Prediction of whole body metabolic clearance of drugs through the combined use of slices from rat liver, lung, kidney, small intestine and colon

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submitted
Abstract

The aim of the current study was to investigate whether precision-cut rat tissue slices can be used to predict drug clearance in vivo. In order to obtain a complete picture, slices not only from liver, but also from lung, kidney, small intestine and colon were investigated.

For this, metabolic clearance of 7-ethoxycoumarin, 7-hydroxycoumarin, testosterone, and three candidate drugs were calculated after measuring disappearance of these compounds during the incubation by slices prepared from liver, lung, kidney, small intestine and colon. To be sure that the clearance was independent of the substrate concentration, incubations were performed not only at relatively low, but also at two- to tenfold higher concentrations.

The total in vitro metabolic clearance was determined by summing the individual in vitro organ clearance values from the slices. Prediction on the basis of the in vitro clearance was found to be reasonably accurate, being 0.14 to 1.78 fold of the corresponding in vivo values. Interestingly, the relative contribution of extra-hepatic metabolic clearance of the studied compounds to total clearance was remarkably high, ranging from 50% to 87% of the total metabolic clearance.

It is concluded that the model of multi-organ precision-cut slices is an useful in vitro tool for prediction of in vivo metabolic clearance, and that it provides information about the relative contribution of the liver, lung, kidney, small intestine and colon to the total metabolic clearance.

Introduction

The removal of drugs from the body is normally expressed in the parameter clearance. Plasma clearance is defined as the volume of plasma that is completely cleared from drug per unit of time. Several in vitro approaches have proven to be useful to predict in vivo clearance [146]. These approaches are based on the determination of the metabolic clearance, which can be calculated from the rate of metabolism over the unbound drug concentration. The classical and most popular in vitro method, used to assess the rate of metabolic conversion, involves the incubation of liver-derived microsomes with a range of parent drug concentrations. At each concentration, the formation rate of one or more metabolites is quantified. To calculate the overall drug conversion in the case of multiple biotransformation pathways, a so-called “metabolic fraction factor” is used that represents the percentage of the particular metabolite formed to the total amount of metabolite formation [137]. From the relationship between the particular rate of parent drug conversion and the (unbound) concentration, the \( V_{\text{max}} \) and \( K_m \) values of the particular process can be determined. Dividing \( V_{\text{max}} \) by \( K_m \) will result in the intrinsic clearance [138]. These values are then
multiplied by a scaling factor that takes into account the fraction of the total liver that was used in the experiment. After taken into account plasma protein binding, liver blood flow and physiologically based pharmacokinetic modeling, the hepatic in vivo metabolic clearance can be calculated.

There are three important disadvantages to this approach. Firstly, many incubations and bioanalyses are required to obtain sufficient data for reliable a Michaelis-Menten plot. Secondly, detailed knowledge about the relative importance of the particular metabolic pathway under study to the overall metabolic clearance of the compound is required if the rate of metabolite formation is used. For many drugs, in particular new candidate drugs within the pharmaceutical industry, such data are not available off hand. In addition, the “metabolic fraction factor” may vary in different organs and between species, reflecting the organ- and species dependent metabolic profile of the compound under study. Finally, poor solubility and low metabolic turnover very often does not allow us to create an appropriate Michaelis-Menten plot.

A much simpler approach, that circumvents these disadvantages, is based on one incubation at a single, relatively low initial concentration (see below) and monitoring the depletion of the compound of interest [138]. This does not require a priori knowledge of the metabolic fate of the drug. This approach is reported much less frequently compared with the V_{max}/K_{m} approach. The latter may be partly due to the relatively long incubations that were necessary to measure sufficient drug depletion, making it unsuitable for microsomal incubations because of the inherent instability of this membrane preparation.

Recently, the drug depletion approach has gained popularity, due to the development of other long-term incubation systems, together with the use of more sensitive analytical techniques, such as LC-MS. This is illustrated by five papers in which this technique is used and compared with in vivo data for seven to as much as 1163 compounds [47,211,220,221,284]. However, only liver preparations were used.

