PREDICTING THE ORAL BIOAVAILABILITY OF 19-NORTESTOSTERONE PROGESTINS IN VIVO FROM THEIR METABOLIC STABILITY IN HUMAN LIVER MICROSONAL PREPARATIONS IN VITRO

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ABSTRACT:
It was the aim of this study to investigate whether assessment of the metabolic stability of selected progestins of the 19-nortestosterone type in human microsomal liver preparations was a suitable approach to predict the oral bioavailability of these drugs in humans. The Michaelis-Menten parameters Vmax and Km, int for norethisterone, levonorgestrel, gestodene, desogestrel, 3-keto-desogestrel, norgestimate, and dienogest were determined in in vitro incubations with human liver microsomes. Using these data, both the in vitro intrinsic clearance (Cl, int) and, after application of a suitable scaling factor, the scaled in vivo Cl, int were calculated. For progestins for which human in vivo data were available, the in vitro results were correlated with in vivo Cl, int values and oral bioavailability.

Progestins of the 19-nortestosterone type, such as norethisterone, levonorgestrel, gestodene, desogestrel, norgestimate, and dienogest, are still the most widely used progestogenic components in combination oral contraceptives. They differ in potency and are also characterized by different pharmacological profiles. There is an ongoing search for new progestins that not only provide sufficient contraceptive efficacy without affecting lipoproteins, coagulation factors, or carbohydrate metabolism in an unfavorable manner but also offer the potential for additional therapeutic benefits. At early stages in the drug development process, a suitable candidate must be selected from a limited number of structurally related drugs that may have similar pharmacological properties in the various in vitro systems and animal models used. At that point, information on the oral bioavailability of these drugs in humans would provide an important and particularly useful selection criterion. However, at that early stage in drug development, results from clinical studies are not yet available; therefore, there is a need for in vitro methods that would allow prediction of the oral bioavailability in humans. For these progestins, which are almost completely metabolized by the liver and which are not excreted unchanged to a significant extent, the metabolic stability in human liver preparations may represent a suitable tool to obtain this information. There have been many attempts to use in vitro methods for the prediction of human pharmacokinetic parameters, such as CL and bioavailability, and the underlying concepts have been reviewed only recently (Houston, 1994; Hoener, 1994; Obach et al., 1997).

The aim of the present study was to investigate whether assessment of the metabolic stability of selected 19-nortestosterone progestins in human microsomal liver preparations is a suitable approach to predict the oral bioavailability of these drugs in humans. Because the bioavailability of a number of these progestins was determined in previous clinical pharmacokinetic studies, comparison of these results obtained in vivo with the corresponding metabolic stability in vitro would allow assessment of the predictive value of the in vitro system for this particular class of compounds. In addition, different methods of data evaluation were applied to identify the most efficient approach during early drug candidate selection.

Materials and Methods

Chemicals and Microsomal Preparations. The following progestins, which were synthesized at Schering AG, were included in this study: norethisterone, levonorgestrel, gestodene, desogestrel, 3-keto-desogestrel, norgestimate, and dienogest. For protein binding analysis, [3H]levonorgestrel (1.81 Tbk/mmol) and [3H]gestodene (2.05 Tbk/mmol), with radiochemical purities of at least 99%, were obtained from the Department of Isotope Chemistry (Schering AG). All other chemicals and organic solvents were of analytical grade and were obtained from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), Boehringer Mannheim (Mannheim, Germany), Baker (Groß-Gera, Germany), or Riedel de Haen (Berlin, Germany).

Pooled liver microsomes from 31 Caucasian subjects and 1 Hispanic subject (17 female and 15 male donors) were obtained as a stock suspension (20 mg of protein/ml) from the International Institute for the Advancement of Medicine (Exton, PA). The microsomal preparation was characterized with respect to the activity of individual CYP isozymes.

Microsomal Studies. Dependence of the Turnover Rate on the Incubation
Period and Protein Concentration. To establish conditions that are linear with respect to time and enzyme concentration, each progestin was incubated, at low (2 μM) and intermediate (15 μM) concentrations, with 0.7 mg of microsomal protein for 0, 15, 30, 45, and 60 min. In addition, the compounds were incubated with protein concentrations ranging from 0.05 to 1.0 mg/ml, for a fixed time period of 15 or 30 min.

