Empirical validation of a rat in vitro organ slice model as a tool for in vivo clearance prediction

de Graaf, IAM; de Kanter, R; de Jager, MH; Camacho, R; Langenkamp, E; van de Kerkhof, EG; Groothuis, GMM

[Link to publication in University of Groningen/UMCG research database](#)

EMPIRICAL VALIDATION OF A RAT IN VITRO ORGAN SLICE MODEL AS A TOOL FOR IN VIVO CLEARANCE PREDICTION

Inge A. M. de Graaf, Ruben de Kanter, Marina H. de Jager, Raquel Camacho,
Elise Langenkamp, Esther G. van de Kerkhof, and Geny M. M. Groothuis

*Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration, Groningen, The Netherlands
(I.A.M.d.G., M.H.d.J., R.C., E.L., E.G.v.d.K., G.M.M.G.); and Nerviano Medical Sciences, Nerviano, Italy (R.d.K.)*

ABSTRACT:

Tissue slices have been shown to be a valuable tool to predict metabolism of novel drugs. However, besides the numerous advantages of their use for this purpose, some potential drawbacks also exist, including reported poor penetration of drugs into the inner cell layers of slices and loss of metabolic capacity during prolonged incubation, leading to underprediction of metabolic clearance. In the present study, we empirically identified (and quantified) sources of underprediction using rat tissue slices of lung, intestine, kidney, and liver and found that thin liver slices (±100 μm) metabolized model substrates (7-hydroxycoumarin, testosterone, warfarin, 7-ethoxycoumarin, midazolam, haloperidol, and quinidine) as rapidly as isolated hepatocytes. Furthermore, it was found that organ slices remain metabolically active for sufficient periods of incubation, enabling study of the kinetics of low clearance compounds. In addition, we determined the influence of albumin on the clearance prediction of six model substrates. For three of these substrates, the intrinsic clearance in the presence of albumin was approximately 3 times higher than that obtained from incubations without albumin, but corrected for unbound fraction. This resulted in a much more accurate prediction of in vivo whole body metabolic clearance for these compounds. Collectively, these results show that drawbacks of the use of slices for clearance prediction are largely surmountable. Provided that thin liver slices and physiological albumin concentration are used, whole body metabolic clearance is predicted with acceptable (2-fold) accuracy with organ slices. These results emphasize the applicability of organ slices in this field of research.

Many potential drug candidates are rejected in drug discovery because of undesirable in vivo pharmacokinetics. In vitro tools can serve to predict pharmacokinetics, enabling selection of lead compounds with favorable properties. A major advantage of the use of in vitro models is the opportunity to use human tissue for predictions, overcoming difficulties that may occur with extrapolation of animal data because of large interspecies differences in metabolism. Until recently, liver-derived models were solely used for this purpose, whereas only a few researchers have included in vitro models from extrahepatic organs into predictions (de Kanter et al., 2002; De Graaf et al., 2002; De Kanter et al., 2004). However, the intestine has especially been proven to be metabolically very active; moreover, its metabolism has been shown to be readily influenced by drug interactions on enzyme and transporter activity (Pelkonen et al., 2001). However, kidney [e.g., furosemide (Wallin et al., 1977)] and lung [e.g., naphthol (Mistry and Houston, 1985)] also may take part significantly in the metabolic clearance of some compounds.

Recently, we have developed an in vitro model using tissue slices

from liver, small intestine, colon, kidney, and lung to predict whole body metabolic clearance (De Kanter et al., 2004) and showed that, dependent on the substrate used, extrahepatic organs may account for a significant part of metabolic clearance of xenobiotics. An advantage of the use of slices for clearance prediction, which they share with isolated cells like hepatocytes, is that they exhibit the natural interplay between cofactors, transporters, and metabolizing enzymes that is lost when subcellular fractions are used. Unlike in hepatocytes, however, cell-to-cell contacts and normal tissue microarchitecture remain intact in slices, and they are prepared according to a rapid, reproducible, and relatively simple method (Krumdieck et al., 1980) from various organs and animal species. However, potential drawbacks also exist, including reported poor penetration of high turnover drugs into the inner cell layers of slices (Worboys et al., 1997) and loss of metabolic capacity during prolonged incubation (VandenBranden et al., 1998). Both these phenomena can potentially influence the predictability of metabolic clearance in a negative manner by introducing an underestimation of metabolic rate.

Another potential source of error in clearance prediction, which is not necessarily limited to the use of slices but is also relevant for other in vitro models, is calculation of in vivo organ clearances from in vitro intrinsic organ clearances. In this calculation, it should be taken into account that clearance in vivo is limited by blood flow and binding of the substrate to plasma proteins. Although it is generally accepted that

ABBREVIATIONS: 7-HC, 7-hydroxycoumarin; 7-EC, 7-ethoxycoumarin; L-15, Leibovitz's L-15 medium; WME, Williams' E medium; UW, University of Wisconsin organ preservation solution; BSA, bovine serum albumin; LC-MS, liquid chromatography/mass spectrometry; P450, cytochrome P450; CL_{int}, intrinsic clearance; $CL_{int,org}$, intrinsic metabolic organ clearance; CL_{org}, metabolic organ clearance; f_u, unbound fraction.

in vivo organ clearance is directly related to the free fraction of the compound in plasma, which has been confirmed in vitro (Shibata et al., 2000; Bachmann et al., 2003), in some publications it is reported that in vivo clearance is underpredicted in vitro when intrinsic clearance is corrected for plasma protein binding (Ludden et al., 1997; Blanchard et al., 2004).

In the present study, we empirically identified and designed strategies to eliminate potential sources of underprediction of in vivo clearance using tissue slices of rat lung, intestine, kidney, and liver. Intrinsic clearance of model compounds with variable intrinsic liver clearance [7-hydroxycoumarin (7-HC), testosterone, warfarin, 7-ethoxycoumarin (7-EC), midazolam, haloperidol, and quinidine] was compared between liver slices of various thicknesses and with freshly isolated hepatocytes. In this manner, the extent to which cells in the inner layers of liver slices take part in metabolic conversion of high and low clearance drugs was examined. Intrinsic slice metabolic clearances of 7-HC, testosterone, and 7-EC in organ slices also were determined after various periods of preincubation (0, 4, 8, and 24 h) to assess the stability of metabolism during prolonged culturing. In addition, organ slices were incubated with model compounds either without or in the presence of albumin in the incubation medium to determine the influence of the free fraction on calculated organ clearances. Finally, an improved in vitro method using organ slices for whole body metabolic clearance prediction is proposed.

Materials and Methods

Chemicals. Quinidine, haloperidol, 7-HC, warfarin, testosterone, Leibovitz's L-15 medium (L-15), insulin, trypan blue, and low gelling temperature agarose (type VII-A) were purchased from Sigma Chemie (Deisenhofen, Germany). Williams' E medium (WME) with glutamax-I, gentamicin, fungizone (amphotericin B), and penicillin/streptomycin were products from Invitrogen (Carlsbad, CA). Liver perfusion medium, liver digest medium, and glutamine also were from Invitrogen. Sodium thiopental was from Pfizer, Inc. (New York, NY). D-Glucose, NaHCO₃, and NaOH were purchased from Merck (Darmstadt, Germany). University of Wisconsin organ preservation solution (UW) (DuPont, Wilmington, DE) and midazolam were gifts from the University Medical Center Groningen, The Netherlands. Bio-Rad protein assay reagent was from Bio-Rad Laboratories GmbH (Munich, Germany), and 7-EC was from Fluka (Buchs, Switzerland). Hepes and bovine serum albumin (BSA; acid-free) were obtained from MP Biomedicals (Irvine, CA).

