0090-9556/00/2812-1518–1523$03.00/0
DRUG METABOLISM AND DISPOSITION
DMD 28:1518–1523, 2000

Vol. 28, No. 12
87/868486
Printed in U.S.A.

A CONVENIENT IN VITRO SCREENING METHOD FOR PREDICTING IN VIVO DRUG METABOLIC CLEARANCE USING ISOLATED HEPATOCYTES SUSPENDED IN SERUM

YOSHIHIRO SHIBATA, HIROYUKI TAKAHASHI, AND YASUYUKI ISHII

Drug Metabolism, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan

(Received April 29, 2000; accepted September 14, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

A novel and convenient in vitro method for predicting in vivo metabolic clearance in the liver (CL_H) was developed. The CL_H of a drug is usually predicted by using both the unbound fraction in serum and the intrinsic hepatic clearance of the unbound fraction, but this procedure is labor-intensive. We simplified the method by directly measuring intrinsic hepatic clearance using isolated rat hepatocytes suspended in rat serum and called this "the serum incubation method". Sixteen commercially available compounds reported to be mainly excreted by liver metabolism were evaluated using our method. The remaining ratio of the unchanged drug after incubation was measured to calculate the rate of metabolism, and then CL_H was predicted based on the dispersion model. The predicted CL_H values of the drugs estimated by the serum incubation method were in good agreement with their in vivo plasma clearance values. In addition, the intrinsic hepatic clearance values obtained by the serum incubation method were comparable with those obtained by conventional methods. Furthermore, oral bioavailability values were equal to or lower than hepatic availability values predicted from the serum incubation method. These results indicate that compounds showing poor oral bioavailability can be excluded before in vivo pharmacokinetic study by using this method. In conclusion, the serum incubation method is a convenient and useful tool at the early stage of drug discovery.

The importance of pharmacokinetic studies at the early stage of drug discovery is increasing, and the progress of chemical synthetic techniques such as combinatorial chemistry and other parallel syntheses is increasing the number of candidates to be evaluated. As in vivo pharmacokinetic studies are time-consuming and labor-intensive, an in vitro approach for the quantitative prediction of in vivo parameters is desirable as a primary screen. Since avoidance of first-pass metabolism is one of the important issues in developing an orally available drug, a screening method that could predict metabolic hepatic clearance and/or availability would be useful in the drug discovery stage. Theoretical backgrounds for in vitro to in vivo extrapolation of hepatic clearance of a drug have been developed (Pang and Rowland, 1977; Rane et al., 1977; Wilkinson, 1987), and successful prediction of in vivo hepatic metabolic clearance has been reported (Houston, 1994; Houston and Carlile, 1997; Iwatsubo et al., 1997). Parameters such as unbound fraction in serum (f_{ub}[1]) and intrinsic hepatic clearance of unbound fraction ($CL_{H,\ u\ int}$), which can be obtained under experimental conditions that properly reflect the situation in vivo, are necessary for this type of prediction, making the procedure labor-intensive for screening many compounds at the early stage of drug discovery. Therefore, we developed a more convenient method, called "the serum incubation method", that can quantitatively predict in vivo metabolic clearance in the liver. The method involves direct measurement of intrinsic hepatic clearance ($CL_{H,\ int}$) of a drug by using serum as an incubation medium for hepatocytes, which allows a prediction of hepatic clearance (CL_H) and hepatic availability (F_H) without considering serum protein binding.

In this method, we used isolated rat hepatocytes to reflect $CL_{H,\ u\ int}$ and rat serum to reflect f_{ub}. Hepatocytes possess not only phase I metabolic activity but also phase II metabolic activity in the liver and cell membranes in which drug transporters are functioning. In these respects, isolated hepatocytes are regarded as a more appropriate in vitro source for reflecting metabolic clearance in the liver than are liver microsomes, which mainly represent the microsomal phase I metabolic activity (Houston, 1994; Houston and Carlile, 1997; Iwatsubo et al., 1997). In addition, serum contains major drug-binding proteins. From these considerations, a method using hepatocytes and serum would be ideal for prediction. In this report, we describe the usefulness of our method for the prediction of in vivo CL_H and F_H of drug candidates.

Experimental Procedures

Chemicals. Ibuprofen and 7-ethoxy coumarin were purchased from Aldrich (Milwaukee, WI). Tolbutamide and (*S*)-warfarin were obtained from Salford Ultrafine Chemical and Research Ltd. (Manchester, England). Alprenolol,

[1] Abbreviations used are: f_{ub}, unbound fraction in serum; CL, clearance; $CL_{u\ int,\ WE}$, intrinsic clearance of unbound fraction obtained from incubation in WE; $CL_{int,\ serum}$, intrinsic clearance obtained from incubation in serum; CL_H, hepatic clearance; $CL_{H,\ u\ int}$, hepatic intrinsic clearance of unbound fraction; $CL_{H,\ u\ int,\ WE}$, hepatic intrinsic clearance of unbound fraction obtained from incubation in WE; $CL_{H,\ int}$, hepatic intrinsic clearance; $CL_{H,\ int,\ WE}$, hepatic intrinsic clearance obtained from incubation in WE; $CL_{H,\ int,\ serum}$, hepatic intrinsic clearance obtained from incubation in serum; CL_p, plasma clearance; DMSO, dimethyl sulfoxide; D_N, dispersion number; E_H, hepatic extraction ratio; F_H, hepatic availability; F_{po}, oral bioavailability; SF, scaling factor; Q_H, hepatic blood flow rate; R, ratio of intact drug remaining after incubation; R_B, blood to plasma concentration ratio; WE, William's E medium (pH 7.4).