Final Incubation Conditions to Determine \( K_{M,app} \) and \( V_{max} \) and Sample Preparation. The incubation mixture (final volume, 1 ml of 100 mM phosphatase buffer, pH 7.4) contained 0.2 mg of microsomal protein and an NADPH-regenerating system (0.6 mM NADP, 8 mM glucose-6-phosphate, 1.4 unit of glucose-6-phosphate dehydrogenase, 38 mM KCl, and 5 mM MgCl₂). After a preincubation period of 3 min at 37°C, the reaction was started by the addition of 25 μl of a methanolic solution of the progestin. Each substrate concentration was incubated in triplicate for 15 min at 37°C, except for norgestimate, which was incubated for only 10 min. The reaction was stopped by the addition of 2 ml of the precipitating solution (ice-cold acetone/methanol, 3:1, v/v). Samples were extracted for 20 min by end-over-end rotation and, after centrifugation and decantation, the liquid phase was dried under a gentle stream of nitrogen. The dried residues were stored at −18°C. The recovery was examined for each progestin over a range of 0.1 to 100 μM and was between 76 and 101%.

Analysis of Progestins in Microsomal Preparations by HPLC. HPLC Conditions. HPLC analyses were performed using a Spherisorb ODS-2 column (125 x 4.6 mm, 5 μm; Merck). The mobile phase consisted of a mixture of acetonitrile and water, and the composition was adjusted for each progestin (between 35:65 and 80:20, v/v). The eluent was monitored by UV detection at a flow rate of 0.8–1.5 ml/min.

Quantitative Determination of Progestins. The dry residues were redissolved in 150–500 μl (typically 250 μl) of mobile phase, and aliquots of 50–150 μl were injected into the HPLC system. For each compound, calibration standards were prepared from methanolic stock solutions (0.25 mg/ml). These stock solutions were further diluted with buffer, yielding final calibration standards ranging from 0.1 to 100 μM. Calibration standards were extracted in the same way as the samples. In one case (desogestrel), the standard curve was prepared from incubation medium containing 0.2 mg of microsomes but no NADP, because of differences in the recovery of the progestin from standard and test samples. External standardization based on peak areas was used for quantification. Standards and samples were each analyzed in duplicate.

Quality Control. Each sample set included a standard curve, samples from the in vitro experiments, and three or four quality control samples for one progestin. The quality controls were prepared by spiking the phosphate buffer that was used in the incubation experiments with defined amounts of the progestins to be analyzed. The following quality controls were analyzed: 2, 15, 30, and 100 μM (norethisterone and dienogest); 2, 15, and 30 μM (3-keto-desogestrel, gestodene, and norgestimate); 2, 30, and 40 μM (levonorgestrel); or 8 and 15 μM (desogestrel). The quality controls were processed in the same way as the samples and were analyzed singly. The deviation of the measured values from the nominal concentrations (accuracy) was between 89 and 108%. The lower limit of quantitation was set at 0.1 μM for each of the progestins investigated. This was also the lowest concentration of each standard curve, and the signal/noise ratio was >3 in each case.

Testosterone Metabolism. Each assay was controlled for CYP enzyme activity using testosterone (100 μM) as the substrate and using the same incubation conditions as for the investigated progestins. Enzyme activity was evaluated qualitatively as the production of 6β-hydroxytestosterone, which was analyzed by reverse-phase HPLC using a Spherisorb ODS-2 column (250 x 4.6 mm, 3 μm; Merck) and acetonitrile/water (44:55, v/v) as the mobile phase. A flow gradient (0.5–1.0 ml/min) was applied, and the eluent was monitored by UV detection at 248 nm.

Protein Binding of Levonorgestrel and Gestodene in the Incubation Medium. Two milliliters of an incubation mixture containing 0.2 mg of microsomes/ml but no NADPH were spiked with gestodene (or levonorgestrel), at a 50 μM (40 μM) concentration, plus 1 H-labeled gestodene (or levonorgestrel) (approximately 50,000 cpm). The mixture was maintained at 37°C for 1 hr. Aliquots of 300 μl were transferred to an MPS2 ultrafiltration unit (Amicon) and centrifuged for 20 min at 1500g, using a fixed-angle rotor. Aliquots of 50 μl of both incubation medium and ultrafiltrate were each mixed with 4.5 ml of scintillation cocktail (Atomlight; NEN), and the total radioactivity was measured in a liquid scintillation counter. Each sample was analyzed repeatedly (incubation medium, three times; ultrafiltrate, five times). The free fraction of each progestin was calculated from the radioactivity present in the ultrafiltrate and the incubation medium. No correction was made for nonspecific binding to the ultrafiltration device.