In the present study, the drug depletion approach was used to determine metabolic clearances of three commonly used model compounds, 7-ethoxycoumarin, 7-hydroxycoumarin, testosterone, and three candidate drugs that are currently under clinical development and of which data on body clearance were available (figure 1). These compounds were selected on the basis of their unbound fractions and clearance values in the rat aiming at a broad range of binding and clearance values in this study.

Metabolism of xenobiotica is often seen as an exclusive function of the liver, but more recent findings support the notion that the lungs, kidneys and intestine may contribute considerably [61]. Therefore, it is important to incorporate not only liver, but also lung, kidney, and intestinal tissue to predict the overall metabolic clearance.

There are several in vitro models to study metabolism of xenobiotica in these organs, all with their own distinct advantages and disadvantages. Ideally, an in
vitro model should be simple, applicable in a similar way for each organ, and also should include all metabolic pathways that are defined in vivo. The disadvantage of the use of microsomes lies in the lack of cytosolic enzymes and in the non physiological co-factors supply. When using isolated cells, only one cell type is considered in most cases, while in most organs several cell types can contribute to the metabolic clearance, or can influence each other in this regard. In contrast, in precision-cut organ slices all relevant cell types, metabolic enzymes, transporters, and co-factors are present. Slices are easy to scale up and applicable to liver, kidney, lung as well as to the intestines [63]. In the present study we used precision-cut slices from liver, kidney, lung, small intestine and colon to incorporate all organs that reasonably can be expected to contribute to the total metabolic clearance. The aim of the current study was to determine if organ slices can be used to adequately predict the in vivo metabolic clearance using the drug depletion method. Apart from the liver, the relative

<table>
<thead>
<tr>
<th>substrate</th>
<th>substrate concentration 1</th>
<th>substrate concentration 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>testosterone</td>
<td>1 µM</td>
<td>2 µM</td>
</tr>
<tr>
<td>7-ethoxycoumarin</td>
<td>1 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>7-hydroxycoumarin</td>
<td>1 µM</td>
<td>2 µM</td>
</tr>
<tr>
<td>compound A</td>
<td>0.3 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>compound B</td>
<td>0.3 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>compound C</td>
<td>0.3 µM</td>
<td>1 µM</td>
</tr>
</tbody>
</table>

Figure 1. The four stage strategy for the determination of clearance in this study. Substrate concentration 1 is used as drug depletion concentration, while substrate concentration 2 is used as a control concentration (see discussion).
contribution of the lung, kidney and intestines to metabolism of the 6 compounds was assessed.

Materials and Methods

Materials. The following compounds were obtained from the sources indicated: 7-ethoxycoumarin from Fluka (Buchs, Germany); testosterone, 7-hydroxycoumarin, cremophor EL and low melting agarose (type VII-A) from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands); Viaspan organ preservation solution (UW) from Barr Laboratories (Pomona, NY, USA); amphotericin B (Fungizone), penicillin-streptomycin solution and Williams’ medium E (with Glutamax) from Gibco (Breda, the Netherlands). Three candidate drugs indicated as compound A, B and C were kindly provided by Yamanouchi Europe B.V. (Leiderdorp, the Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