Send reprint requests to: Yoshihiro Shibata, Drug Metabolism, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Okubo 3, Techno-park Oho, Tsukuba, Ibaraki 300-2611, Japan.

quinidine, propranolol and trypsin inhibitor (Type II-S: Soybean) were obtained from Sigma Chemical Co. (St. Louis, MO). Lidocaine, theophylline and hexobarbital were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Antipyrine, caffeine, chlorpromazine, diazepam, phenytoin, and collagenase were obtained from Wako Pure Chemical Industries (Osaka, Japan). Compounds X and Y were synthesized in our laboratories. William's E medium was from Life Technologies (Grand Island, NY). All other chemicals were obtained from either Wako Pure Chemical Industries (Osaka, Japan) or Junsei Chemical Co. (Tokyo, Japan).

Serum Preparation. Male Sprague-Dawley rats, 7 to 10 weeks of age, purchased from Charles River Japan, Inc. (Kanagawa, Japan), were used in all of the experiments. Rat blood was collected via the abdominal aorta under ether anesthesia. After stabilization for 3 h at room temperature to coagulate the blood, samples were centrifuged (10 min, 1800*g*). Serum was collected as the clear supernatant and stored at −80°C until use.

Hepatocyte Preparation. Hepatocytes were freshly isolated from rats by a procedure similar to that described by Baur et al. (1975). After isolation, the hepatocytes were suspended in William's E medium (WE), pH 7.4, and kept on ice until use. Cell viability was routinely checked by the 0.4% Trypan Blue exclusion test, and only hepatocytes with viability greater than 90% were used in this study.

Effect of Serum on Antipyrine Metabolism. Incubation media containing 0, 25, 50, and 100% serum were prepared by mixing rat serum and William's E medium (v/v). Hepatocytes were resuspended at a density of 2×10^6 cells/ml in each incubation medium at ice-cold temperature. An aliquot of DMSO solution of 12.5 mM antipyrine was pipetted at a volume of 1 μl per well into a 48-well plate. Each hepatocyte suspension was added at a volume of 250 μl per well at ice-cold temperature (the final concentration of antipyrine was 50 μM, DMSO was 0.4%). The final antipyrine concentration was set lower than its Michaelis-Menten constant, K_m = 2.2 mM (Buters and Reichen, 1990). The samples were incubated at 37°C with shaking at 150 rpm under an atmosphere of 95% O_2/5% CO_2. After 0.5-, 1-, and 2-h incubation times, the reaction was terminated by adding 500 μl of ice-cold CH_3CN/MeOH solution (2:1, v/v). The samples were centrifuged (10,000*g* × 10 min), and the amount of antipyrine remaining in the supernatant was measured by HPLC-UV (254 nm) (SPD10A; Shimadzu, Tokyo, Japan).

Serum Incubation Method. The metabolism studies were typically performed as follows. Hepatocytes were resuspended at a density of 1×10^6 cells/ml in rat serum at ice-cold temperature. An aliquot of DMSO solution of a compound was pipetted at a volume of 1 μl per well into a 48-well plate with the exception of (*S*)-warfarin, which was added as an aliquot of water solution. The hepatocyte suspension was added in a volume of 250 μl per well at ice-cold temperature (final concentration of DMSO: 0.4%). The samples were incubated at 37°C with shaking at 150 rpm under an atmosphere of 95% O_2/5% CO_2. After a 1-h incubation, the reaction was terminated by adding 500 μl of ice-cold CH_3CN/MeOH solution (2:1, v/v). The samples were centrifuged (10,000*g* × 10 min), and the amount of the compound remaining in the supernatant was measured by HPLC-UV or liquid chromatography-tandem mass spectrometry. For low-clearance compounds such as antipyrine, caffeine, ibuprofen, theophylline, tolbutamide, and (*S*)-warfarin, cell density and incubation time were modified to 3×10^6 cells/ml and 2 h, respectively. All substrate concentrations were set lower than their Michaelis-Menten constants (K_m values). Compounds with unknown K_m values, such as chlorpromazine, propranolol, verapamil, compound X, compound Y, and phenobarbital, were incubated at 1 μM, while theophylline was incubated at 5 μM.

Conventional Hepatocyte Incubation Method. Hepatocytes were resuspended at a density of 0.2×10^6 cells/ml in William's E medium, pH 7.4, at ice-cold temperature. Incubation and subsequent steps were performed as described under *Serum Incubation Method*. For low-clearance compounds such as antipyrine, caffeine, ibuprofen, theophylline, tolbutamide, and (*S*)-warfarin, cell density and incubation time were changed to 1×10^6 cells/ml and 2 h, respectively. Compounds with unknown K_m values, such as chlorpromazine, verapamil, and phenobarbital, were incubated at 1 μM, while propranolol was incubated at 0.2 μM, compounds X and Y at 0.1 μM, and theophylline at 5 μM.

Theory and Calculation

The in vitro clearance of a drug is commonly expressed as follows:

$$CL = \text{rate of metabolism}/C_E \quad (1)$$

where C_E = the unbound drug concentration available at the enzyme site.

When the C_E of a drug is much lower than its K_m value, the CL is calculated by the following eq. 2, using cell density (D), incubation time (T), and the ratio of unchanged compound remaining after incubation (R).

$$CL = (-\log_e R)/(D \times T) \quad (2)$$

Derivation of eq. 2: when the cell density (D) is used for an in vitro metabolism study, the rate of metabolism (dC/dt) at the concentration C(t) is expressed from eq. 1 as follows:

$$dC/dt = -CL \times D \times C(t)$$

By solving this differential equation, the amount of drug remaining after the incubation time (T) is expressed as follows:

$$C(T) = C(0) \times e^{(-CL \times D \times T)}$$

As C(T)/C(0) = R, the equation is converted to $R = e^{(-CL \times D \times T)}$; then applying $\log_e$ to both sides, eq. 2 is produced.

When the compound is metabolized by hepatocytes suspended in William's E medium (the conventional incubation method), the intrinsic clearance of unbound fraction in William's E medium ($CL_{u\ int,\ WE}$) is calculated by eq. 2. Also, when an incubation is performed with hepatocytes suspended in serum (the serum incubation method), the intrinsic clearance in serum ($CL_{int,\ serum}$) is also calculated by eq. 2. To scale these in vitro clearance values up to in vivo liver values, the hepatocyte number per kilogram of body weight calculated from a report by Houston (1994) is used as a scaling factor (SF). The calculation is as follows:

SF = liver weight × hepatocyte number per gram of liver = 45 (g/kg of body weight) × 1.35×10^8 (cells/g of liver) = 6075×10^6 (cells/kg).