Preparation of Precision-Cut Organ Slices. Male Wistar rats (body weight between 300 and 350 g) (Harlan, Horst, The Netherlands) were housed in standard cages with food (Harlan chow) and tap water available ad libitum. They were anesthetized with isoflurane and N_2O/O_2 before organs were excised and placed in either ice-cold UW solution (lung, kidney, and liver) or in oxygenized ice-cold Krebs-Henseleit buffer supplemented with 10 mM Hepes, 25 mM D-glucose, and 25 mM NaHCO₃ (small intestine and colon). Subsequently, liver and kidney cores of 8 mm were prepared with a coring tool of 9-mm diameter (R&D Systems, Minneapolis, MN) attached to a drilling machine with a variable rotation speed (Kinzo, Ede, The Netherlands). For some experiments a coring tool of 6 mm was used to produce cores with a 5-mm diameter because in this manner the amount of liver and kidney tissue used for incubations was comparable with that of the other organ slices. No differences between metabolic rates between 8- and 5-mm slices were observed (data not shown). In this case, 12-well plates were used for incubations (see below). To prepare 8-mm cores of the lung, this organ was first filled with a 37°C solution of 1.5% (w/v) low melting agarose and 0.9% (w/v) NaCl via the trachea. The agarose then was allowed to solidify in ice-cold Krebs-Henseleit buffer.

From the small intestine, the first 25 cm measured from the stomach was rejected, and the following 15 cm, which corresponds to the jejunum, was cut into 3- to 4-cm pieces and flushed thoroughly with ice-cold Krebs-Henseleit buffer to remove its contents. The colon underwent the same treatment. The flushed parts then were filled with 3% (w/v) low melting agarose solution with

0.9% (w/v) NaCl of 37°C as described previously (de Kanter et al., 2005) and subsequently embedded in the same solution to produce agarose cylinders with a diameter of 16 mm using a tissue-embedding unit (R&D Systems).

From the organ cores, slices were cut using a Krumdieck tissue slicer (R&D Systems) filled with ice-cold and oxygenated Krebs-Henseleit buffer as described previously (De Kanter et al., 2004). Liver and kidney precision-cut slices with an 8-mm diameter were cut to have a wet weight of 10 to 15 mg, whereas lung slices were cut to have a wet weight of about 40 mg because less tissue is present as a result of the agarose filling. For some experiments, thickness and diameter of liver slices were varied. Slices of variable thicknesses were produced by varying the blade-to-tissue distance in the tissue slicer. Thickness was monitored indirectly by measuring the wet weight of five to six representative slices (with a fixed diameter). In this manner, for the experiments in which the influence of slice thickness on clearance was determined, five groups of slices with varying thicknesses were cut. Retrospectively, the number of cell layers of three slices of each group was estimated on histomorphological examination (see later). After preparation, slices were kept on ice-cold UW (lung, liver, and kidney slices) or Krebs-Henseleit buffer (colon and small intestinal slices) until incubation. The whole procedure from organ excision until incubation typically took maximally 2 h.

Incubation of Slices. Slices were cultured individually in six-well culture plates (Greiner Bio-One, Kremsminster, Austria) that were filled with WME (3.2 ml/well), supplemented with glucose (25 mM) and antibiotics/fungicides, and prewarmed inside a plastic box in an incubation cabinet under a constant flow of humidified 95% O_2/5% CO_2. Sometimes (when 5-mm liver and kidney slices were used, and/or in the experiments with albumin in the medium, where the reduction of volume/tissue ratio made substrate depletion more readily measurable) 12-well plates (Corning Life Sciences, Acton, MA) filled with 1.3 ml of medium per well were used. For liver, lung, colon, and intestinal slices, gentamicin (50 μg/ml) was used as antibiotic, whereas for kidney, penicillin (100 U/ml) and streptomycin (100 μg/ml) were used to avoid kidney slice toxicity by gentamicin. In addition, fungizone (2.5 μg/ml) was added as an antifungal agent to the medium used for slices from small intestine and colon. These agents are poorly metabolized themselves and therefore should not interfere with biotransformation. Model compounds were added to the medium directly in the beginning of the incubation as a 100 or 1000× stock solution (in methanol) to reach a final concentration of 1 or 2 μM. To test the influence of albumin on clearance, 4% albumin was added to the incubation medium when appropriate. In some experiments, slices were first preincubated in medium without the compounds. Before transferring into the culture plates (one slice/ well), liver, lung, and kidney slices were washed briefly (<1 min) in WME. As a control, compounds were incubated without slice to determine whether spontaneous degradation or adherence to the wells had occurred. During incubation, plates were shaken (90 times/min), whereas the slices were continuously submerged in the medium. (Medium) samples were taken at various time points and/or at the end of incubation.

Preparation of Hepatocytes. For the isolated hepatocyte studies, male Sprague-Dawley rats (200–220 g) from Charles River Italica (Calco, Italy) were maintained under a 12-h light/dark cycle in standard cages and bedding with free access to standard commercial food pellets and tap water. Although a different rat strain was used for the hepatocyte studies than for the slices (because these studies were performed in different laboratories), we do not expect this to influence the experimental outcome because previous (unpublished) studies with microsomes from Wistar and Sprague-Dawley rats within our laboratories did not show differences in metabolic rates of 20 tested compounds (including warfarin, quinidine, midazolam, and testosterone) between the two strains. After i.p. anesthesia with sodium thiopental (100 μg/kg), the liver was perfused in situ with prewarmed (37°C) and pregassed (95% O_2/5% CO_2) liver perfusion medium for about 5 min and then with prewarmed (37°C) and pregassed (95% O_2/5% CO_2) liver digest medium for about 10 min. Then the liver was excised and hepatocytes were harvested, by removing the capsule, into L-15 (4°C), pregassed (95% O_2/5% CO_2), containing 1 μM insulin and 2 mM glutamine and 1% BSA. After filtration, washing with L-15 (4°C), and centrifugation (four times at 50g for 5 min at 4°C), the viability of the hepatocytes was determined using the trypan blue (0.4%) exclusion method. Only preparations with viability greater than 80% were used. Hepatocytes were resuspended in L-15 at a trypan blue viable concentration of 10^6 cells/ml.

Incubation of Hepatocytes. Hepatocyte incubations were performed in 24-well plates under continuous shaking at 37°C under room atmosphere. In each well, 10^6 hepatocytes were incubated in a final volume of 1 ml of L-15 containing 1 μM insulin and 2 mM glutamine. The amount of solvent dimethyl sulfoxide present in the incubation was 0.1%. From each well containing hepatocytes, an aliquot of 80 μl of the incubation suspension was taken at 0, 5, 10, 20, 30, 45, 60, and 90 min.

Sample Analysis. Medium samples taken from the incubations without albumin were immediately mixed with an equal amount of ice-cold methanol (slices) or acetonitrile (hepatocytes). In case albumin was used in the incubation medium, samples were mixed at a ratio of 1:4 with methanol/acetonitrile to precipitate all the protein. The medium/solvent mixture was then centrifuged ($700g$ for 20 min at 4°C) and stored at −20°C until analysis. The supernatants then were used for liquid chromatography/mass spectrometry (LC-MS) analysis. To exclude substrate depletion by unspecific binding of compounds to the slice, in some cases substrate concentrations in slice incubations were also determined at the end of incubation in the slice/medium homogenate (obtained by sonification), which was then mixed with methanol and centrifuged (2 min, $2000g$). Because in previous studies testosterone was shown to bind to the slices, the latter approach was used as standard for this compound. The supernatant then was used for LC-MS analysis, whereas the precipitates were used to determine the protein content of the slices. For hepatocyte incubations, suspension samples were taken in all cases.

LC-MS Analysis. The samples were analyzed by LC-MS using a turbo ion spray source and a triple quadrupole API 2000 instrument (PerkinElmer Life and Analytical Sciences, Boston, MA). A 4.6 (inner diameter) × 12.5 mm C8 column (Agilent Technologies, Palo Alto, CA) was applied using a mobile phase containing 10 mM ammonium formate (pH 4.0) and acetonitrile increasing from 5% to 95% within 0.4 min and then back to 5% in 1.4 min. The flow rate was 1.5 ml/min for the first 0.2 min to equilibrate the column quickly, and the flow rate was reduced to 0.6 ml/min 0.2 min after injection. The eluent from the column was led into the mass spectrometry system only between 0.3 min and 1.05 min after injection of the sample. The ion spectra were acquired by injecting 20 μl of the sample, and quantification was performed by comparing the peak areas with authentic standards of each compound.