In vivo data. In vivo clearance under linear conditions and plasma protein binding data, were obtained from literature data from Sprague-Dawley rats for 7-ethoxycoumarin [39] and 7-hydroxycoumarin [267]. Animal handling was in accordance with the national laws on animal experimentation for the protection of vertebrate animals used for experimental and other scientific purposes. The in vivo total clearance of testosterone was determined in castrated, overnight fasted, male Wistar (HsdCpb:WU) rats (Harlan, Horst, the Netherlands) of 275-350 g after can-nulation of the vena cava under isofurane and N₂O/O₂ anesthesia. A single bolus of 1 mg/kg testosterone was administrated via the vena cava. Testosterone was dissolved in 80% ethanol, after which the solution was first diluted 1:2 with cremophor EL and than diluted (1:5,5) with a solution containing 5% (w/v) mannitol (final concentration 1 mg/ml). Nine blood samples were taken via the vena cava, blood was collected in EDTA coated syringes and plasma was prepared by centrifugation at 24000 N×kg⁻¹ for 15 min. at 4°C, stored at –20°C and analyzed by LC-MSMS. The in vivo total clearances for compounds A, B and C were determined after a single bolus intravenous administration via the tail vein of 0.3 to 1.0 mg/kg to non-fasted male F344 rats (Charles River Japan, Co., Ltd.) weighing 120-160 g. Blood samples were taken from the vena cava inferior under ether anesthesia with a heparin-added syringe at nine time points between 0.1 h and 8 h and plasma was prepared as described for testosterone. The plasma samples were stored frozen at –20°C until analysis. The amount of compound in the plasma was determined via validated HPLC-UV or fluorescence methods. The pharmacokinetic parameters of testosterone and compound A, B and C were calculated by a non-compartment analysis using mean concentration values of 3 animals at each sampling point. The in vivo clearance value was calculated by dividing the dose by the area under the plasma concentration curve.

In vitro experiments. Male Wistar (HsdCpb:WU) rats, mean body weight 362 g (Harlan, Horst, the Netherlands) and F344 (CrlBR) rats, mean body weight 380 g (Charles River, Someren, the Netherlands) were housed in standard cages and had free access to food (RMH-chow, Hope Farms Inc, Woerden, the Netherlands) and tap water. Rats were anaesthetised by isofurane and N₂O/O₂, and the liver, lungs, kidneys and intestines were excised and placed in ice-cold Krebs-Henseleit buffer containing 10 mM Hepes and 25 mM NaHCO₃, pH=7.4. The intestines were flushed thoroughly with ice-cold Krebs-Henseleit buffer to remove the contents. The lungs were filled with agarose solution as described earlier [64].

Preparation of slices. Tissue cylinders from liver, agarose-filled lung and kidney were prepared with a coring tool, i.d. 8 mm (Alabama R&D, Munford, AL USA), attached to a drilling machine with a variable rotation speed. The organ cylinders were stored in ice-cold UW until slicing.

To prepare agarose filled slices, the intact intestines were first cut in 10 cm parts that were subsequently ligated on one side. These parts were then filled with 3% (w/v) low melting agarose solution in 0.9% (w/v) NaCl at 37°C and allowed to gel in ice-cold Krebs-Henseleit buffer. The agarose-filled intestines were cut in 1 cm parts and were embedded in the low melting agarose solution at 37°C using the Tissue Embedding Unit from Alabama R&D so that agarose gel cylinders with a diameter of 16 mm were formed. Organ cylinders from liver, lung, kidney, and intestines were used to prepare precision-
cut slices (about 0.25 mm thickness, but for lung slices 0.50 mm thickness), using a Krumdieck tissue slicer (Alabama R&D), pre-cooled and filled with oxygenated, ice-cold Krebs Henseleit buffer. Kidney slices were derived mainly from cortex tissue. All slices were stored in ice-cold UW until incubation. The time gap between coring and incubation was max. 2 hr.

**Incubation of slices.** Slices were incubated in 3.2 ml Williams medium E that was pre-warmed at 37°C, gassed with 95% O₂/5% CO₂ and supplemented with glucose (final concentration 25 mM). For liver and lung slices gentamicin (50 µg/ml) was added. For kidney slices penicillin (100 U/ml) plus streptomycin (100 µg/ml) was used, while for intestine slices gentamicin (50 µg/ml) and amphotericin B (2.5 µg/ml) was added. These agents are poorly metabolized themselves and therefore assumed not to interfere with biotransformation enzymes. Slices were individually incubated in 6-well culture plates, which were placed in a plastic container, continuously gassed with humidified 95% O₂/5% CO₂, and shaken back and forth (90 times/min) in a cabinet at 37°C. During incubation, the gel surrounding the slices, but not inside the ring of intestinal tissue, separated from the slices.