Then, $CL_{H,\ int}$ is calculated as follows:

In the case of the conventional method:

$$CL_{H,\ u\ int,\ WE} = CL_{u\ int,\ WE} \times SF \quad (3)$$

By multiplying the unbound fraction in serum (f_{ub}, measured value),

$$CL_{H,\ int,\ WE} = f_{ub} \times CL_{H,\ u\ int,\ WE} \quad (4)$$

In the case of the serum incubation method, without using the f_{ub} value:

$$CL_{H,\ int,\ serum} = CL_{int,\ serum} \times SF \quad (5)$$

CL_H is predicted from $CL_{H,\ int,\ WE}$ or $CL_{H,\ int,\ serum}$ by using the dispersion model:

$$CL_H = Q_H \times R_B \times (1 - 4a/((1 + a)^2 \exp[(a - 1)/(2 \times D_N)] - (1 - a)^2 \exp[-(a + 1)/(2 \times D_N)]))$$

$$R_N = (CL_{H,\ int,\ (WE\ or\ serum)})/(Q_H \times R_B) \quad a = (1 + 4 \times R_N \times D_N)^{1/2} \quad (6)$$

$$F_H = 1 - E_H = 1 - CL_H/(Q_H \times R_B) \quad (7)$$

where Q_H is liver blood flow rate (70 ml/min/kg) (an average value from the literature: Lin et al., 1982; Houston, 1994), D_N is dispersion

TABLE 1

Effects of serum on antipyrine metabolic activity in isolated rat hepatocytes

Results are expressed as the mean ± S.D. (%) of three incubations. Cell density, 2.0×10^6 cells/ml; incubation medium used, William's E medium, pH 7.4. Remaining (%) antipyrine after incubation.

Incubation Time (h)	Serum Content			
	0%	25%	50%	100%
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.5	96 ± 0	95 ± 0	94 ± 1	96 ± 1
1	93 ± 0	90 ± 1	89 ± 0	92 ± 2
2	83 ± 1	78 ± 1	77 ± 1	80 ± 2

number (0.17) (Roberts and Rowland, 1986), and R_B is blood to plasma concentration ratio (in compounds with unknown R_B values, a unity is used).

Results

Effects of Serum on Antipyrine Metabolic Activity in Isolated Rat Hepatocytes. The effects of serum on the intrinsic metabolic potency of isolated rat hepatocytes were investigated using antipyrine as a test compound because antipyrine was reported not to bind to serum proteins ($f_{ub} = 1$; Singh et al., 1991). Aliquots of serum were added to the incubation medium (William's E medium) containing hepatocytes to give 0, 25, 50, and 100% serum. The metabolism of antipyrine in these incubation media was studied as described under *Experimental Procedures*.

No marked difference was observed in the metabolic rate of antipyrine in the different incubation media (Table 1). Moreover, a linear metabolic reaction was observed over the 2 h following the start of the reaction. These observations demonstrate that serum has no effect on the intrinsic metabolic potency of antipyrine and can be used as an incubation medium for suspended isolated rat hepatocytes, at least during short-term metabolism studies.

Prediction of Metabolic Clearance and Availability in Liver by the Serum Incubation Method. Eighteen compounds eliminated mainly by liver metabolism, including 16 commercial and 2 novel compounds, were evaluated by the serum incubation method in terms of prediction of CL_H and F_H in the liver. The following parameters of the compounds are listed in Table 2: Michaelis-Menten constant (K_m), f_{ub}, in vivo plasma clearance (CL_p), and oral bioavailability (F_{po}). In vivo $CL_{H, int}$ was calculated by the dispersion model with the assumption that CL_p was the same as the metabolic clearance. Incubation was performed as described under *Experimental Procedures*. The following parameters were calculated by the equations described under *Theory and Calculation*: $CL_{int, serum}$, $CL_{H, int, serum}$, CL_H, and F_H. As shown in Fig. 1, a good correlation ($r^2 = 0.94$) was observed between the predicted CL_H and in vivo CL_p. Low-clearance drugs ($CL_p < 15$ ml/min/kg), such as antipyrine, caffeine, ibuprofen, phenytoin, tolbutamide, theophylline, and (*S*)-warfarin, were predicted to be low-clearance compounds. Moderately cleared drugs ($15 < CL_p < 40$ ml/min/kg), such as compounds X and Y, were predicted to be moderate-clearance compounds. Moreover, drugs showing high clearance ($CL_p > 40$ ml/min/kg), such as alprenolol, chlorpromazine, diazepam, 7-ethoxy coumarin, hexobarbital, lidocaine, propranolol, quinidine, and verapamil, were predicted to be high-clearance compounds. These results indicate that the serum incubation method is able to predict in vivo hepatic clearance with appropriate rank order.

Figure 2 shows the correlation between the predicted F_H and in vivo F_{po} values. Compounds such as alprenolol, hexobarbital, lidocaine, propranolol, and verapamil, defined as having low oral bioavailability in vivo ($F_{po} < 0.2$), were predicted to have poor hepatic availability because of extensive hepatic metabolism (predicted $F_H < 0.2$). This finding indicates that compounds extensively metabolized by first-pass metabolism in the liver can be predicted by the serum incubation method. Also, compounds reported to have good oral bioavailability ($F_{po} > 0.7$), such as antipyrine, caffeine, ibuprofen, tolbutamide, and theophylline, were predicted to have good hepatic availability (predicted $F_H > 0.7$). However, phenytoin was also predicted to have $F_H > 0.7$, while the in vivo F_{po} is reported as 0.26 (Scriba et al., 1995). Phenytoin was reported to show poor absorption in the intestine because of low solubility (Scriba et al., 1995). For such compounds with insufficient availability in the intestine, F_{po} cannot be predicted only by F_H because F_{po} is calculated as the product of F_H and intestinal availability. Therefore, for compounds with excellent availability in the intestine, the serum incubation method predicts in vivo F_{po} values in rank order.