Protein Determination. Protein content of the samples was determined using a Bio-Rad protein assay dye reagent and a BSA standard curve. Slice protein was either determined (from the albumin-free incubations) after dissolving the precipitate left after methanol extraction (when homogenate samples were taken for LC-MS analysis, see above) or in slices taken at the end of incubation (when medium samples were used for LC-MS analysis, see above). Either the precipitate or the slices were dissolved in 5 M NaOH at 37°C for 30 min, sonicated, and subsequently diluted to 0.1 M NaOH. The Bio-Rad reagent then was added, and finally the protein content was determined by an enzyme-linked immunosorbent assay reader (ThermoMax microplate reader, Molecular Devices, Sunnyvale, CA) at 450 and 650 nm.

Calculation of Clearance. The intrinsic clearance (CL_{int}), which is defined as clearance without the physiological limitation by blood flow or plasma protein binding, such as is the case in the current in vitro situations, was determined according to the substrate depletion method as described previously (Houston, 1994; De Kanter et al., 2004). Briefly, CL_{int} was calculated by multiplying the slope (k) of the natural logarithm of the concentration of compound remaining against the time curve with the incubation volume and dividing this value by the number of hepatocytes in the incubation volume (eq. 1) or the protein content of the slices (eq. 2):

$$CL_{int} = \frac{-k \cdot volume}{number\ of\ hepatocytes} \quad (1)$$

$$CL_{int} = \frac{-k \cdot volume}{slice\ protein\ content\ (mg)} \quad (2)$$

For all the compounds it was found that the slope of the natural logarithmic concentration of compound remaining against time was constant when the initial concentration was 2 μM. This implies that the (overall) apparent metabolic K_m was ≫ 2 μM.

If in previous slice experiments metabolic rates had appeared to be constant and no aspecific adhesion to the slices had occurred, samples were taken only

TABLE 1

Determination of scaling factors

	Organ Weight	Organ Protein Content[a]	Scaling Factor[b]
	g/kg body weight	mg/g organ weight	
Liver	39.9	141	5660
Lung (agarose filled)	18.4	9	163
Kidney	7.7	170	1320
Small intestine	18.9	120	2258
Colon	2.9	122	354

[a] Determined by measuring the protein content of an organ sample with a known wet weight.
[b] Calculated by multiplying organ weight with the protein content per gram organ.

at the end of incubation instead of at different time points. Then k was determined from the slope of the natural logarithm of the initial concentration (in this case substrate incubated without a slice) and the end-concentration in the medium (or slice homogenate in the case of testosterone) against time.

CL_{int} obtained in this way (in ml/min/10^6 cells for hepatocytes or ml/min/mg protein for slices) then was up-scaled to obtain the whole organ intrinsic clearance ($CL_{int,\ org}$) by multiplying with a scaling factor. Scaling factors for slice data were calculated by multiplying the organ weight (per kilogram body weight) with the protein content (in milligrams) per gram organ wet weight (Table 1), thus obtaining the total organ protein content per kilogram rat. Scaling factors differ somewhat from previous data published by our laboratory (De Kanter et al., 2004) because of differences in procedures of solubilizing the protein from slices and organs. From data obtained from the hepatocyte incubations (ml/min/10^6 cells), intrinsic organ clearance (per kilogram body weight) was calculated assuming a hepatocellularity of 109 million hepatocytes/g liver (Carlile et al., 1997) and 39.9 g liver/kg body weight, which was determined previously in our laboratory (see Table 1).

To calculate metabolic organ clearance (CL_{org}) from $CL_{int,\ org}$, the organ blood flow and the unbound fraction of the compound in plasma in vivo (f_u) and the unbound fraction of the compound in the medium (f_u') in vitro were considered according to eq. 3, which is based on the venous equilibration model (or well-stirred model). For blood flow rates, see the previous report from our laboratory (De Kanter et al., 2004). The f_u' of the various compounds in plasma were obtained from literature data (references in Table 2). When slices were incubated without albumin, the compound was supposed to be unbound ($f_u' = 1$), whereas in the presence of 4% albumin, f_u' was supposed to be equal to f_u; therefore, f_u/f_u' becomes equal to 1 in the equation.

$$CL_{org} = \frac{blood\ flow \times f_u/f_u' \times CL_{int,org}}{blood\ flow + f_u/f_u' \times CL_{int,org}} \quad (3)$$

From individual organ clearances, the whole body clearance was calculated as described previously, taking into account the prehepatic extraction ratio on liver clearance (De Kanter et al., 2004).

Determination of ATP Content and Histomorphological Examination of Slices. For the experiments determining the influence of slice thickness on clearance, the ATP content of liver slices with various thicknesses was determined as described previously (de Kanter, 2005) with the only difference that the slice homogenate was centrifuged (at $16,000g$ for 2 min), and the supernatant was used for measuring the ATP content with a luminometer (Lumicounter; PerkinElmer Life and Analytical Sciences) instead of the homogenate. ATP content was determined relative to the protein content of the slices.

For histomorphological examination, slices were fixed in 70% ethanol and further processed as described previously (de Graaf et al., 2000). Sections were stained with H&E. The number of cell layers of which slices consisted was estimated by counting the number of cells in their cross-sections.

Results

Influence of Slice Thickness on Clearance. Intrinsic organ clearance of model compounds was determined with liver slices of several thicknesses and with hepatocytes in suspension. As shown in Fig. 1, there is a strong relationship between the wet weight and the protein content of liver slices (Fig. 1A) and the wet weight and the number of cell layers in a slice, as determined on histomorphological examina-

TABLE 2

The unbound fraction (f_u) and in vivo clearances of used compounds

	f_u	CL	Reference
		ml/min/kg	
Warfarin	0.11	0.18	Shibata et al., 2000
7-EC	0.22	40	Carlile et al., 1998
7-HC	0.10	97.4	Ritschel et al., 1992
Testosterone	0.40	62	Pardridge, 1986; De Kanter et al., 2004
Midazolam	0.04	79	Kotegawa et al., 2002
Quinidine	0.325	33.8	Sawada et al., 1985
Haloperidol	0.08	78	Lombardo et al., 2002; Cheng and Paalzow, 1992

tion (Fig. 1B). Therefore, wet weight was further used as a measure for slice thickness. As shown in Fig. 2, for all the compounds with a high (>blood flow) liver intrinsic clearance (midazolam, 7-EC, 7-HC, testosterone, quinidine, and haloperidol), $CL_{int, liver}$ was significantly lower (1.5–4 times) when calculated from slices with a "standard format" (9–12 mg wet weight/11 cell layers = ~220 μm) than when thin slices of 2 to 5 mg (5 cell layers = ~100 μm) were used.

$CL_{int, liver}$ calculated for these compounds with (thin) liver slices were not significantly different from those obtained with hepatocytes, indicating that metabolism was not limited by penetration of the compounds into these thin slices. $CL_{int, liver}$ of warfarin, a low turnover compound with $CL_{int, liver} \ll$ liver blood flow, was not different in slices of various thicknesses. Metabolism of warfarin was too slow to be measured during the 6-h culture period with hepatocytes in suspension. As indicated by slice ATP levels (Fig. 3), decrease of $CL_{int, org}$ calculated with thicker slices appeared not to be caused by a lower viability: standard slices of 9 to 12 mg had a somewhat higher ATP content per milligram protein than the thinnest slices, but this difference was not statistically different, although they exhibited a lower intrinsic clearance of most compounds.

Metabolic Activity during Prolonged Culturing. Intrinsic clearance of 7-HC, testosterone, and 7-EC by organ slices was determined after various periods (0, 4, 8, or 24 h) of preincubation. It appeared that intrinsic metabolic clearance of 7-HC (Fig. 4A), which is conjugated with glucuronide and sulfate, was unchanged during at least 24 h of incubation of liver, kidney, colon, and lung slices. Only in small intestinal slices was the clearance reduced to about 50% of initial values after 24 h of preincubation.