Data Analysis. Calculation of \( K_{M,app} \) and \( V_{max} \). Values. Because, in the present study, only disappearance rates for the progestins were evaluated, the Michaelis-Menten constant was designated \( K_{M,app} \), because more than one enzyme is involved in the metabolism of these drugs. \( K_{M,app} \) and \( V_{max} \) were determined by nonlinear regression analysis according to the Michaelis-Menten method (rate of substrate degradation as a function of substrate concentration).

Calculation of In Vitro CL_{int} from Michaelis-Menten Parameters. The ratio of \( V_{max} \) and \( K_{M,app} \) was used to calculate in vitro CL_{int}, which was expressed as milliliters per minute per milligram of microsomal protein.

\[ \text{In vitro } CL_{int} = \frac{V_{max}}{K_{M,app}} \]  

Calculation of In Vitro CL_{int} from Dose and AUC. With initial drug concentrations of 2 and 15 μM, the incubation reactions were stopped after 10, 15, 20, 30, and 60 min, and the concentrations of the progestins were analyzed. The concentration-time curves that were obtained for each progestin were evaluated model-independently (TOPFIT 2.1; Goedecke, Schering AG, Thoma) to calculate the necessary pharmacokinetic parameters. The terminal rate constant λ was calculated by regression analysis, and the corresponding in vitro half-life (\( t_{1/2} \)) was obtained according to the following equation:

\[ t_{1/2} = \ln \frac{2}{\lambda} \]  

The AUC was calculated as

\[ AUC = AUC_{app} + \frac{C_{int}}{\lambda} \]  

where \( t_{int} \) is the total incubation time (60 min) and \( C_{int} \) is the drug concentration measured at \( t_{int} \). The in vitro CL_{int} (expressed as milliliters per minute per milligram of microsomal protein) was calculated as

\[ \text{In vitro } CL_{int} = \frac{D}{AUC} \]  

where \( D \) is the initial amount of drug in the incubation medium.

Scaling of In Vitro CL_{int} to In Vivo CL_{int}. To transform the microsomal in vitro CL_{int} to an in vivo CL_{int} value, a suitable scaling factor is required. Assuming that 1 g of liver contains approximately 50 mg of microsomal protein and the liver of a 70-kg human weighs approximately 1,400 g, one obtains a scaling factor (SF) of 70,000 mg. Multiplication of the in vitro CL_{int} by this scaling factor yields the scaled in vivo CL_{int}, expressed as milliliters per minute or liters per hour.

\[ \text{Scaled in vivo } CL_{int} = SF \times \text{in vitro } CL_{int} \]  

Calculation of CL_{H} in Humans In Vivo Total CL. The CL_{H} in humans can be derived from the total in vivo CL after the iv administration of drug, assuming that CL is identical to CL_{H}. Based on the well-stirred (venous equilibrium) liver model, CL_{H} is given by the following equation:

\[ CL_H = \frac{Q_H \times f_A \times CL_{int}}{Q_H + f_A \times CL_{int}} \]  