In conclusion, these results indicate that the serum incubation method can be used to predict in vivo CL_H and maximum F_{po} values without the f_{ub} value.

Comparison with the Conventional Method. Conventional hepatocyte incubation was performed as described under *Experimental Procedures*. The following parameters were calculated by the equations described under *Theory and Calculation*: $CL_{int, WE}$, $CL_{H, u int, WE}$, and $CL_{H, int, WE}$. The correlation coefficient value in this section is calculated based on low- to moderate-clearance compounds with in vivo CL_p values under 40 ml/min/kg [phenytoin, compounds X and Y, antipyrine, caffeine, ibuprofen, tolbutamide, theophylline, and (*S*)-warfarin] to focus on the relationship of intrinsic clearance and free fraction in serum.

Figure 3A shows the correlation between in vivo $CL_{H, int}$ and in vitro $CL_{H, u int, WE}$ ($r^2 = 0.55$). Because f_{ub} was not reflected, the values of $CL_{H, u int, WE}$ were greater than those of in vivo $CL_{H, int}$, especially in the case of compounds with high serum protein binding ($f_{ub} < 0.02$), such as ibuprofen, tolbutamide, and (*S*)-warfarin, while the values of compounds with low serum protein binding, such as antipyrine, caffeine, theophylline, and hexobarbital, were in good agreement. On the other hand, when $CL_{H, u int, WE}$ was converted to $CL_{H, int, WE}$ by multiplying the f_{ub}, the values were well correlated with in vivo $CL_{H, int}$ (Fig. 3B, $r^2 = 0.86$). These observations suggest that the f_{ub} value is necessary for precise prediction of in vivo hepatic clearance if the conventional incubation method is adopted.

Figure 3C shows the correlation between in vivo $CL_{H, int}$ and in vitro $CL_{H, int, serum}$, which was directly predicted by the serum incubation method. As in Fig. 3B, a good correlation was observed between the in vitro and in vivo values ($r^2 = 0.98$). This finding shows that the serum incubation method gives comparable prediction results without considering the f_{ub}.

Discussion

Many methods for predicting the in vivo metabolic hepatic clearance of drugs from in vitro values have been reported. These methods usually require two independent in vitro parameters, f_{ub} and $CL_{H, u int}$ (Houston, 1994; Houston and Carlile, 1997; Iwatsubo et al., 1997). Because we used isolated rat hepatocytes to reflect $CL_{H, u int}$ and rat serum to reflect f_{ub}, parameters of in vitro $CL_{H, u int}$ obtained from incubation with isolated hepatocytes and f_{ub} are essential to predict in vivo metabolism.

However, the conventional methodology is inadequate as a screening technique in the early stage of drug discovery because independent measurements of these two parameters are time-consuming. Moreover, it is difficult to measure f_{ub} and/or $CL_{H, u int}$ accurately. Although f_{ub} is usually obtained by ultrafiltration or equilibrium dialysis methods, some compounds adsorb to the membrane and/or

TABLE 2

Pharmacokinetic parameters and K_m values for the metabolism of the compounds tested

All values were quoted from the literature as follows: alprenolol (Borg et al., 1974; Skanberg, 1980); chlorpromazine (Sato and Koshiro, 1995a,b); diazepam (Igari et al., 1984; Tsang and Wilkinson, 1982; Zomorodi et al., 1995); 7-ethoxy coumarin (Carlile et al., 1998); hexobarbital (Igari et al., 1982; Rane et al., 1977; Sawada et al., 1985; Van der Graaff et al., 1985; Vermeulen et al., 1991); lidocaine (Kawai et al., 1985; Nakamoto et al., 1997; Shibasaki et al., 1988; Supradist et al., 1984); phenytoin (Ashforth et al., 1995; Colburn and Gibaldi, 1977; Scriba et al., 1995; Tsuru et al., 1982); propranolol (Iwamoto and Watanabe, 1985; Singh et al., 1991; Terao and Shen, 1983); quinidine (Rakhit and Mico, 1985; Watari et al., 1989); verapamil (Bhatti and Foster, 1997; Manipisitkul and Chiou, 1993); antipyrine (Buters and Reichen, 1990; Pollack et al., 1984; Singh et al., 1991; Torres-Molina et al., 1992); caffeine (Bonati et al., 1984, 1985; Hayes et al., 1995); ibuprofen (Kata and Tekle, 1992; Satterwhite and Boudinot, 1991); tolbutamide (Ashforth et al., 1995; Sugita et al., 1981; Yamao et al., 1994); theophylline (Gomita et al., 1991; Matthew and Houston, 1990; Ramzan and Levy, 1987); (*S*)-warfarin (Baars et al., 1990; Pohl et al., 1976).

No.	Compound	K_m Value[a]	f_{ub}	R_B	i.v. Dose	CL_p	In Vivo $CL_{H,\ int}$[c]	F_{po}
		μM			*mg/kg*		*ml/min/kg*	
1	Alprenolol	9.5		1.71[b]	2	79	148	0.04
2	Chlorpromazine		0.10	1.48[b]	4	76	162	
3	Diazepam	3	0.22	1.04	1.5	52	108	0.35
4	7-Ethoxy coumarin	0.8	0.22	0.98	(infusion)	48	97	
5	Hexobarbital	105	0.62	1.00	25	62	195	0.18
6	Lidocaine	1.7[a]	0.38	1.27	10	88	591	0.01
7	Phenytoin	4.6	0.18	0.99	10	13	15	0.26
8	Propranolol		0.075	0.78	1.5	62		0.08
9	Quinidine	2.6[a]	0.20	1.87	5	93	193	
10	Verapamil		0.054	0.85	1	40	78	0.06
11	Compound X		0.038[b]		1	32[b]	46	0.46[b]
12	Compound Y		0.067[b]		1	36[b]	56	0.10[b]
13	Antipyrine	2200	1.00	1.00	20	3.6	3.7	0.74
14	Caffeine	150	0.90		2.5	13	14	1.00
15	Ibuprofen	70[a]	0.02		2.5	2.8	2.9	0.90
16	Tolbutamide	650	0.031	0.75	13	0.5	0.5	0.90
17	Theophylline		0.72		3.25	3.7	3.8	1.00
18	(*S*)-Warfarin	32	0.0112		2 (s.c.)	0.18	0.18	

