead>
<tr>
<th>No.</th>
<th>compound</th>
<th>f_b</th>
<th>R_b</th>
<th>Cl_int, in vivo</th>
<th>F_blood</th>
<th>Cl_int, in vivo, mean</th>
<th>SF_mean</th>
<th>Cl_int, predicted, mean</th>
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<th>Cl_int, predicted, 10^7 cells/kg</th>
<th>F_blood, predicted, 10^7 cells/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naloxone</td>
<td>0.559</td>
<td>1.22*</td>
<td>0.02</td>
<td>24.8</td>
<td>154.9</td>
<td>34.7 ± 8.2</td>
<td>4.5</td>
<td>25.3 ± 0.1</td>
<td>0.00 ± 0.00</td>
<td>23.8 ± 0.7</td>
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<tr>
<td>2</td>
<td>Buspirone</td>
<td>0.050</td>
<td>0.81*</td>
<td>0.04 ± 0.04</td>
<td>21.8</td>
<td>79.1</td>
<td>12.1 ± 6.9</td>
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<tr>
<td>3</td>
<td>Verapamil</td>
<td>0.100</td>
<td>0.77</td>
<td>0.20 ± 0.12</td>
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average SF_mean 8.5 ± 4.0

f_b, unbound fraction in plasma; R_b, blood-to-plasma concentration ratio (reported); Cl_int, in vivo, plasma clearance in humans (reported); F_blood, oral bioavailability in humans (reported); Cl_int, in vivo, hepatic intrinsic clearance values calculated from F_blood, in vivo, by the 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, 70<i>, in vivo, mean; Cl_int, in vitro, for 10 individual lots; Cl_int, predicted, mean, predicted hepatic clearance from Cl_int, in vitro, 70<i>, and average SF_mean (8.5 x 10^7 cells/kg) as a scaling factor; F_blood, predicted, mean, predicted hepatic availability from Cl_int, in vitro, 70<i>, and SF_mean (8.5 x 10^7 cells/kg) as a scaling factor; F_blood, predicted, 10^7 cells/kg, predicted hepatic availability from Cl_int, in vitro, mean, and biologically based scaling factor of hepatic availability (SF_fixed = 3.1 x 10^7 cells/kg).

* In house data.

### TABLE 2

Profiles of 14 compounds tested with the pooled preparation of human hepatocytes lots 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 [Gammians et al. (1986)]; verapamil [Ginos et al. (1986); 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 Caradaj 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 [Ellis and Lindgren (1978); Vesell et al. (1975)]; diazepam [Divillo et al. (1983); Greenblatt et al. (1980); Maguire et al. (1980)]; quinidine [Guenet 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 [Chen et al. (1988); Obach (1999); Willins et al. (2000)]; phenacetin [Raalwath and Dubach (1975); Vesell et al. (1975)].

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average SF_mean 10.8 ± 2.4

f_b, unbound fraction in plasma; R_b, blood-to-plasma concentration ratio (reported); Cl_int, in vivo, plasma clearance in humans (reported); F_blood, oral bioavailability in humans (reported); Cl_int, in vivo, hepatic intrinsic clearance values calculated from F_blood, in vivo, by the dispersion model using the Goal Seek method attached to Microsoft Excel; D, cell density of hepatocytes suspended in serum; SF_mean, mean of scaling factor calculated from Cl_int, in vitro, 70<i>, in vivo, mean; Cl_int, in vitro, for 10 individual lots; Cl_int, predicted, mean, predicted hepatic clearance from Cl_int, in vitro, 70<i>, and average SF_mean (10.8 x 10^7 cells/kg) as a scaling factor; F_blood, predicted, mean, predicted hepatic availability from Cl_int, in vitro, 70<i>, and average SF_mean (10.8 x 10^7 cells/kg) as a scaling factor.

* In house data.
among 10 cryopreserved preparations of human hepatocytes. The prediction of CL\textsubscript{H, int, in vivo} resulted in a marked underestimation when the biologically based scaling factor \((3.1 \times 10^9 \text{ cells/kg})\) was used to extrapolate CL\textsubscript{H, int, in vitro} to CL\textsubscript{H, int, in vivo} (Fig. 1). The empirical scaling factor \((3.1 \times 10^9 \text{ cells/kg})\), which was approximately 3 times larger than that of biologically based value \((3.1 \times 10^9 \text{ cells/kg})\), was used for the extrapolation.

The averaged results from 10 cryopreserved 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) suspended in serum appeared to provide more reliable predictions for the human liver metabolism, whereas it was less convenient and cost-effective.

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., 1999). 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 \((8.5 \times 10^9 \text{ cells/kg})\) from 10 individual preparations in Table 1; \(10.8 \times 10^9 \text{ cells/kg}\) from pooled preparations in Table 1) were approximately 3 to 4 times larger than the anatomically calculated value \((3.1 \times 10^9 \text{ cells/kg})\). In addition, the variation of 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.
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