Testosterone, which in rat is metabolized via several cytochrome P450 isoenzymes (Arlotto et al., 1991) and 17β-hydroxysteroid dehydrogenase (Farthing et al., 1982), was cleared at constant rates in all the organs after various periods of preincubation (Fig. 4B). In contrast, slice intrinsic clearance of 7-EC (Fig. 4C) decreased to signif-

icantly lower values after 8 h (liver) and 24 h (liver and lung) of preincubation. In colon and small intestinal slices, metabolic rates tended to decrease as well, although the decrease was not statistically significant.

Influence of Albumin on Clearance Prediction. Clearance is limited in vivo by protein binding because only the free fraction (f_u) of a given compound can be taken up by the cell and metabolized. Therefore, intrinsic clearances in vitro should be corrected for protein binding in vivo when no protein is present in the incubations. This also means that for compounds that in vivo predominantly bind to albumin, intrinsic clearances found in vitro in the presence of 4% albumin should be lower, proportionally to f_u, than those of albumin-free incubations. In Fig. 5, intrinsic clearances of six model compounds from incubations with liver slices in the presence of 4% albumin are shown as a fraction of those obtained from albumin-free incubations (set to 1). According to the foregoing, this fraction should be equal to the literature f_u in vivo. In fact, this is the case for testosterone, quinidine, and 7-EC, but not for haloperidol, 7-HC, and midazolam. For the latter compounds, intrinsic clearances were approximately 3 times higher than expected based on protein binding data from literature. Correspondingly, liver clearances were also higher for these compounds when calculated from intrinsic clearances obtained from incubations in the presence of 4% albumin.

Strikingly, the whole body clearance, calculated with the venous equilibrium model from albumin-free incubations with the different organ slices, was underestimated for the same compounds (haloperidol, 7-HC, and midazolam), whereas the whole body clearance of testosterone, quinidine, and 7-EC was correctly predicted (Fig. 6). Importantly, when whole body clearance was calculated from organ slice incubations (using thin liver and kidney slices) with 4% albumin, predicted values were in the desired range of 0.5- to 2-fold in vivo values for *all* the compounds under study.