[a] K_m values were obtained from incubation with hepatocytes except for Nos. 6, 9, and 15, which were obtained from microsomal incubation.
[b] In-house data.
[c] In vivo $CL_{H,\ int}$ was calculated by the dispersion model assuming that hepatic clearance was the same as CL_p. No. 8 was calculated to be infinite because the CL_p value was greater than the hepatic blood flow rate.

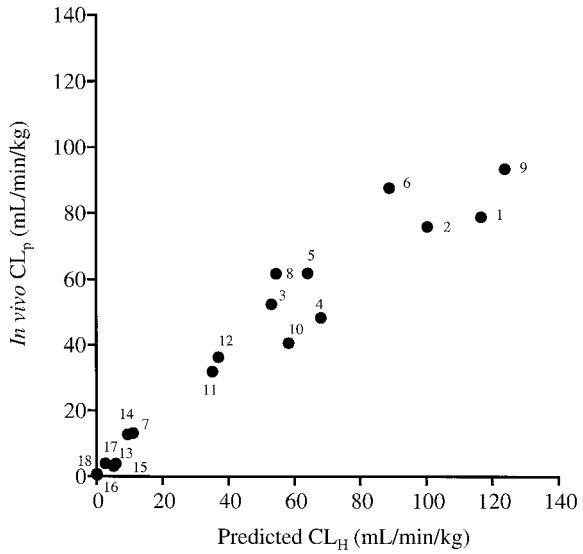

FIG. 1. *Prediction of metabolic clearance in the liver by the serum incubation method.*

CL_H was determined by the serum incubation method as described under *Experimental Procedures*. Each value represents the mean of three incubations. In vivo CL_p in rats was quoted from references. $r^2 = 0.94$ [1, alprenolol; 2, chlorpromazine; 3, diazepam; 4, 7-ethoxy coumarin; 5, hexobarbital; 6, lidocaine; 7, phenytoin; 8, propranolol; 9, quinidine; 10, verapamil; 11, compound X; 12, compound Y; 13, antipyrine; 14, caffeine; 15, ibuprofen; 16, tolbutamide; 17, theophylline; 18, (*S*)-warfarin].

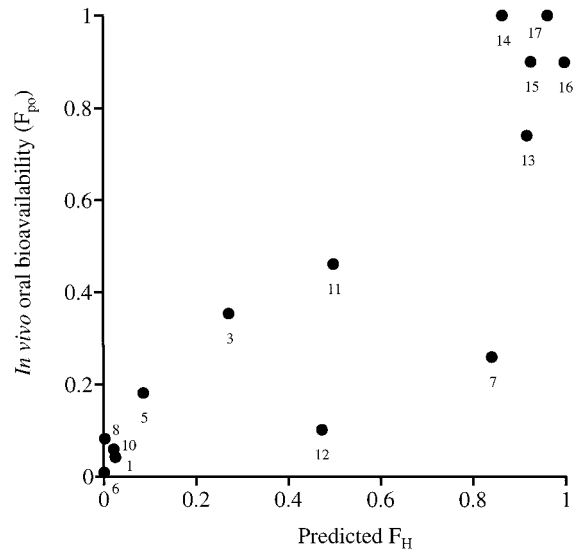

FIG. 2. *Comparison of F_H and in vivo oral bioavailability F_{po}.*

F_H was determined by the serum incubation method as described under *Experimental Procedures*. Each value represents the mean of three incubations. In vivo F_{po} in rats was quoted from references. [1, alprenolol; 3, diazepam; 5, hexobarbital; 6, lidocaine; 7, phenytoin; 8, propranolol; 10, verapamil; 11, compound X; 12, compound Y; 13, antipyrine; 14, caffeine; 15, ibuprofen; 16, tolbutamide; 17, theophylline].

apparatus, which causes an inaccurate estimation of the results (Bertilsson et al., 1979; Desoye, 1988). The same problem would also affect the calculation of $CL_{H,\ u\ int}$ because some drugs may not only adsorb to the apparatus but may also bind to microsomes or hepatocytes in the incubates (Obach, 1997, 1999). Therefore, for accurate calculation of $CL_{H,\ u\ int}$, the concentration of a drug in the incubate should be corrected as the free fraction of the drug (Obach, 1997, 1999). Thus, additional studies would be required for this purpose.

However, with the serum incubation method, direct measurement of $CL_{H,\ int}$ at the expected concentration can be performed without considering the unbound fraction in serum as demonstrated in Fig. 3C, and quantitative prediction of in vivo CL_H and F_H can be performed as shown in Figs. 1 and 2. Because the number of samples in the