which can be transformed into

\[ \text{In vivo } CL_{int} = \frac{CL_H \times CL_{H}}{f_A (1 - \frac{CL_H}{Q_H})} \]
TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>In Vivo CL&lt;sub&gt;n&lt;/sub&gt;</th>
<th>f&lt;sub&gt;E&lt;/sub&gt;</th>
<th>In Vivo CL&lt;sub&gt;int&lt;/sub&gt;</th>
<th>E</th>
<th>f</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>liters/hr</td>
<td>%</td>
<td>liters/hr</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>3-Keto-desogestrel</td>
<td>8.7</td>
<td>1.8</td>
<td>588</td>
<td>0.18</td>
<td>76</td>
<td>Back et al., 1987b; Kuhnz et al., 1992c,e</td>
</tr>
<tr>
<td>Levonorgestrel</td>
<td>9.5</td>
<td>1.8</td>
<td>655</td>
<td>0.19</td>
<td>62</td>
<td>Orme et al., 1991</td>
</tr>
<tr>
<td>Gauldine</td>
<td>5.8</td>
<td>1.3</td>
<td>506</td>
<td>0.12</td>
<td>100</td>
<td>Back et al., 1987a</td>
</tr>
<tr>
<td>Gestodene</td>
<td>7.1</td>
<td>1.3</td>
<td>639</td>
<td>0.14</td>
<td>87</td>
<td>Humpel et al., 1987; Kuhnz et al., 1992a,b, 1994b</td>
</tr>
<tr>
<td>Norethisterone</td>
<td>3.4</td>
<td>1.3</td>
<td>281</td>
<td>0.07</td>
<td>87</td>
<td>Kuhnz et al., 1992d,e, 1993; Orme et al., 1991; Dibbelt et al., 1992; Heuner et al., 1995</td>
</tr>
<tr>
<td>Dienogest</td>
<td>2.7</td>
<td>1.3</td>
<td>220</td>
<td>0.06</td>
<td>99</td>
<td>Tauber et al., 1989</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.7</td>
<td>1271</td>
<td>0.49</td>
<td>64</td>
<td>Back et al., 1973, 1978; Hammond et al, 1982</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>10</td>
<td>37</td>
<td>0.07</td>
<td>91</td>
<td>Oettel et al., 1995</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>10</td>
<td>44</td>
<td>0.08</td>
<td>96</td>
<td>Oettel et al., 1995</td>
</tr>
</tbody>
</table>

If more than one study was published, the mean values for the parameters (f and in vivo CL<sub>n</sub>) were used for the in vitro-in vivo correlations (figs. 2, 3, and 5).

f<sub>E</sub>, unbound fraction; E, extraction ratio.

where f<sub>E</sub> is the free fraction of the drug and Q<sub>E</sub> is either the hepatic blood flow (90 liters/hr) or the hepatic plasma flow (49 liters/hr), depending on whether CL<sub>int</sub> was determined from whole blood or plasma. The pharmacokinetic parameters for the progestins obtained during pharmacokinetic studies in healthy young women are summarized in table 1.

Calculation of Bioavailability Based on Scaled In Vivo CL<sub>n</sub>. In cases where the first-pass effect is solely or mainly attributable to hepatic metabolism, the oral bioavailability (f) of a drug is almost exclusively dependent on the hepatic extraction ratio (E).

\[ f = 1 - E \tag{8} \]

where E is given by eq. 9.

\[ E = \frac{\text{CL}_{\text{int}} \times f_e}{Q_E + \text{CL}_{\text{int}} \times f_e} \tag{9} \]

For CL<sub>int</sub> ≪ Q<sub>E</sub>, eq. 9 can be reduced to

\[ E = \frac{\text{CL}_{\text{int}}}{Q_E} \tag{10} \]

Under the same assumption, eq. 6 for CL<sub>app</sub> can be reduced to

\[ \text{CL}_{\text{int}} = f_e \times \text{CL}_{\text{int}} \tag{11} \]

and, combining eqs. 10 and 11,

\[ E = \frac{f_e \times \text{CL}_{\text{int}}}{Q_E} \tag{12} \]

Thus, f can be calculated from eqs. 8 and 12, using the free fraction of the drug in serum (table 1), the scaled in vivo CL<sub>int</sub> and the hepatic plasma flow (table 2). The parameters derived from in vitro studies (scaled in vivo CL<sub>int</sub> in vitro CL<sub>n</sub> and in vitro half-lives) were correlated with in vivo parameters (in vivo CL<sub>int</sub> and bioavailability) by linear regression analysis.

Results

Optimal conditions, ensuring linearity of rates of drug disappearance from the incubation medium with respect to time and microsomal protein concentration, were established at a microsomal protein content of 0.2 mg/sample and an incubation period of 15 min (except for norgestimate, which was incubated for 10 min). The CYP enzyme activity of the microsomal incubations was assessed by incubation with testosterone and was qualitatively confirmed in each assay by the presence of 6β-hydroxytestosterone at the end of the incubation period.

To examine whether binding of the progestins to microsomal proteins is likely to have an influence on the rate of metabolism in vitro, the free fractions of gestodene and levonorgestrel in the incubation medium were determined by ultrafiltration. These two drugs were chosen because they are the ones that are most extensively bound to serum proteins in vivo. In vivo, approximately 82% of gestodene was free and only approximately 18% was bound to protein. For levonorgestrel, the free and protein-bound fractions were approximately 47 and 53%, respectively. Because the two progestins were not extensively bound to microsomal proteins, total instead of free concentrations were used for further calculation of CL<sub>n</sub> values.