**Metabolic activity of slices.** Metabolism of the compounds was studied by the addition of 32 µl stock-solution in methanol to 3.2 ml Williams’ medium E, but for compound B, a 0.01 M NaOH stock-solution was used. For incubations with 7-ethoxycoumarin, 7-hydroxycoumarin and testosterone, slices from three Wistar rats were used, while for compound A, B and C three F344 rats were used. In each experiment three individual slice incubations, from each organ, were analyzed as described below, after the incubation periods indicated in the legends of figures 3 to 8.

Medium incubated with substrates, but without slices, served as controls for spontaneous metabolism or adherence to the 6-wells plates and showed no depletion of the particular substrates. All samples were stored at –20°C until analysis. Control experiments showed that 7-ethoxycoumarin or 7-hydroxycoumarin were not significantly retained in the slices, at the concentration used (results not shown) and therefore only medium samples were used for analysis. For analyses of testosterone and the compounds A, B and C, slices and incubation media (3.2 ml) were harvested together after incubation and homogenized using sonication to extract the compounds from the slice. Medium samples (1 ml) of 7-ethoxycoumarin incubations were acidified by adding 10 µl 2 M HCl after sampling, in order to prevent spontaneous degradation of metabolites that occurred if the pH of the incubation exceeds 8.0. After thawing of 7-ethoxycoumarin and 7-hydroxycoumarin samples, sodium azide was added to inhibit bacterial contamination (final concentration 0.1 mg/ml) and 50 µl of the centrifuged sample was analyzed using HPLC as described earlier [327]. For testosterone analysis, 11β-hydroxytestosterone (5 µl, dissolved in methanol) was added after thawing as internal standard to 1 ml of the homogenate and 6 ml dichloromethane was then added. After removal of the water phase and protein interphase, the organic phase was evaporated and re-dissolved in 150 µl 50% (v/v) methanol which was analyzed using HPLC as described earlier [313].

For analysis of compounds A, B, and C, 25 µl of a selected internal standard (200 ng/ml) was added to 100 µl sample. An LC-MS/MS column switching system was used, consisting of an Alkyl-Diol-Silica (ADS) column (25 x 2.0 mm, Merck, Darmstadt, Germany) operated with a mobile phase consisting of 20 mM ammoniumacetate/MeOH (90:10) for compound A and C, and 1% formic acid/MeOH (90:10) for compound B, at a flow of 2.5 ml/min. The analytical column was a Symmetry C₁₈ column (100 x 2.1 mm, Waters, Etten-Leur, the Netherlands) operated with a mobile phase consisting of 20 mM ammoniumacetate/MeOH (25:75) for compounds A and C and 1% formic acid/MeOH (25:75) for compound B, at a flow of 0.3 ml/min. Aliquots of 20 µl of the sample mixtures were injected, using a Gilson215 96-well plate autosampler, into the ADS column of the appropriate column switching system for sample clean-up. One minute after injection on the ADS column, the column was back-flushed and the elute was directed to the analytical column for final separation followed by quantification using a Finnigan TSQ7000 APi2 mass spectrometer in MS/MS mode. Sample introduction and ionisation was done using Atmospheric Pressure Chemical Ionisation (APCI) in the positive mode for all three compounds. The vaporizer temperature was 550°C and the heated capillary temperature 275°C. Ions were collisionally activated at a collision energy of 25eV and at an argon pressure of 2.5 mTorr. The mass spectrometer was operated at unit mass resolution and set to select the [M+H]⁺ ions via the first quadrupole, the signal of a suitable fragment ion was monitored via the third quadrupole mass filter. Data were collected using Xcalibur software (Thermoquest, San Jose, CA, USA).
Protein content of organs and slices. The organ weight was determined of each liver, lung (after filling with agarose), kidney, small intestine, and colon (after removing the contents) from each animal that was used in the in vitro studies. After incubation, the wet weight of five slices from each organ was determined to determine average wet weight and these slices were subsequently homogenized in their own incubation medium by sonication and stored at −20°C until analysis. After thawing, the homogenates were diluted with 0.1 M NaOH and the protein content was determined using the Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) against a bovine serum albumin standard curve. All metabolic rates were expressed per µg protein, and not by wet weight. This is because in lung and intestinal slices agarose is present that contributes to the wet weight.