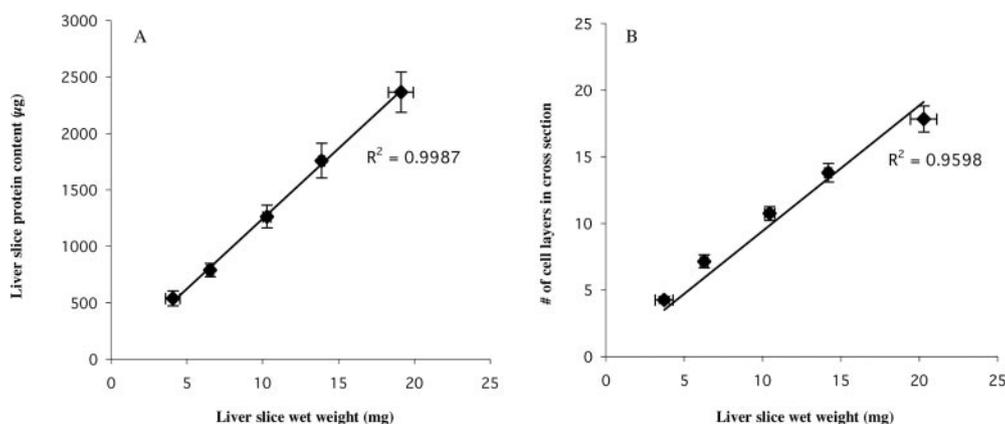
FIG. 1. Relation between slice wet weight and protein content (A) and slice wet weight and the number of cell layers in the slice cross-section (B). Values represent the mean of three (2- to 5-mg slices) to eight (all the other groups) experiments, three to five slices per experiment (±S.E.M.).
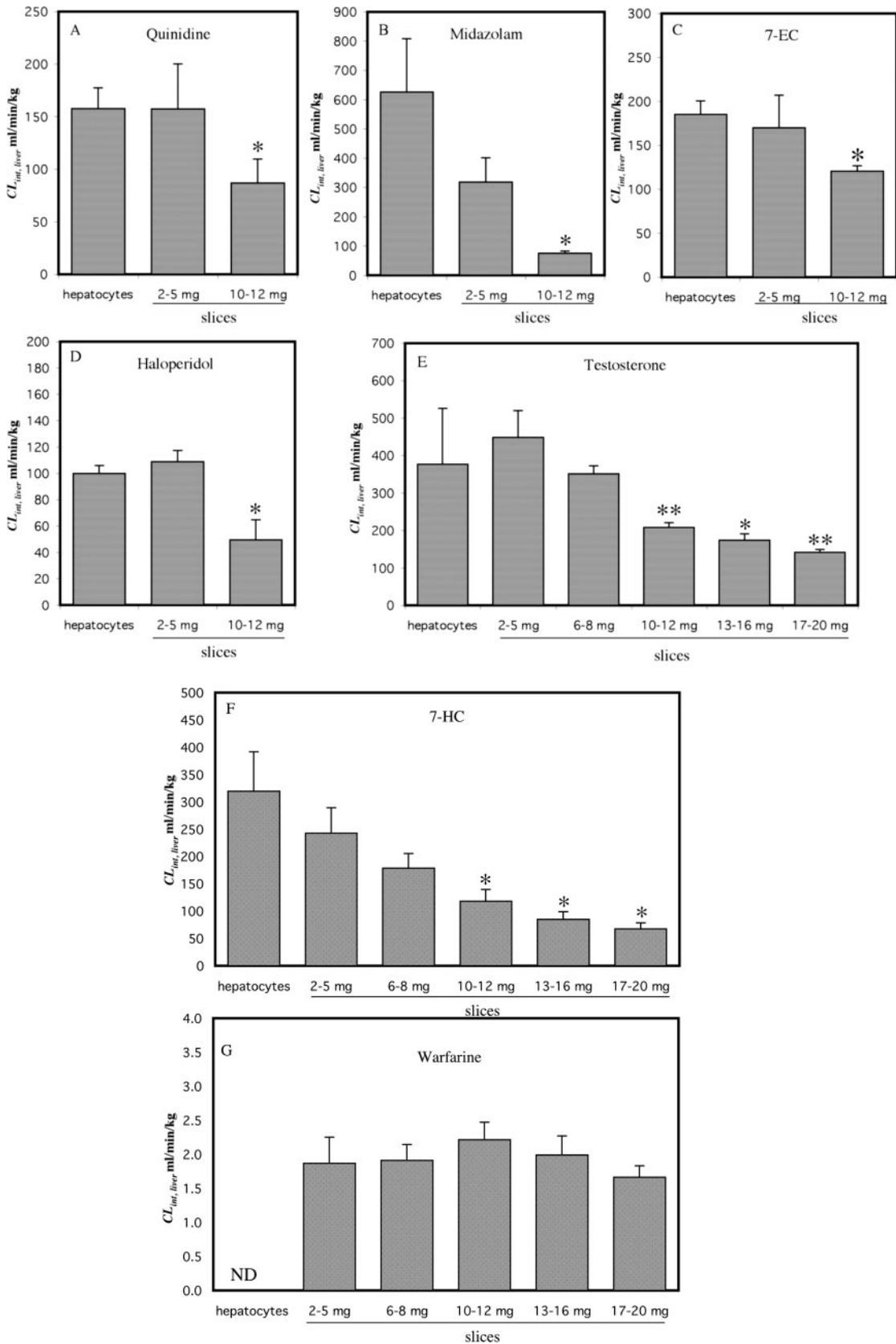
FIG. 2. CL$_{int, liver}$ of model compounds quinidine (A), midazolam (B), 7-EC (C), haloperidol (D), testosterone (E), 7-HC (F), and warfarin (G) calculated with hepatocytes or slices of several thicknesses. Bars represent the mean of three to eight experiments (+S.E.M.), three to five slices per substrate per experiment, one hepatocyte incubation per substrate per experiment. Slices were incubated with 1 μM substrate for 1 h (testosterone), 3 h (7-HC, 7-EC), 6 h (quinidine, haloperidol, and midazolam), or 24 h (warfarin). Hepatocytes were incubated for 6 h. Neither slices nor hepatocytes were preincubated. Medium samples were taken at various time points and/or at the end of incubation. ∗, values differ significantly ($p < 0.05$) from those obtained with the thinnest slices; ∗∗, values differ significantly with $p < 0.01$. ND (not detectable): CL$_{int, liver}$ was lower than 5 ml/min/kg, but the exact amount could not be determined because this compound was hardly metabolized by hepatocytes.
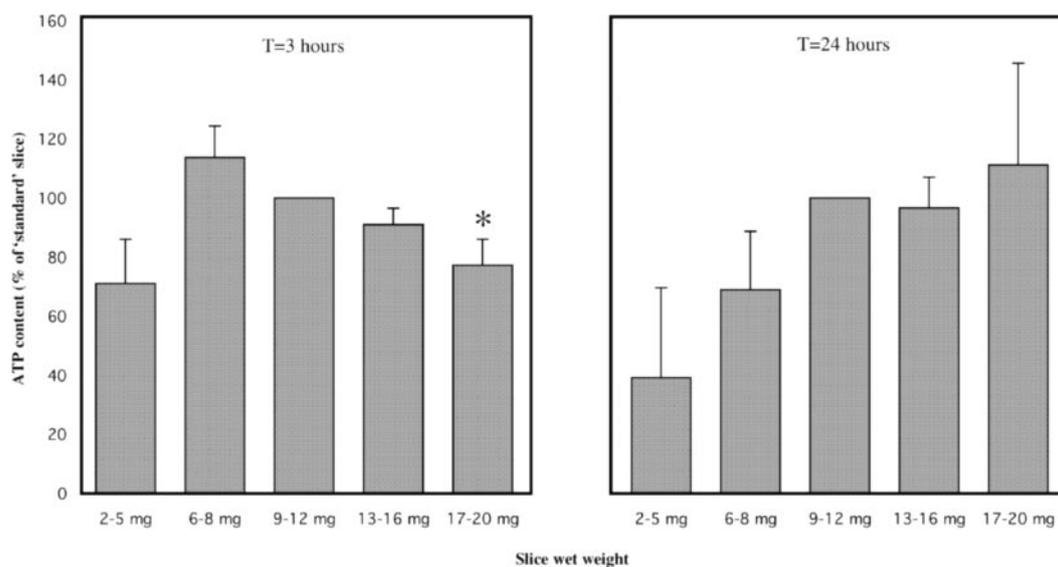
FIG. 3. ATP content of liver slices after 3 and 24 h of incubation. Values are given as percentage of the values of standard 9- to 12-mg liver slices (+S.E.M.) of three to eight experiments (three slices per experiment). Mean ATP content of slices with 9 to 12 mg wet weight was 6.2 ($\pm$0.6) nmol/mg protein after 3 h of incubation and 8.7 ($\pm$1.6) nmol/mg protein after 24 h of incubation. $*$, values differ significantly ($p < 0.05$) from those of the standard slices.
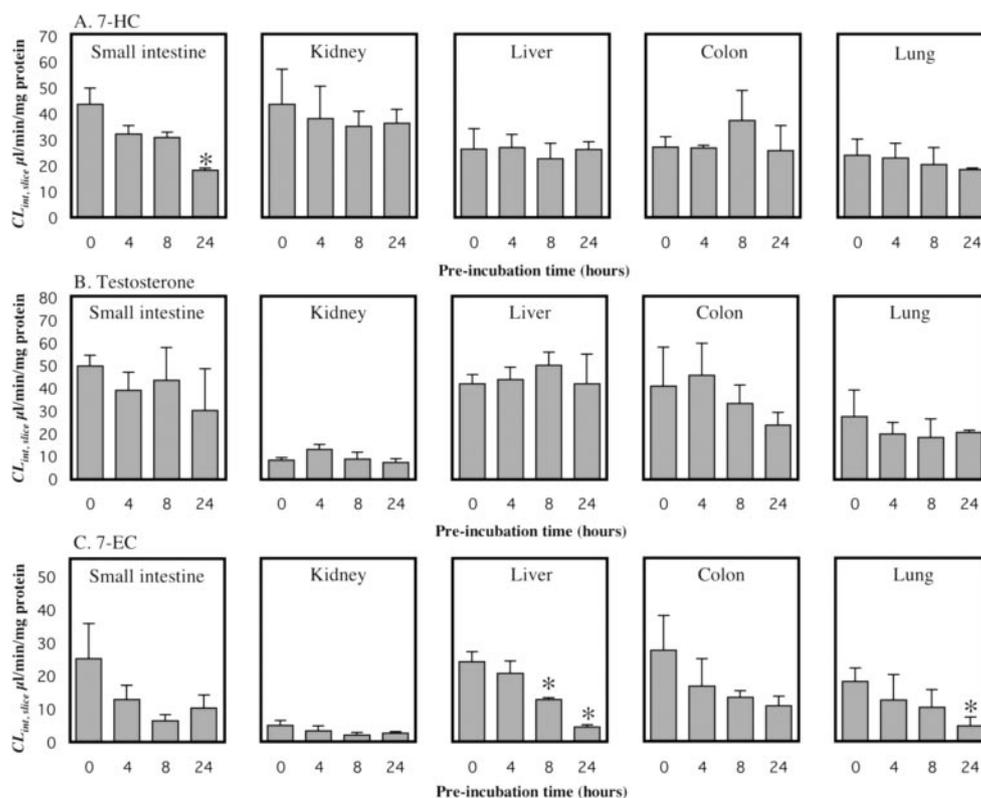