The parameters KM<sub>app</sub> and V<sub>max</sub> obtained for the different progestins, as well as the corresponding in vitro CL<sub>n</sub> and scaled in vivo CL<sub>n</sub> values, are presented in table 2. The in vitro CL<sub>n</sub> values were in the range of 0.046 (dienogest) to 0.309 ml/min/mg (norgestimate). For those progestins that had been administered iv to women, the in vivo CL<sub>n</sub> was calculated from total CL values (table 1) according to eq. 7. The correlation of these in vivo CL<sub>n</sub> values with the scaled in vivo CL<sub>n</sub> values is presented in fig. 1. There was a linear relation (r = 0.769) between scaled in vivo CL<sub>n</sub> and corresponding in vivo CL<sub>n</sub> values; however, the latter were approximately twice as high as the scaled values.

A correlation between the in vitro CL<sub>n</sub> values and the oral bioavailability of the investigated progestins in humans is presented in fig. 2. When desogestrel was excluded, the regression line was characterized by a correlation coefficient of r = 0.986.

In a second approach, CL<sub>n</sub> values were calculated on the basis of dose and AUC at two different drug concentrations (table 3). At a 2 μM drug concentration, the in vitro CL<sub>n</sub> values were in the range of 0.013 (desogestrel) to 0.066 ml/min/mg (norgestimate); at a concentration of 15 μM, the in vitro CL<sub>n</sub> values were in the range of 0.009 (dienogest) to 0.027 ml/min/mg (norgestimate). The correlation between the in vitro CL<sub>n</sub> values and the oral bioavailability in vivo is presented in fig. 3. When desogestrel was excluded, the regression line was characterized by correlation coefficients of −0.922 and −0.989 at drug concentrations of 2 and 15 μM, respectively. The results obtained with this approach at a 15 μM concentration were comparable to those obtained before using the Michalis-Menten parameters (fig. 2); however, the absolute CL<sub>n</sub> values differed by a factor of approximately 10 between the two data sets. Another way of assessing the metabolic stability of the progestins is to determine the disappearance half-life of drug in vitro. This was performed for each progestin at two initial concentrations (2 and 15 μM). Drug levels in the incubation medium declined, in a logarithmic-linear fashion, over the complete incubation time of 60 min; this allowed straightforward determination of the corresponding half-lives. Only for desogestrel, a somewhat more irregular time course for the drug levels was observed at a 2 μM concentration (fig. 4). The results are presented in table 3, and the correlation between in vitro half-lives and oral bioavailability...
is shown in fig. 5. Obviously, only a poor correlation was obtained between the two parameters at the low concentration of 2 \( \mu M \) \( (r = 0.439) \); a somewhat better correlation was obtained at 15 \( \mu M \) \( (r = 0.843) \).

**Discussion**

Human liver microsomes have become readily available, and they can be used for _in vitro_ studies of drug metabolism at early stages in drug development. However, it must be kept in mind that microsomal preparations may provide a suitable model only for drugs for which mainly phase I reactions are involved in drug disposition and conjugation reactions are not rate-limiting. These requirements are met by most of the progestins of the 19-nortestosterone type. Because the oral bioavailability in humans is known for a number of these progestins (Back et al., 1978, 1987a,b; Orme et al., 1991; Hümpe et al., 1987;
Tauber et al., 1989; Oettel et al., 1995), these data can be compared with the results obtained from in vitro metabolism studies, to assess the value of the in vitro model for predicting bioavailability.

A suitable parameter for the in vitro-in vivo correlation is $CL_{int}$, which can be defined as the maximal metabolic capacity of the eliminating organ (liver) for a particular drug in the absence of any flow or diffusion restrictions of drug supply. $CL_{int}$ can be expressed as the quotient of the Michaelis-Menten enzyme parameters $V_{max}$ and $K_{m}$.

\[
\text{TABLE 3}
\]

CL$_{int}$ values and in vitro half-lives for selected progestins, derived from human liver microsomal incubations with either 2 or 15 µM drug concentrations and an incubation time of 60 min