Calculation. From the difference between initial amount of substrate added and the amount of compounds left-over after incubation, the rate of metabolic conversion was quantified and expressed per µg protein. In addition, from the initial \( C_{\text{initial}} \) and final concentrations \( C_{\text{end}} \) the mean concentration \( C_{\text{mean}} \) during the experiment was calculated using equation 1.

\[
C_{\text{mean}} = \frac{C_{\text{initial}} - C_{\text{end}}}{\ln(C_{\text{initial}} / C_{\text{end}})}
\]

The intrinsic clearance \( CL_{\text{int}} \) was calculated from the rate of degradation \( v \) divided by the mean concentration during the experiment, as depicted in equation 2.

\[
CL_{\text{int}} = \frac{v}{C_{\text{mean}}}
\]

The intrinsic clearance by the slices was multiplied with a scaling factor, which was derived by multiplying wet organ weights taking into account the protein percentage of the slices, as depicted in table 1. Using the venous equilibration (“well-stirred”) model (equation 3) the in vivo metabolic clearance was calculated from intrinsic clearance, blood flow data from figure 2, and unbound fraction \( (fu) \) as shown in table 2, assuming blood-to-plasma ratios to be unity.

\[
CL_{\text{org}} = \frac{\text{bloodflow} \cdot fu \cdot CL_{\text{int}}}{\text{bloodflow} + fu \cdot CL_{\text{int}}}
\]

Equation 3.

Finally, total metabolic clearance \( CL_{\text{calc}} \) was calculated by adding the clearance of liver, lung, kidney, small intestine, and colon (equation 4) and expressing this value per kg body weight. Because the liver is supplied by the portal vein, collecting blood from the stomach, spleen, small intestine and colon, organs where already drug elimination takes place [265], we corrected the calculated contribution of the liver clearance. In fact this organ operates at a lower drug concentration due to pre-hepatic extraction of blood drained by the liver. The contribution of the liver clearance by the supply of the portal vein (80% of total liver bloodflow) is corrected by a factor \( F \) (equation 5), while the contribution by the arterial supply (20%) is not corrected since this part does not pass eliminating organs prior to the liver (equation 4 and figure 2).

\[
CL_{\text{calc}} = 20\% \cdot CL_{\text{liver}} + 80\% \cdot F \cdot CL_{\text{liver}} + CL_{\text{lung}} + CL_{\text{kidney}} + CL_{\text{small intestine}} + CL_{\text{colon}}
\]

Where \( F \) is calculated according to equation 5.

\[
F = \frac{(1-E_s) \cdot Q_s + (1-E_c) \cdot Q_c}{Q_s + Q_c}
\]

Where \( Q_s \) and \( Q_c \) are the blood flows through the small intestine and colon respectively (see figure 2), \( E \) is the extraction ratio which is described by \( E_s = CL_{\text{SI}} / Q_s \) for the small intestine and \( E_c = CL_{\text{IC}} / Q_c \) for the colon.
Results

Prediction of metabolic clearance by slice incubations. Three model compounds and three candidate drugs were used to evaluate the possibility of using rat organ slices prepared from liver, lung, kidney, small intestine and colon to predict total clearance in vivo. The concentrations of the incubated substrates left over after a certain incubation period were determined and the intrinsic clearances of the slices were calculated using equations 1 and 2.

Table 1. Organ weights, slice protein percentage and scaling factors

<table>
<thead>
<tr>
<th>organ weight (g)</th>
<th>slice protein percentage</th>
<th>scaling factor (x1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>15.3</td>
<td>10.8%</td>
</tr>
<tr>
<td>lung (agarose filled)</td>
<td>7.2</td>
<td>1.0%</td>
</tr>
<tr>
<td>kidney</td>
<td>2.9</td>
<td>7.5%</td>
</tr>
<tr>
<td>small intestine</td>
<td>6.9</td>
<td>12.1%</td>
</tr>
<tr>
<td>colon</td>
<td>1.0</td>
<td>12.1%</td>
</tr>
</tbody>
</table>

\(^a\) determined through wet weight and protein content of the slices.