FIG. 4. Intrinsic clearance of model compounds 7-HC (A), testosterone (B), and 7-EC (C) by organ slices during prolonged culturing. Slices were preincubated 0, 4, 8, or 24 h before adding the drug (2 μM) and then 1 to 3 h with the drug. Samples of medium and/or medium-slice homogenate were taken at the end of incubation. Each bar represents at least three experiments, three to five slices per experiment (+S.E.M.). $*$, values differ significantly ($p < 0.05$) from those obtained without preincubation.

Discussion

The major advantage of using precision-cut slices for prediction of in vivo metabolic clearance is that metabolically active extrahepatic organs can easily be taken into account besides the liver. A disadvantage of slices is that not all the cells are in direct contact with the medium containing the drug, making it necessary for the drug to travel through the slice into the inner cell layers. This phenomenon may not play a role in intestinal and lung slices because metabolizing cells are

in direct contact with the easily permeable agarose gel, but metabolism may be limited to the outer cell layers in kidney and liver slices. Intrinsic clearances of drugs were reported to be structurally lower in liver slices than in hepatocytes (Worboys et al., 1997). It was suggested that this could be partly because of poor penetration of oxygen into the slice. However, although in the present study we also found, for most of the tested compounds found, a 2- to 4-fold lower CL_{int}/mg protein in thicker slices than in thinner slices (and hepatocytes), ATP
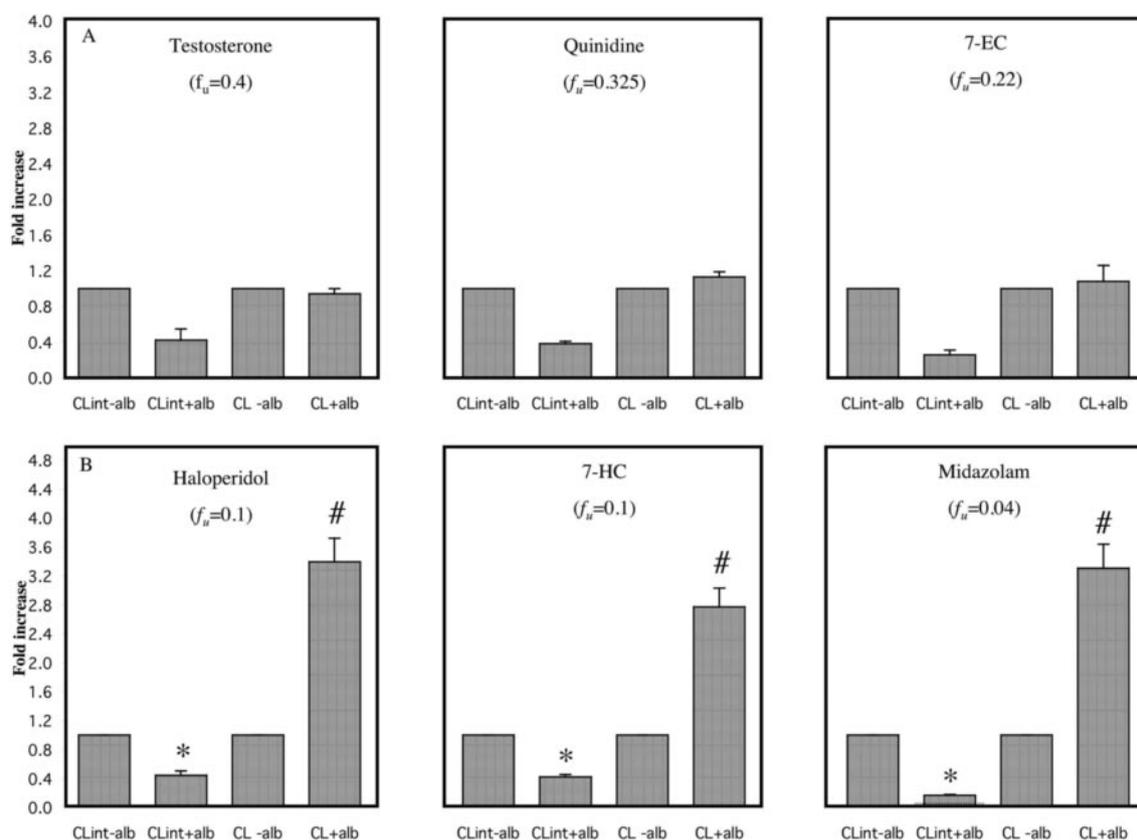
FIG. 5. The influence of the presence of albumin in the incubation on intrinsic clearance and clearance of model compounds (A) testosterone, quinidine, and 7-EC and (B) haloperidol, 7-HC, and midazolam. $CL_{int, liver}$ (without albumin) and CL_{liver} without albumin were normalized to 1. Values represent the mean of three to five experiments (+S.E.M.), three or four slices per experiment per substrate. Substrate concentration was 1 μM. Slices were incubated for 1 h (testosterone), 3 h (7-HC, 7-EC), or 6 to 8 h (quinidine, haloperidol, and midazolam) with the model compound. *, $CL_{int, liver}$ differs significantly ($p < 0.05$) from those obtained from incubations without albumin. #, CL_{liver} differs significantly ($p < 0.05$) from those obtained from incubations without albumin.

content of thick (approximately 20-cell layer) liver slices was not lower than that of thin (5-cell layer) slices. Moreover, the intrinsic clearance of warfarin was not different in thick and thinner slices, and no signs of cell damage in the inner cell layers of slices were observed histomorphologically (data not shown). Thus, comprised metabolic function by lack of oxygen seems not to be the cause of lower clearance in slices. It seems more likely that metabolism of lipophilic high and intermediate turnover drugs like testosterone, haloperidol, midazolam, quinidine, 7-EC, and 7-HC is limited by delayed penetration of the drug into thicker slices, preventing establishment of equilibrium concentrations. However, the solution for this problem seems obvious: when thin slices (~2–5 mg wet weight/100-μm thickness/4- to 6-cell layers) were used, CL_{int} was found to be equal or higher than in hepatocytes, thus preventing underprediction.

Stability of metabolic rate on culturing is a prerequisite to adequately predict metabolic clearance of low clearance compounds (such as warfarin) in vitro, for which up to 24 h of incubation is required to reliably measure substrate depletion. With hepatocytes in suspension, as used in the present study, viability is not sufficient (approximately 50% after 6 h of culturing) to accurately measure clearance of such compounds. With organ slices, metabolic activity toward testosterone [metabolized via several P450s (Arlotto et al., 1991) and 17β-hydroxysteroid dehydrogenase (Farthing et al., 1982)] was maintained during culturing for 24 h. Conjugation of 7-HC with glucuronide and sulfate was only significantly decreased in small intestinal slices after 24-h preincubation. Metabolism of 7-EC [via CYP1A1 (Zhang et al., 1997), CYP1A2, CYP2A1, and CYP2B1/2 (Bayliss et al., 1994)] decreased on incubation. Calculated over 24 h,

the mean activity toward 7-EC was minimally 50% of initial values. This implies that for highly stable compounds, clearance may be underestimated by maximally 50%, depending on the P450 isoform involved. The fact that conjugation reactions, which presumably are highly energy-consuming and require maintained cellular viability, were barely affected implies that slice quality is not the cause of this loss. Loss of P450 activity on culturing has been reported repeatedly using conventional (monolayer) cultures of isolated hepatocytes (Sherratt and Damani, 1989; Rogiers et al., 1990; Wortelboer et al., 1990; McMillan et al., 1991; Bayliss et al., 1994) and has been ascribed to activation of NO-synthesis as a result of collagenase treatment (Lopez-Garcia, 1998) and dedifferentiation of hepatocytes by loss of cell-to-cell or cell-to-matrix interactions. Indeed, culturing of hepatocytes in cocultures with other liver-derived cells and/or in combination with extracellular matrix has expanded the time course in which hepatocytes stay viable (Rogiers et al., 1990) and to some extent has also led to better preserved P450 levels (Koebe et al., 1994; Evans, 1995; Kern et al., 1997). However, damage by collagenase, loss of intercellular communication, and cell-matrix interactions cannot play a role in the loss of P450 activity in the present study and those of others (Wright and Paine, 1992; VandenBranden et al., 1998; Renwick et al., 2000) because no disruptive isolation procedure is used to prepare slices and cellular interactions are presumably maintained. The observed decrease of P450 activity toward 7-EC may be because of the loss of (endogenous or exogenous) inductive stimuli, which are constantly present at basal levels in vivo but not in vitro. Differential sensitivity of P450 isoenzymes to inactivation has been
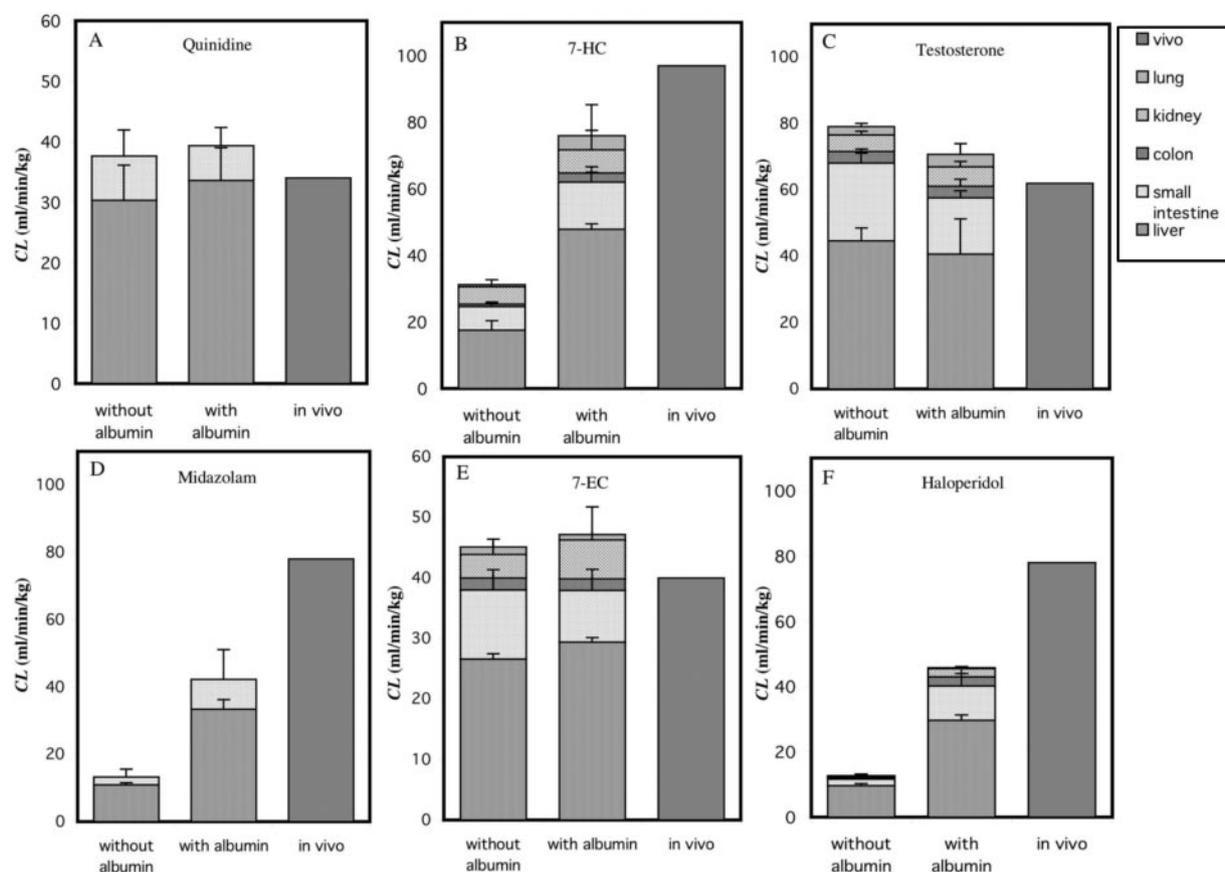
FIG. 6. Whole body clearances of model compounds quinidine (A), 7-HC (B), testosterone (C), midazolam (D), 7-EC (E), and haloperidol (F) calculated from incubations with or without albumin with organ slices compared with in vivo values. Values represent the mean of three to eight experiments (+S.E.M.), three or four slices per substrate per experiment. Slices were generally incubated with testosterone for 1 h (liver) to 3 h (other organs), with 7-HC for 3 h, with 7-EC for 3 h (liver) to 8 h (other organs), and with quinidine, haloperidol, and midazolam for 6 h (liver) to 8 h (other organs), substrate concentration 1 μM. In vivo data were from literature (see Table 2). For quinidine and midazolam, clearance in kidney, colon, and lung could not be detected.