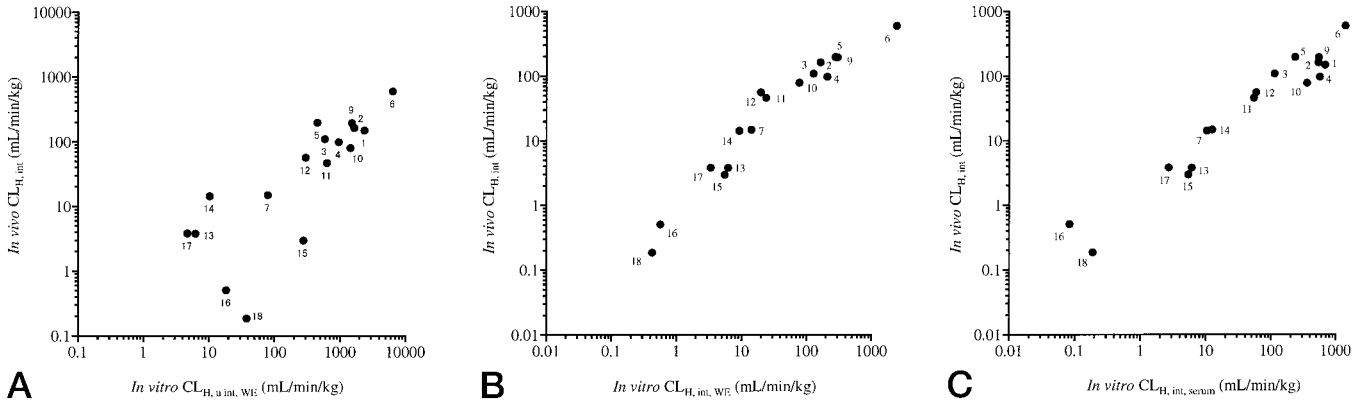

FIG. 3. *Comparison of in vitro and in vivo intrinsic clearance values obtained by the conventional and the serum incubation methods.*

A, $CL_{H,\ u\ int,\ WE}$ versus in vivo $CL_{H,\ int}$. B, $CL_{H,\ int,\ WE}$ versus in vivo $CL_{H,\ int}$. C, $CL_{H,\ int,\ serum}$ versus in vivo $CL_{H,\ int}$. In vivo $CL_{H,\ int}$ values were calculated by the dispersion model assuming that hepatic clearance was the same as in vivo CL_p. [1, alprenolol; 2, chlorpromazine; 3, diazepam; 4, 7-ethoxy coumarin; 5, hexobarbital; 6, lidocaine; 7, phenytoin; 9, quinidine; 10, verapamil; 11, compound X; 12, compound Y; 13, antipyrine; 14, caffeine; 15, ibuprofen; 16, tolbutamide; 17, theophylline; 18, (*S*)-warfarin]. Phenytoin, compounds X and Y, antipyrine, caffeine, ibuprofen, tolbutamide, theophylline, and (*S*)-warfarin are classified as low- to moderate-clearance compounds. A, $CL_{H,\ u\ int,\ WE}$ was compared with in vivo $CL_{H,\ int}$. In vitro $CL_{H,\ u\ int,\ WE}$ was determined using the conventional hepatocyte incubation method under serum-free conditions as described under *Experimental Procedures*. $r^2 = 0.55$ in low- to moderate-clearance compounds; $r^2 = 0.89$ in all compounds. B, $CL_{H,\ int,\ WE}$ was compared with in vivo $CL_{H,\ int}$. In vitro $CL_{H,\ int,\ WE}$ was calculated by multiplying $CL_{H,\ u\ int,\ WE}$ and f_{ub} as quoted from references. $r^2 = 0.86$ in low- to moderate-clearance compounds; $r^2 = 0.90$ in all compounds. C, $CL_{H,\ int,\ serum}$ was compared with in vivo $CL_{H,\ int}$. In vitro $CL_{H,\ int,\ serum}$ was determined by the serum incubation method as described under *Experimental Procedures*. $r^2 = 0.98$ in low- to moderate-clearance compounds; $r^2 = 0.85$ in all compounds.

analysis step is thereby reduced at least by half compared with the conventional method, we can obtain the prediction result much faster. In addition, serum can reduce the adsorption of compounds to the apparatus or to hepatocytes because serum proteins such as albumin are sometimes used to prevent adsorption of compounds to the apparatus. There are several reports of in vitro metabolism studies with hepatocytes or microsomes performed with media containing purified albumin or other serum proteins (Gariepy et al., 1992; Obach, 1997). But as in vivo serum includes many drug-binding proteins, it would be difficult to reflect in vivo f_{ub} correctly using only purified serum proteins. Lavé et al. (1997) ranked the compounds according to the ratio of hepatic extraction into three groups (low, middle, and high) after calculation of $CL_{H,\ u\ int}$ based on human hepatocyte suspensions, and their observations correspond fundamentally to those shown in Fig. 3A. Thus, the serum incubation method seems to be ideal for predicting in vivo metabolic CL_H and F_H, and the simplicity of the method may save considerable time and labor in the drug discovery stage. As far as we know, this is the first report to describe a method that can quantitatively predict in vivo metabolic CL_H and F_H using isolated hepatocytes directly suspended in serum.

It is true that serum protein binding improves F_H and CL_H, but the unbound drug concentration in serum also has significant effects on many aspects of pharmacodynamics and should also receive attention at the drug screening stage. Therefore, the f_{ub} should be confirmed by an appropriate method such as ultrafiltration or equilibrium dialysis after screening by the serum incubation method. The f_{ub} can be roughly estimated according to the difference in metabolic rate of $CL_{H,\ int,\ serum}$ versus $CL_{H,\ u\ int,\ WE}$ by a procedure similar to that reported by Gariepy et al. (1992). However, the relevance of this approach remains unclear because the activation of metabolism by serum protein was reported (Ludden et al., 1997), and adsorption of a drug to the apparatus and/or hepatocytes, especially in serum-free condition, should be considered. A high unbound fraction is desirable for accessibility to the target, but a low unbound fraction may not always be unfavorable if the drug has good pharmacokinetic properties and good pharmacodynamic effects, for the in vivo drug effect is also dependent on its intrinsic potency to the target.

When orally available compounds are screened in the drug discovery stage, the prediction of in vivo CL_H and/or F_H by the serum incubation method can facilitate the exclusion of undesirable candidates that have high clearance and low oral bioavailability due to extensive hepatic metabolism, without the performance of labor-intensive and time-consuming in vivo pharmacokinetic studies. In addition, when an in vivo pharmacokinetic result has already been obtained, predicted CL_H and F_H values obtained from in vitro experiments may help to estimate the contribution of hepatic metabolism to in vivo oral bioavailability and systemic clearance.