\(^b\) these factors were calculated by multiplying organ weight with the protein content.
To convert the results of the individual slices into data for the whole organ, scaling factors were used that take into account the fraction of the organ that was used in the experiments. The scaling factors for rat liver, lung, kidney, small intestine, and colon are depicted in table 1.

The calculated contribution of the organs to the total metabolic clearance was calculated using equation 3, 4 and 5 using the blood flow values depicted in figure 2 and the unbound fractions as indicated in table 2. Data for rat blood flow through the liver are from [39], while liver and kidneys receive 25%, and 20% respectively of the total bloodflow. 80% of the of the bloodflow to the liver is supplied by the portal vein and 20% by the hepatic artery [265]. The ratio of blood-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Unbound fraction in blood (fu)</th>
<th>In vivo prediction (%)</th>
<th>% hepatic contribution</th>
<th>CL vivo (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-ethoxycoumarin</td>
<td>0.22(^a)</td>
<td>109%</td>
<td>27%</td>
<td>40(^a)</td>
</tr>
<tr>
<td>7-hydroxycoumarin</td>
<td>0.10(^b)</td>
<td>25%</td>
<td>37%</td>
<td>97(^b)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.40(^c)</td>
<td>114%</td>
<td>50%</td>
<td>62(^f)</td>
</tr>
<tr>
<td>Compound A</td>
<td>0.01(^d)</td>
<td>44%</td>
<td>20%</td>
<td>42(^d)</td>
</tr>
<tr>
<td>Compound B</td>
<td>0.021(^d)</td>
<td>178%</td>
<td>13%</td>
<td>2.0(^d)</td>
</tr>
<tr>
<td>Compound C</td>
<td>0.33(^d)</td>
<td>14%</td>
<td>45%</td>
<td>112(^d)</td>
</tr>
</tbody>
</table>

\(\(^a\)\) from [39]  
\(\(^b\)\) from [267]  
\(\(^c\)\) from [241]  
\(\(^d\)\) from Yamanouchi Europe B.V. (Leiderdorp, the Netherlands)  
\(\(^e\)\) CL\(_{calc} / CL_{vivo}\)  
\(\(^f\)\) from Organon N.V. (Oss, the Netherlands)

To convert the results of the individual slices into data for the whole organ, scaling factors were used that take into account the fraction of the organ that was used in the experiments. The scaling factors for rat liver, lung, kidney, small intestine, and colon are depicted in table 1.

The calculated contribution of the organs to the total metabolic clearance was calculated using equation 3, 4 and 5 using the blood flow values depicted in figure 2 and the unbound fractions as indicated in table 2. Data for rat blood flow through the liver are from [39], while liver and kidneys receive 25%, and 20% respectively of the total bloodflow. 80% of the of the bloodflow to the liver is supplied by the portal vein and 20% by the hepatic artery [265]. The ratio of blood-

Figure 3. 7-ethoxycoumarin clearance  
In vitro clearance of 7-ethoxycoumarin determined with slices at 1 µM and 10 µM and compared to the in vivo value. Incubation period was 3 h.
flow through the small intestine and colon corresponds to the ratio of the wet weight of these organs in this study.

Metabolic clearances are shown in figures 3 to 8 for the compounds studied. Of note, the amount of compound that was taken up into, or, was secreted from the slices, but not metabolized, was not included in the measured clearances. This because we disrupted the slices in their own incubation medium and extracted the parent compounds for analysis.

It is shown in figures 3 to 8 that the in vivo clearances were reasonably well predicted by the present data, although both under- and over predictions were observed (see also table 2).

Incubation at the two different concentrations that were used, gave about the same clearance predictions for all compounds studied (figures 3 to 8). The

**Figure 4.** 7-hydroxycoumarin clearance
In vitro clearance of 7-hydroxycoumarin determined with slices at 1 µM and 2 µM and compared the in vivo value. Incubation period was 3 h.