reported before (Utesch et al., 1991; Renwick et al., 2000) and may be caused by differences in turnover times and/or inducibility.

Protein binding decreases the free concentration and thereby the intracellular concentration of drugs. Because the rate of metabolic conversion of drugs in concentrations under K_m is directly related to the concentration that is "seen" by the metabolic enzyme, metabolic rate is expected to decrease proportionally to the extent of protein binding in the presence of plasma proteins. After adding 4% albumin to the incubation medium, we observed, as expected, that the metabolic rate of model compounds was decreased. For testosterone, 7-EC, and quinidine, this decrease was exactly proportional to their literature f_u. However, CL_{int} of midazolam, 7-HC, and haloperidol was much less affected by albumin than was expected based on their extent of protein binding, resulting in an approximately 3 times higher predicted metabolic clearance for the latter compounds in the presence of albumin. Several explanations could account for this observation. First, BSA, which was used in the present study, might not exhibit the same drug-binding properties as rat plasma. This explanation may not be unlikely because plasma also contains other drug-binding proteins, although albumin is the most abundant. Besides this, drugs may bind with affinity to a rat serum albumin other than BSA. To exclude this for the model compounds used, we performed a pilot study in which we compared intrinsic clearance of these compounds in the presence of 20% rat plasma and an equivalent amount of 0.8% BSA. No differences were found, indicating that the use of BSA in the present study was valid (data not shown). Despite this validation, it seems to be more straightforward to use full plasma or serum in future studies

(Shibata et al., 2000). However, the latter method may be difficult to standardize because of the presence of other components (e.g., prostaglandins, growth factors) in serum. Future studies need to elucidate whether this may influence the reproducibility and predictive value of the assay.

A second explanation for our results may be that some compounds may nonspecifically bind to the slices, decreasing their free concentration in the incubation medium. Correcting CL_{int} for in vivo f_u could then have introduced an overcorrection. However, no difference in the concentration of either of these compounds between the incubation medium and the medium/slice homogenate at the end of incubation was found (unpublished observations), indicating that binding to slices was not sufficient to influence medium concentration. Protein "contamination," possibly caused by production of albumin by (liver) slices, "sticking" albumin left after slice preparation (organs were not perfused; therefore, some blood could be left), or release of proteins from dying cells can also be a reason for the culture medium mistakenly being considered as drug-binding protein-free. However, the presence of protein in the medium would imply an impact (proportional to the extent of protein binding) on the clearance of all the tested compounds, which was not the case. Another explanation may be that albumin facilitates (active) transport of a compound into metabolizing cells, thereby (partly) compensating its decreasing effect on intracellular drug concentration. An example is furosemide, of which uptake by specific anion transporters in the kidney is enhanced by albumin binding (Pichette et al., 1996). It has also been shown in vivo that

hepatic extraction of some (lipophilic) compounds (e.g., propanolol) (Gariepy et al., 1992) is not restricted by protein binding.

It has been suggested that the possibly enhancing effect of albumin on the uptake of drugs is rather aspecific and might be explained by the fact that albumin helps to bring the drug more "closely" to the cell by ionic interactions with the plasma membrane, thereby shortening the diffusional distance (Blanchard et al., 2004). Albumin also could improve solubility of compounds that have a low solubility in water. However, this explanation seems less likely for current results because clearance of the compound with the lowest solubility (testosterone) was not increased, whereas that of 7-HC (which has a much higher solubility) was. To further unravel the mechanism by which albumin enhances clearance, more research is required.

Extrahepatic organ clearance of midazolam, haloperidol, and 7-HC was equally affected by the presence of 4% albumin, and it appeared that whole body metabolic clearance was much better predicted. Clearances of all the tested model compounds predicted in the presence of albumin (and using thin liver and kidney slices) lay within a 2-fold range of in vivo metabolic clearances. Thus, this approach seems promising to adequately predict in vivo clearance.

In conclusion, these results confirm the applicability of slices in metabolic clearance prediction. The application of thin slices eliminates the penetration limitation reported for high clearance drugs in conventional slices. Furthermore, organ slices can be kept metabolically active for sufficient periods of incubation, enabling study of the kinetics of low clearance compounds, although (a slight) underestimation of clearance can be expected in some cases because of downregulation of certain P450s. In vitro prediction with organ slices of in vivo metabolic clearance is significantly improved for a number of compounds when incubation is conducted in the presence of albumin, resulting in predictions that lay within a 0.5- to 2-fold range of in vivo values.

References

Arlotto MP, Trant JM, and Estabrook RW (1991) Measurement of steroid hydroxylation reactions by high-performance liquid chromatography as indicator of P450 identity and function. *Methods Enzymol* **206:**454–462.

Bachmann K, Byers J, and Ghosh R (2003) Prediction of in vivo hepatic clearance from in vitro data using cryopreserved human hepatocytes. *Xenobiotica* **33:**475–483.

Bayliss MK, Bell JA, Wilson K, and Park GR (1994) 7-Ethoxycoumarin O-deethylase kinetics in isolated rat, dog and human hepatocyte suspensions. *Xenobiotica* **24:**231–241.

Blanchard N, Richert L, Notter B, Delobel F, David P, Coassolo P, and Lave T (2004) Impact of serum on clearance predictions obtained from suspensions and primary cultures of rat hepatocytes. *Eur J Pharm Sci* **23:**189–199.