<table>
<thead>
<tr>
<th>Compound</th>
<th>2 µM $CL_{int}$ Scaled</th>
<th>15 µM $CL_{int}$ Scaled</th>
<th>$t_{1/2}$ In Vitro</th>
<th>$t_{1/2}$ In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/min/mg/liters/hr</td>
<td>ml/min/mg/liters/hr</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>3-Keto-desogestrel</td>
<td>0.02486</td>
<td>104.4</td>
<td>54.6</td>
<td>0.01600</td>
</tr>
<tr>
<td>Desogestrel</td>
<td>0.01309</td>
<td>55.0</td>
<td>68.8</td>
<td>0.01316</td>
</tr>
<tr>
<td>Norgestimate</td>
<td>0.06600</td>
<td>277.2</td>
<td>20.8</td>
<td>0.02700</td>
</tr>
<tr>
<td>Levonorgestrel</td>
<td>0.01729</td>
<td>72.6</td>
<td>78.8</td>
<td>0.01024</td>
</tr>
<tr>
<td>Gestodene</td>
<td>0.02471</td>
<td>103.8</td>
<td>47.5</td>
<td>0.00943</td>
</tr>
<tr>
<td>Norethisterone</td>
<td>0.01929</td>
<td>81.0</td>
<td>73.7</td>
<td>0.01743</td>
</tr>
<tr>
<td>Dienogest</td>
<td>0.01914</td>
<td>80.4</td>
<td>64.9</td>
<td>0.00900</td>
</tr>
</tbody>
</table>

**FIG. 3.** Correlation between the oral bioavailability of selected progestins in humans and the in vitro $CL_{int}$ values derived with initial drug concentrations of 2 µM (A) and 15 µM (B) in the incubation medium.

The regression lines are presented together with the corresponding equations and the correlation coefficients (thick lines, complete data set; thin lines, data set with desogestrel excluded).

Tauber et al., 1989; Oettel et al., 1995), these data can be compared with the results obtained from in vitro metabolism studies, to assess the value of the in vitro model for predicting bioavailability.

A suitable parameter for the in vitro-in vivo correlation is $CL_{int}$, which can be defined as the maximal metabolic capacity of the eliminating organ (liver) for a particular drug in the absence of any flow or diffusion restrictions of drug supply. $CL_{int}$ can be expressed as the quotient of the Michaelis-Menten enzyme parameters $V_{max}$ and $K_{m}$. 

\[
CL_{int} = \frac{V_{max}}{K_{m}}
\]
A \[\text{Concentration [\mu g/ml]}\]

\[
\begin{array}{c}
\text{Incubation time [min]} \\
\hline
0 & 10 & 20 & 30 & 40 & 50 & 60 & 70 \\
\hline
0.1 & 1 &  \text{B} & \text{A}\
\end{array}
\]

B \[\text{Concentration [\mu g/ml]}\]

\[
\begin{array}{c}
\text{Incubation time [min]} \\
\hline
0 & 10 & 20 & 30 & 40 & 50 & 60 & 70 \\
\hline
0.1 & 1 &  \text{B} & \text{A}\
\end{array}
\]

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure}
\caption{Semilogarithmic presentation of the drug concentration-time curves (mean values) for selected progestins in the microsomal incubation medium during the complete incubation period of 60 min (A, 2 \mu M; B, 15 \mu M initial substrate concentration).}
\end{figure}

The concept has been applied to a variety of drugs in an ever-increasing number of studies using microsomes, hepatocytes, or liver slices (Houston, 1994; Hoener, 1994; Obach et al., 1997; Lin et al., 1980; Rane et al., 1977; Sidelmann et al., 1996).

In the present study, these parameters were derived for each progestin in the in vitro experiments under conditions where the metabolic disposition rate was linear with respect to incubation time and amount of microsomal protein. The importance of non-specific binding to microsomal proteins in vitro and its implications for in vitro-in vivo correlations have been emphasized recently (Obach, 1996). In the present study, however, no correction of the \(\text{CL}_{\text{int}}\) values was deemed necessary, because levonorgestrel and gestodene, which are both >95% bound to serum proteins in vivo, showed only negligible or moderate non-specific binding to liver microsomes in vitro (Kuhnz et al., 1994a).