**Figure 5.** Testosterone clearance
In vitro clearance of testosterone determined with slices at 1 µM and 2 µM and compared to the in vivo value. Incubation period for liver slices was 0.5 h. Incubation period for lung, kidney, small intestine, and colon slices was 3 h.
largest difference in clearance at the different substrate concentrations were observed for compound C, however this difference was not statistically significant (t-test value 0.19). Therefore we concluded that the chosen concentrations were well under the total K_{m} of the metabolic reactions involved (see discussion).

As shown in table 2, the in vitro prediction of the total in vivo clearance showed an underprediction for three compounds, and an overprediction for the other three of the six compounds studied.

Figure 6. Compound A clearance
In vitro clearance of compound A determined with slices at 0.3 µM and 1 µM compared to the in vivo value. Incubation period for liver, lung, kidney small intestine, and colon slices was 0.5 h.

Figure 7. Compound B clearance
In vitro clearance of compound B determined with slices at 0.3 µM and 1 µM compared to the in vivo value. Incubation period for liver slices was 0.5 h. Incubation period for lung, kidney small intestine, and colon slices was 3 h.
Discussion

In this study we incubated slices from several organs and measured drug depletion to predict *in vivo* clearance. A systematic account of the factors that differ in the drug metabolism observed *in vitro* with those *in vivo* provided a simple strategy for extrapolation of *in vitro* metabolic clearance. Organ slices resemble the *in vivo* situation more closely than isolated cells or cell fractions, and conse-

**Figure 8.** Compound C clearance

*In vitro* clearance of compound C determined with slices at 0.3 µM and 1 µM and compared to the *in vivo* value. Incubation period for liver slices was 3 h. Incubation period for lung, kidney small intestine, and colon slices was 24 h.

**Figure 9.** *In vitro* – *in vivo* relationship

The relationship between *in vitro* and *in vivo* clearance ($r^2=0.55$) is depicted. Each point represent a compound under study.
quently allow simple scaling procedures. Moreover, slices from different organs are prepared in essentially the same manner making them a convenient tool to study different organs simultaneously. A limitation of the use of slices is that only metabolic clearance can be estimated using the present method, and not excretion of the parent drug.

Our method is based on measuring the rate of drug disappearance under linear conditions. A pre-requisite for the drug depletion approach is that the chosen substrate concentration \( C_{\text{initial}} \) should be well under the overall \( K_m \) of the enzymes involved in the substrate metabolism [138]. We confirmed that this was the case because experiments with higher substrate concentrations did not significantly change the clearance values for all compounds tested (figures 3 to 8). In the case that the chosen substrate concentrations would not be clearly below the overall \( K_m \), a lower intrinsic clearance would be expected with the (higher) control substrate concentration (1 µM, 2 µM or 10 µM in this study), compared to the (lower) depletion substrate concentration (0.3 µM or 1 µM in this study). Curves relating the substrate dependency of the rate of metabolism can be sigmoidal, even in the linear range of Michaelis-Menten kinetics. This is indicative for substrate-caused enzyme activation [140]. In that case, a relatively higher clearance would be observed using the (higher) control substrate concentration compared to the (lower) depletion concentration. This was not the case in the current study (figures 3 to 8). On the other hand, a lower clearance would be expected using the (higher) control substrate concentration if non-competitive substrate-, or product inhibition, takes place [140]. However, also this phenomenon was not observed with the present study.

Interestingly, “sigmoidal kinetics” were not even observed for testosterone in the current study, in spite of the fact that it has been shown before that the formation rate of a metabolite of testosterone, 6β-hydroxytestosterone, follows sigmoidal kinetics in microsomes from a cell line, expressing human CYP3A4 [156]. This reaction is catalyzed by CYP3A, and this reaction is considered to be responsible for the sigmoidal kinetic pattern, both in man and in the rat [140]. Absence of sigmoidal kinetics of testosterone in this study can be explained assuming that the concentration dependent rate of 6β-hydroxytestosterone formation is of minor influence on the total depletion kinetics of testosterone. That CYP3A does not have a significant influence on the total metabolic clearance of testosterone is in agreement with the major importance of direct glucuronidation of testosterone [261].