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.

Carlile DJ, Zomorodi K, and Houston JB (1997) Scaling factors to relate drug metabolic clearance in hepatic microsomes, isolated hepatocytes and the intact liver: studies with induced livers involving diazepam. *Drug Metab Dispos* **25:**903–911.

Cheng YF and Paalzow LK (1992) Linear pharmacokinetics of haloperidol in the rat. *Biopharm Drug Dispos* **13:**69–76.

de Graaf IA, van der Voort D, Brits JH, and Koster HJ (2000) Increased post-thaw viability and phase I and II biotransformation activity in cryopreserved rat liver slices after improvement of a fast-freezing method. *Drug Metab Dispos* **28:**1100–1106.

de Graaf IA, Van Meijeren CE, Pektas F, and Koster HJ (2002) Comparison of in vitro preparations for semi-quantitative prediction of in vivo drug metabolism. *Drug Metab Dispos* **30:**1129–1136.

de Kanter R, Monshouwer M, Draaisma AL, de Jager MH, de Graaf IA, Proost JH, Meijer DK, and Groothuis GM (2004) Prediction of whole-body metabolic clearance of drugs through the combined use of slices from rat liver, lung, kidney, small intestine and colon. *Xenobiotica* **34:**229–241.

de Kanter R, Monshouwer M, Meijer DK, and Groothuis GM (2002) Precision-cut organ slices as a tool to study toxicity and metabolism of xenobiotics with special reference to non-hepatic tissues. *Curr Drug Metab* **3:**39–59.

de Kanter R, Tuin A, van de Kerkhof E, Martignoni M, Draaisma AL, de Jager MH, de Graaf IA, Meijer DK, and Groothuis GM (2005) A new technique for preparing precision-cut slices from small intestine and colon for drug biotransformation studies. *J Pharmacol Toxicol Methods* **51:**65–72.

Evans PJ (1995) Maintenance of cytochrome P-450 levels in hepatocytes preserved on gelatin gels. *Cell Biol Int* **19:**855–860.

Farthing MJ, Vinson GP, Edwards CR, and Dawson AM (1982) Testosterone metabolism by the rat gastrointestinal tract, in vitro and in vivo. *Gut* **23:**226–234.

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.

Houston JB (1994) Utility of in vitro drug metabolism data in predicting in vivo metabolic clearance. *Biochem Pharmacol* **47:**1469–1479.

Kern A, Bader A, Pichlmayr R, and Sewing KF (1997) Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochem Pharmacol* **54:**761–772.

Koebe HG, Pahernik S, Eyer P, and Schildberg FW (1994) Collagen gel immobilization: a useful cell culture technique for long-term metabolic studies on human hepatocytes. *Xenobiotica* **24:**95–107.

Kotegawa T, Laurijssens BE, Von Moltke LL, Cotreau MM, Perloff MD, Venkatakrishnan K, Warrington JS, Granda BW, Harmatz JS, and Greenblatt DJ (2002) In vitro, pharmacokinetic and pharmacodynamic interactions of ketoconazole and midazolam in the rat. *J Pharmacol Exp Ther* **302:**1228–1237.

Krumdieck CL, dos Santos JE, and Ho KJ (1980) A new instrument for the rapid preparation of tissue slices. *Anal Biochem* **104:**118–123.

Lombardo F, Obach RS, Shalaeva MY, and Gao F (2002) Prediction of volume of distribution values in humans for neutral and basic drugs using physicochemical measurements and plasma protein binding data. *J Med Chem* **45:**2867–2876.

Lopez-Garcia MP (1998) Endogenous nitric oxide is responsible for the early loss of P450 in cultured rat hepatocytes. *FEBS Lett* **438:**145–149.

Ludden LK, Ludden TM, Collins JM, Pentikis HS, and Strong JM (1997) Effect of albumin on the estimation, in vitro, of phenytoin Vmax and Km values: implications for clinical correlation. *J Pharmacol Exp Ther* **282:**391–396.

McMillan JM, Shaddock JG, Casciano DA, Arlotto MP, and Leakey JE (1991) Differential stability of drug-metabolizing enzyme activities in primary rat hepatocytes, cultured in the absence or presence of dexamethasone. *Mutat Res* **249:**81–92.

Mistry M and Houston JB (1985) Quantitation of extrahepatic metabolism. Pulmonary and intestinal conjugation of naphthol. *Drug Metab Dispos* **13:**740–745.

Pardridge WM (1986) Serum bioavailability of sex steroid hormones. *Clin Endocrinol Metab* **15:**259–278.

Pelkonen O, Boobis AR, and Gundert-Remy U (2001) In vitro prediction of gastrointestinal absorption and bioavailability: an experts' meeting report. *Eur J Clin Pharmacol* **57:**621–629.

Pichette V, Geadah D, and du Souich P (1996) The influence of moderate hypoalbuminaemia on the renal metabolism and dynamics of furosemide in the rabbit. *Br J Pharmacol* **119:**885–890.

Renwick AB, Watts PS, Edwards RJ, Barton PT, Guyonnet I, Price RJ, Tredger JM, Pelkonen O, Boobis AR, and Lake BG (2000) Differential maintenance of cytochrome P450 enzymes in cultured precision-cut human liver slices. *Drug Metab Dispos* **28:**1202–1209.

Ritschel WA, Johnson RD, Vachharajani NN, and Hussain AS (1992) Prediction of the volume of distribution of 7-hydroxycoumarin in man from in vitro and ex vivo data obtained in rat. *Biopharm Drug Dispos* **13:**389–402.

Rogiers V, Vandenberghe Y, Callaerts A, Verleye G, Cornet M, Mertens K, Sonck W, and Vercruysse A (1990) Phase I and phase II xenobiotic biotransformation in cultures and co-cultures of adult rat hepatocytes. *Biochem Pharmacol* **40:**1701–1706.

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.

Sherratt AJ and Damani LA (1989) Activities of cytosolic and microsomal drug oxidases of rat hepatocytes in primary culture. *Drug Metab Dispos* **17:**20–25.

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.

Utesch D, Molitor E, Platt KL, and Oesch F (1991) Differential stabilization of cytochrome P-450 isoenzymes in primary cultures of adult rat liver parenchymal cells. *In Vitro Cell Dev Biol* **27A:**858–863.

VandenBranden M, Wrighton SA, Ekins S, Gillespie JS, Binkley SN, Ring BJ, Gadberry MG, Mullins DC, Strom SC, and Jensen CB (1998) Alterations of the catalytic activities of drug-metabolizing enzymes in cultures of human liver slices. *Drug Metab Dispos* **26:**1063–1068.

Wallin JD, Ryals P, and Kaplowitz N (1977) Metabolic clearance of furosemide in the rat. *J Pharmacol Exp Ther* **200:**52–57.

Worboys PD, Bradbury A, and Houston JB (1997) Kinetics of drug metabolism in rat liver slices. III. Relationship between metabolic clearance and slice uptake rate. *Drug Metab Dispos* **25:**460–467.

Wortelboer HM, de Kruif CA, van Iersel AA, Falke HE, Noordhoek J, and Blaauboer BJ (1990) The isoenzyme pattern of cytochrome P450 in rat hepatocytes in primary culture, comparing different enzyme activities in microsomal incubations and in intact monolayers. *Biochem Pharmacol* **40:**2525–2534.

Wright MC and Paine AJ (1992) Evidence that the loss of rat liver cytochrome P450 in vitro is not solely associated with the use of collagenase, the loss of cell-cell contacts and/or the absence of an extracellular matrix. *Biochem Pharmacol* **43:**237–243.

Zhang QY, Wikoff J, Dunbar D, Fasco M, and Kaminsky L (1997) Regulation of cytochrome P4501A1 expression in rat small intestine. *Drug Metab Dispos* **25:**21–26.

Address correspondence to: I.A.M. de Graaf, Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: i.a.m.de.graaf@rug.nl