To scale the in vitro \(\text{CL}_{\text{int}}\) to a scaled in vivo \(\text{CL}_{\text{int}}\), an appropriate, biologically meaningful, scaling factor must be chosen. A range of approximately 40–60 mg of microsomal protein/g of liver was found in the literature for microsomal protein recovery from liver tissue; therefore, a value of 50 mg was used in the present study (Houston, 1994; Lin et al., 1980; Rane et al., 1977). Assuming that 1 g of liver tissue contains approximately 50 mg of microsomal protein and the average weight of a human liver is approximately 1,400 g (Lentner, 1981), a scaling factor of 70,000 mg can be calculated. A comparison of the scaled in vivo \(\text{CL}_{\text{int}}\) values and the corresponding in vitro \(\text{CL}_{\text{int}}\) values, which were derived from total \(\text{CL}\) values obtained after iv administration of these progestins to human volunteers, reveals a linear relation with a correlation coefficient of \(r = 0.769\) (fig. 1). However, the slope of the regression line (2.2) indicates an underestimation of in vivo \(\text{CL}_{\text{int}}\), by a factor of approximately 2, with microsomal data. This was noted by other investigators who used microsomal preparations to predict in vivo CL, and the underestimation was thought to be partly attributable to a loss of enzyme activity during microsomal preparation or perhaps to product inhibition as a consequence of the absence of phase II reactions (Houston, 1994). This may also apply to the present study, because in vivo the metabolites of the progestins are mainly conjugated with sulfuric and glucuronic acid before excretion.

There was also a linear relation between in vitro \(\text{CL}_{\text{int}}\) and the oral bioavailability of the progestins. For desogestrel, a \(\text{CL}_{\text{app}}\) value far lower than anticipated from the low oral bioavailability observed in vivo was obtained. If desogestrel was excluded, a reasonably good correlation \((r = -0.986)\) was obtained (fig. 2). It should be noted that accurate human bioavailability data are not available for norgestimate or desogestrel, because both progestins are prodrugs that are readily and almost completely metabolized to their active metabolites. Therefore, an arbitrary oral bioavailability of 5% was assumed for the purpose of the present investigation.

Obviously, in the case of desogestrel, the metabolic stability in human microsomal preparations is not a good predictor of the oral bioavailability in vivo. The explanation for this is probably that, in contrast to the other progestins, desogestrel undergoes considerable first-pass metabolism during passage through the intestinal wall, before the first liver passage. This is supported by a study investigating the metabolic degradation of desogestrel in human gut mucosa in vitro (Madden et al., 1989). Desogestrel undergoes both phase I and phase II metabolism. In addition to the formation of 3-keto-desogestrel and other hydroxylated metabolites, direct sulfation of desogestrel was observed. If this also occurs in vivo, then the present results obtained with liver microsomes only illustrate the fact that this model is not applicable to desogestrel, because one of the requirements described above (no substantial extrahepatic metabolism) is not met. Although there are also reports that demonstrate the in vitro metabolic degra- dation of norgestimate incubated with human gut mucosa (mainly deacetylation of the parent drug and only minor formation of 3-keto-norgestimate), the extent of intestinal first-pass metabolism in vivo is, in contrast to desogestrel, probably negligible, compared with the contribution of the liver to the total first-pass effect (Madden and Back, 1991). In particular, direct sulfation of norgestimate has not been observed and is very unlikely to occur, because the molecule does not contain a free hydroxyl group.

Two other approaches to evaluate the metabolic stability of the progestins in microsomal preparations and to correlate these data with the oral bioavailability were tested. One approach was to use only one initial concentration of the progestin in the incubation medium and to measure the disappearance of the drug at regular time intervals. From the drug concentration-time course, the terminal half-life of disposition was calculated. If this also occurs in vivo, then the present results obtained with liver microsomes only illustrate the fact that this model is not applicable to desogestrel, because one of the requirements described above (no substantial extrahepatic metabolism) is not met. Although there are also reports that demonstrate the in vitro metabolic degra- dation of norgestimate incubated with human gut mucosa (mainly deacetylation of the parent drug and only minor formation of 3-keto-norgestimate), the extent of intestinal first-pass metabolism in vivo is, in contrast to desogestrel, probably negligible, compared with the contribution of the liver to the total first-pass effect (Madden and Back, 1991). In particular, direct sulfation of norgestimate has not been observed and is very unlikely to occur, because the molecule does not contain a free hydroxyl group.