Conclusion

The serum incubation method appears to be more convenient than conventional methodology for predicting in vivo metabolic clearance and availability in the liver because these values are achieved without assessing f_{ub}. This method may be a useful tool for screening orally available compounds in the early stage of drug discovery.

Acknowledgment. We thank Dr. Allen Nielsen Jones, Merck Research Laboratories, for kindly reviewing the manuscript.

References

Ashforth EI, Carlile DJ, Chenery R and Houston JB (1995) Prediction of in vivo disposition from in vitro systems: Clearance of phenytoin and tolbutamide using rat hepatic microsomal and hepatocyte data. *J Pharmacol Exp Ther* **274:**761-766.

Baars LG, Schepers MT, Hermans JJ, Dahlmans HJ and Thijssen HH (1990) Enantioselective structure-pharmacokinetic relationships of ring substituted warfarin analogues in the rat. *J Pharm Pharmacol* **42:**861-866.

Baur H, Kasperek S and Pfaff E (1975) Criteria of viability of isolated liver cells. *Hoppe-Seyler's Z Physiol Chem* **356:**827-838.

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.

Bhatti MM and Foster RT (1997) Pharmacokinetics of the enantiomers of verapamil after intravenous and oral administration of racemic verapamil in a rat model. *Biopharm Drug Dispos* **18:**387-396.

Bonati M, Latini R, Tognoni G, Young JF and Garattini S (1984-85) Interspecies comparison of *in vivo* caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. *Drug Metab Rev* **15:**1355-1383.

Borg KO, Eklund B, Skanberg I and Wallborg M (1974) Metabolism of alprenolol in liver microsomes, perfused liver and conscious rat. *Acta Pharmacol Toxicol* **35:**169-179.

Buters JT and Reichen J (1990) Sex difference in antipyrine 3-hydroxylation. An *in vivo-in vitro* correlation of antipyrine metabolism in two rat strains. *Biochem Pharmacol* **40:**771-777.

Carlile DJ, Stevens AJ, Ashforth EI, Waghela D and Houston JB (1998) *In vivo* clearance of ethoxycoumarin and its prediction from *in vitro* systems. Use of drug depletion and metabolite formation methods in hepatic microsomes and isolated hepatocytes. *Drug Metab Dispos* **26:**216-221.

Colburn WA and Gibaldi M (1977) Plasma protein binding and metabolic clearance of phenytoin in the rat. *J Pharmacol Exp Ther* **203:**500-506.