To estimate the total metabolic clearance for the comparison with in vivo clearance, the individual contribution of the various organs were added. This implies that potential extra-hepatic metabolism is included, in contrast to most other in vitro studies where only hepatic metabolism is considered. Especially, the inclusion of extra-hepatic metabolism in the gut, was considered to be of major importance [179]. This is in agreement with the current study that mimics the situation after intravenous dosage. Of note, it can be important to incorporate
the contribution of intestinal elimination not only after oral dosage, but also after intravenous dosage [178].

Further, we realized that 80% of the liver blood flow originates from the gastrointestinal tract, where already metabolism can take place. Therefore we corrected the contribution of the liver to the total metabolic clearance, for the fraction eliminated in the gastrointestinal tract that consequently is not available for elimination by the liver. As a result, not only blood flow to the liver limits the supply of substrate available for elimination by the liver, but also elimination in the gut can limit the contribution of hepatic clearance. In other words the liver operates at a lower substrate concentration than indicated by the systemic circulation. We conclude that the influence of blood flow of the liver is taken into account in most in vitro studies but that the influence of pre-hepatic elimination in the gut has often been overlooked, most likely because liver and intestine are not frequently studied together.

After intravenous administration, drugs will first reach the lungs as a site of potential clearance. Therefore, in theory it would be more accurate to correct the clearance of the other organs for the extraction ratio of the lung. However, the extraction fraction (CL / Q) in the lung is in general small, as a result of its modest metabolic activity and the fact that the lung blood flow is large (figure 2). However in some cases, drug metabolism in the lungs can be substantial [120]. In the present study, we considered the organs studied as homogenously mixed compartments and consequently used the ‘well-stirred’ model to calculate their clearance. However, it is known that the distribution of drug metabolizing enzymes varies along the length of the small intestine and along the sinusoids of the liver. In this study we could not make a direct comparison with regard to the contribution of the individual organs in vivo. Nevertheless, the in vitro prediction of the clearance by the summed contribution of the studied organs was reasonably close to the in vivo clearance (table 2). Remarkably, the contribution of the liver to total clearance was less than 50% for all compounds studied (table 2).

In the present study, we observed both under- and overprediction of the in vivo clearances. Underprediction of in vivo clearance may be the result of limited diffusion of substrates into the slices, which has been shown to happen at high metabolic turn-over rates [82]. Also underprediction can be the result if excretion of unchanged drug is a major clearance route in the body. On the other hand, an overprediction may result if enterohepatic circulation in vivo occurs. The highest underprediction was observed for compound C, the compound with the highest clearance in this study. Some overprediction was observed for 7-ethoxycoumarin, testosterone and compound B. Due to the limited amount of compounds tested, additional work is necessary to clarify the cause of these under- and overpredictions.

Several in vitro clearance values that were reported until now yielded an underprediction of the in vivo value, e.g. [7], especially when only liver slices were
used [139]. Only taking into account the contribution of liver slices as single metabolizing system in the present study also produces a considerable under-prediction. However, when extra-hepatic clearance is included, \textit{in vivo} prediction is considerably better (table 2). Therefore, we conclude that slices are a useful preparation for \textit{in vitro} prediction of clearance, when the contribution of the lung, kidney and, especially the intestines, are included. When only the liver is considered for \textit{in vitro} prediction of metabolic clearance, the use of isolated hepatocytes, predicts \textit{in vivo} clearance more closely [139]. It remains to be studied if the use of isolated hepatocytes, is to be preferred above liver slices (because slices give lower clearances), or that isolated hepatocytes actually overpredict the liver clearance, which is masked by the omitting the contribution of the other organs.

In conclusion, we show the usefulness of a straightforward approach to predict clearance using precision-cut slices from liver, lung, kidney, small intestine and colon. This approach also gives important information about the contribution of the various organs towards total metabolic clearance. Most importantly, this technique can be applied to human tissues, allowing prediction of metabolic clearance in man in an early phase of development. Such \textit{in vitro} approach may contribute to the reduction of the use of experimental animals in research on drug disposition.