It is a prerequisite for this kind of study that the drug concentration in the microsomal incubation is well below the apparent \(K_{\text{M,app}}\) value. However, for the characterization and selection of new drug candidates during early development, certain limitations must be accepted. Firstly, the \(K_{\text{M,app}}\) values of new drug candidates are not known \(a\ priori\); secondly, a specific and highly sensitive analytical method (for contraceptive steroids, usually radioimmunoassay) is not available at this stage of development. A realistic approach must take into account these limitations; therefore, we chose HPLC as an analytical tool that is readily available for drug analysis and allows rapid throughput of samples. Because the limit of quantitation of this method was \(0.1\) \mu M for the progestins investigated, two different initial drug concentra-
tions (2 and 15 μM), which were thought to be high enough to allow drug concentrations to be measured over the entire incubation period, were chosen. For the progestins investigated, maximal serum concentrations observed in women are between 15 and 200 nM, well below the chosen in vitro concentrations (Kuhnz et al., 1992a,c, 1993; Oettel et al., 1995). If one also takes into account the fact that these progestins are highly bound to serum proteins and only 1–10% are present in free form, it becomes obvious that the in vitro concentrations of the progestins were approximately 1000-fold higher than the free in vivo concentrations. On the other hand, 1–20 nM concentrations of the progestins can be measured only by highly sensitive and specific analytical methods (e.g., radioimmunoassay or GC/MS), which are usually not available and cannot be easily established within a reasonable time for each drug candidate at an early stage of development.

The quality of the correlation between the oral bioavailability and the in vitro CL\text{int} values was strongly dependent on the initial drug concentration used (fig. 3). When desogestrel was excluded from the evaluation, a similarly good correlation \( r = -0.989 \) was obtained at the 15 μM drug concentration, compared with that previously established from \( V_{\text{max}} \) and \( K_{M,\text{app}} \) values. At the 2 μM concentration, however, the progestins with intermediate bioavailability (norethisterone and 3-keto-desogestrel) were almost indistinguishable in their in vitro CL\text{int} values from the progestins with almost complete bioavailability (gestodene, levonorgestrel, and dienogest).

The situation becomes even less favorable when only the in vitro half-lives are correlated with the corresponding oral bioavailabilities of the progestins. The drug concentrations used (2 and 15 μM) were, except for 3-keto-desogestrel and norgestimate, well below the \( K_{M,\text{app}} \) values of the progestins, but even when these two drugs were excluded from the evaluation only a poor correlation was obtained (fig. 5). At the 2 μM concentration, for example, the half-life of norethisterone (a progestin that exhibits a considerable first-pass effect in vivo) is longer than the half-lives of gestodene and dienogest, both of which are almost completely bioavailable. Furthermore, levonorgestrel and gestodene differ in their half-lives by almost a factor of 2, although both progestins are nearly completely bioavailable. At the initial drug concentration of 15 μM, there is at least a gross separation
between progestins with high, intermediate, and low bioavailabilities; however, the data points are more scattered than with the previous two approaches using in vitro CL_{int} values.

The principal problem in using in vitro concentrations that are close to or even above the K_{Mapp} value has already been mentioned; however, it remains unclear why, in the present study, better correlations with in vivo data were obtained with the 15 μM concentration, compared with the 2 μM concentration. Adequate metabolic turnover of substrate was achieved at both concentrations for each of the progestins. One possible explanation may be that at 2 μM substrates bind preferentially to high-affinity enzymes with low turnover rates, whereas at a higher substrate concentration other enzymes with lower affinities but higher turnover rates may also be saturated and contribute significantly to the total turnover rate. In any case, there was not a satisfactory correlation between in vitro half-life and oral bioavailability in vivo with either 2 or 15 μM concentrations. On the other hand, positive experiences have been reported by others, who determined the in vitro half-lives for a large variety of compounds at a single low initial concentration of 1 μM and used these data to predict in vivo CL in humans. Their results based on in vitro half-life data were fairly comparable to those obtained from enzyme kinetic data (Obach et al., 1997).

In conclusion, the present study has shown that the in vitro model of human hepatic microsomal preparations is a suitable tool for the prediction of the oral bioavailability of 19-nortestosterone progestins in vivo. This applies for progestins with low, moderate, and high bioavailabilities, which are predominantly metabolized by the liver. The limits of the model are reached in cases (like desogestrel) where there is a substantial contribution of intestinal metabolism to the first-pass effect in vivo. To obtain reliable and meaningful results, it is recommended that CL_{int} values be calculated from Michaelis-Menten enzyme parameters, which were determined under linear conditions. Other approaches, such as simple half-life determinations at single initial drug concentrations, are less time-consuming but may lead to erroneous results if the single drug concentration chosen is inappropriate.

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