Desoye G (1988) Error analysis in equilibrium dialysis: Evaluation of adsorption phenomena. *J Biochem Biophys Methods.* **17:**3-16.
Gariepy L, Fenyves D and Villeneuve JP (1992) Propranolol disposition in the rat: Variation in hepatic extraction with unbound drug fraction. *J Pharm Sci* **81:**255-258.
Gomita Y, Furuno K, Eto K, Okazaki M, Suemaru K and Araki Y (1991) Effect of cigarette smoking on theophylline pharmacokinetics in rats. *J Pharm Pharmacol* **43:**621-624.
Hayes KA, Brennan B, Chenery R, and Houston JB (1995) *In vivo* disposition of caffeine predicted from hepatic microsomal and hepatocyte data. *Drug Metab Dispos* **23:**349-353.
Houston JB (1994) Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. *Biochem Pharmacol* **47:**1469-1479.
Houston JB and Carlile DJ (1997) Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metab Rev* **29:**891-922.
Igari Y, Sugiyama Y, Awazu S and Hanano M (1982) Comparative physiologically based pharmacokinetics of hexobarbital, phenobarbital and thiopental in the rat. *J Pharm Biopharm* **10:**53-75.
Igari Y, Sugiyama Y, Sawada Y, Iga T and Hanano M (1984) *In vitro* and *in vivo* assessment of hepatic and extrahepatic metabolism of diazepam in the rat. *J Pharm Sci* **73:**826-828.
Iwamoto K and Watanabe J (1985) Dose-dependent presystemic elimination of propranolol due to hepatic first-pass metabolism in rats. *J Pharm Pharmacol* **37:**826-828.
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.
Kaka JS and Tekle A (1992) Bioavailability of ibuprofen from oral and suppository preparations in rats. *Res Commun Chem Pathol Pharmacol* **76:**171-182.
Kawai R, Fujita S and Suzuki T (1985) Simultaneous quantitation of lidocaine and its four metabolites by high-performance liquid chromatography: Application to studies on *in vitro* and *in vivo* metabolism of lidocaine in rats. *J Pharm Sci* **74:**1219-1224.
Lavé T, Dupin S, Schmitt C, Valles B, Ubeaud G, Chou RC, Jaeck D and Coassolo P (1997) The use of human hepatocytes to select compounds based on their expected hepatic extraction ratios in humans. *Pharm Res* **14:**152-155.
Lin JH, Sugiyama Y, Awazu S and Hanano M (1982) Physiological pharmacokinetics of ethoxybenzamide based on biochemical data obtained *in vitro* as well as on physiological data. *J Pharmacokinet Biopharm* **10:**649-661.
Ludden LK, Ludden TM, Collins JM, Pentikis HS and Strong JM (1997) Effect of albumin on the estimation, *in vitro*, of phenytoin V_{max} and K_m values: Implications for clinical correlation. *J Pharmacol Exp Ther* **282:**391-396.
Manitpisitkul P and Chiou WL (1993) Intravenous verapamil kinetics in rats: Marked arteriovenous concentration difference and comparison with humans. *Biopharm Drug Dispos* **14:** 555-566.
Matthew DE and Houston JB (1990) Drug metabolizing capacity in vitro and in vivo. I. Correlations between hepatic microsomal monooxygenase markers in beta-naphthoflavone-induced rats. *Biochem Pharmacol* **40:**743-749.
Nakamoto T, Oda Y, Imaoka S, Funae Y and Fujimori M (1997) Effect of phenobarbital on the pharmacokinetics of lidocaine, monoethylglycinexylidide and 3-hydroxylidocaine in the rat: Correlation with P450 isoform levels. *Drug Metab Dispos* **25:**296-300.
Obach RS (1997) Nonspecific binding to microsomes: Impact on scale-up of *in vitro* intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab Dispos* **25:**1359-1369.
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.
Pang KS and Rowland M (1977) Hepatic clearance of drugs. I. Theoretical considerations of a 'well-stirred' model and a 'parallel tube' model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *J Pharmacokinet Biopharm* **5:**625-653.
Pohl LR, Nelson SD, Porter WR, Trager WF, Fasco MJ, Baker FD and Fenton JW 2nd (1976) Warfarin-stereochemical aspects of its metabolism by rat liver microsomes. *Biochem Pharmacol* **25:**2153-2162.
Pollack GM, Li RC and Shen DD (1984) Dose-dependent pharmacokinetics of antipyrine in the rat. *Res Commun Chem Pathol Pharmacol* **44:**69-81.
Rakhit A and Mico B (1985) Kinetics of microsomal metabolism of quinidine in rats. *Res Commun Chem Pathol Pharmacol* **49:**109-124.
Ramzan IM and Levy G (1987) Kinetics of drug action in disease states. XVIII. Effect of experimental renal failure on the pharmacodynamics of theophylline-induced seizures in rats. *J Pharmacol Exp Ther* **240:**584-588.
Rane A, Wilkinson GR and Shand DG (1977) Prediction of hepatic extraction ratio from *in vitro* measurement of intrinsic clearance. *J Pharmacol Exp Ther* **200:**420-424.
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.
Satterwhite JH and Boudinot FD (1991) Effects of age and dose on the pharmacokinetics of ibuprofen in the rat. *Drug Metab Dispos* **19:**61-67.
Sato S and Koshiro A (1995a) Protein binding of chlorpromazine *in vivo* and *in vitro*: Effect of chlorpromazine metabolite on chlorpromazine protein binding in rat. *Biol Pharm Bull* **18:**586-592.
Sato S and Koshiro A (1995b) Pharmacokinetic analysis of chlorpromazine in rat serum, cerebrospinal fluid and striatum. *Biol Pharm Bull* **18:**593-599.
Sawada Y, Hanano M, Sugiyama Y and Iga T (1985) Prediction of the disposition of nine weakly acidic and six weakly basic drugs in humans from pharmacokinetic parameters in rats. *J Pharmacokinet Biopharm* **13:**477-492.
Scriba GK, Lambert DM and Poupaert JH (1995) Bioavailability of phenytoin following oral administration of phenytoin-lipid conjugates to rats. *J Pharm Pharmacol* **47:**945-948.
Shibasaki S, Kawamata Y, Ueno F, Koyama C, Itho H, Nishigaki R and Umemura K (1988) Effects of cimetidine on lidocaine distribution in rats. *J Pharmacobiodyn* **11:**785-793.
Singh K, Tripp SL, Dunton AW, Douglas FL and Rakhit A (1991) Determination of *in vivo* hepatic extraction ratio from *in vitro* metabolism by rat hepatocytes. *Drug Metab Dispos* **19:**990-996.
Skanberg I (1980) Metabolism of two beta-adrenoceptor antagonists, alprenolol and metoprolol, in different species. *Acta Pharm Suec* **17:**295-296.
Sugita O, Sawada Y, Sugiyama Y, Iga T and Hanano M (1981) Prediction of drug-drug interaction from in vitro plasma protein binding and metabolism. A study of tolbutamide-sulfonamide interaction in rats. *Biochem Pharmacol* **30:**3347-3354.
Supradist S, Notarianni LJ and Bennett PN (1984) Lignocaine kinetics in the rat. *J Pharm Pharmacol* **36:**240-243.
Terao N and Shen DD (1983) Effect of experimental renal failure on the disposition kinetics of l-propranolol in rats. *J Pharmacol Exp Ther* **227:**295-301.
Torres-Molina F, Aristorena JC, Garcia-Carbonell C, Granero L, Chesa-Jimenez J, Pla-Delfina J and Peris-Ribera JE (1992) Influence of permanent cannulation of the jugular vein on pharmacokinetics of amoxycillin and antipyrine in the rat. *Pharm Res* **9:**1587-1591.
Tsang CF and Wilkinson GR (1982) Diazepam disposition in mature and aged rabbits and rats. *Drug MetabDispos* **10:**413-416.
Tsuru M, Erickson RR and Holtzman JL (1982) The metabolism of phenytoin by isolated hepatocytes and hepatic microsomes from male rats. *J Pharmacol Exp Ther* **222:**658-661.
Van der Graaff M, Vermeulen NP and Breimer DD (1985) Route- and dose-dependent pharmacokinetics of hexobarbitone in the rat: A re-evaluation of the use of sleeping times in metabolic studies. *J Pharm Pharmacol* **37:**550-554.
Vermeulen AM, Rosseel MT and Belpaire FM (1991) High-performance liquid chromatographic method for the simultaneous determination of *R*-(−)- and *S*-(+)-hexobarbital in rat plasma. *J Chromatogr* **567:**472-479.
Watari N, Wakamatsu A and Kaneniwa N (1989) Comparison of disposition parameters of quinidine and quinine in the rat. *J Pharmacobiodyn* **12:**608-615.
Wilkinson GR (1987) Clearance approaches in pharmacology. *Pharm Rev* **39:**1-47.
Yamao T, Nakagami H, Furuhama K, Onodera T, Kurosaki Y, Nakayama T and Kimura T (1994) Pharmacokinetics of tolbutamide following intravenous and oral administrations in rats with obstructive jaundice. *Biol Pharm Bull* **17:**691-695.
Zomorodi K, Carlile DJ and Houston JB (1995) Kinetics of diazepam metabolism in rat hepatic microsomes and hepatocytes and their use in predicting *in vivo* hepatic clearance. *Xenobiotica* **25:**907-916.