The weak opioid codeine is widely used in the management of pain. The major metabolic pathway of codeine is the formation of codeine-6-glucuronide, a minor extent codeine is metabolized via O-demethylation to morphine and via N-demethylation to norcodeine (Yue et al., 1989; Chen et al., 1991; Vree and Verwey-van Wissen, 1992). There is good evidence that the analgesic effect of codeine is mediated by its O-demethylated metabolite morphine. This biotransformation step is catalyzed by cytochrome P450 2D6, which exhibits a genetic polymorphism. Five to 10% of the population who are designated as “poor metabolizers” do not express the functional enzyme. As a consequence after the administration of codeine no analgesia is observed since only trace amounts of morphine are formed (Poulsen et al., 1996; Eckhardt et al., 1998). The World Health Organization proposes a three-stage approach for the treatment of chronic pain, where on step two weak opioids are to be combined with nonsteroidal anti-inflammatory drugs (NSAIDs). There are several studies demonstrating the benefit of the combination of NSAIDs with opioids in comparison to opioids alone (Hodsmann et al., 1987; Strobel, 1992; Björkman et al., 1993; Mercadante et al., 1997; Montgomery et al., 1996). The additive effect is thought to be caused by known different pharmacodynamic mechanisms as opioids displaying their analgesic activity in the central nervous system via opioid receptors, NSAIDs affecting the synthesis of inflammatory prostaglandins via inhibition of the enzyme cyclooxygenase. However, pharmacokinetic interactions cannot be excluded although now only limited knowledge about pharmacokinetic interactions between opioids and NSAIDs is available. Recently, in vitro studies with human liver microsomes revealed that glucuronidation of the structural analog dihydrocodeine was inhibited by diclofenac and naproxen (Kirkwood et al., 1998). The aim of the present study was to investigate whether codeine glucuronidation can be inhibited by diclofenac in vitro and to assess the contribution of the gut wall and the liver to the presystemic glucuronidation of codeine. Because tissue samples of liver and gut wall were obtained from the same individual, interindividual variability in expression of the UGTs involved in codeine-6-glucuronidation could be excluded.

Materials and Methods

Chemicals and Reagents. Codeine phosphate-hemihydrate was purchased from Merck (Germany), codeine-6-glucuronide and codeine-6-glucuronide-d3, from Lipomed, via Inresa Arzneimittel GmbH, Freiburg, Germany; diclofenac sodium, Tris-HCl, Tris, and uridine 5′-diphosphoglucuronic acid (UDPGA), peptatin, leupetin, and EDTA were purchased from Sigma (Taufkirchen, Germany). Pefabloc was purchased from Roth (Karlsruhe, Germany). All other reagents and solvents were purchased from commercial sources and were of analytical reagent grade.

Human Liver and Small Intestine Tissue. Liver tissue was obtained from three male donors (age range 68–79 years) undergoing duodenopancreaectomy; in two of them, additional tissue of small intestine was obtained. The tissue sampling was approved by the local Ethics Committee and each donor gave written informed consent before study entry. The study was carried out in accordance to the Declaration of Helsinki. 

Organ Procurement. After resection, liver biopsies were directly stored on ice and frozen in liquid nitrogen within 10 min. Frozen liver tissue was ground in a ball mill (Braun Biotech, Melsungen, Germany) at 2500 rpm for 2 min. Ground tissue was suspended in protein storage buffer (100 mM Tris, pH 7.4, 1 mM Pefabloc, 1 mM EDTA, 1 μg/ml leupetin, and 1 μg/ml peptatin) and homogenized with a motor-driven glass Teflon homogenizer at 1000 rpm for 2 min. The homogenate was further sonicated at 12 W for 30 s (Sonopuls HD 200, Bandelin, Berlin, Germany). Homogenates were stored at –80°C until
Enzyme Kinetic Studies. Incubations were performed using a modification of the assay described by Yue et al. (1990). Incubations were carried out in a final volume of 250 μl containing 5 μM to 10 mM codeine phosphate, 50 μg of protein homogenate, 5 mM MgCl₂, and 100 mM Tris, pH 7.4. Reactions were started by the addition of 10 mM UDPGA and was stopped after 60 min by adding 1.5 ml of ice-cold ethanol. The incubation tubes were vortexed and placed on ice, 100 pmol of internal standard (codeine-6-glucuronide-d₃) was added. After centrifugation (10,000 g, 10 min), supernatant was taken and evaporated under nitrogen gas at 37°C. The residue was dissolved in mobile phase containing 94% distilled water, 5% acetonitrile, and 1% acetic acid. Formation of codeine-6-glucuronide in relation to time and protein concentration was linear under given conditions. Production of codeine-6-glucuronide was not observed in negative controls, which did not contain UDPGA. The formation of codeine-6-glucuronide was calculated as picomoles formed per minute per milligram of protein.

Inhibition Studies with Diclofenac. Inhibition studies were performed on liver homogenates with a final concentration of 0.5, 5, 10, 50, and 100 μM diclofenac (methanolic solution) on three different concentrations of codeine phosphate (0.1, 1, and 10 mM). In the final concentration 1% methanol produced an average inhibition of glucuronidation of less than 5%.

Analysis of Codeine-6-Glucuronide. Codeine-6-glucuronide was determined with HPLC electrospray mass spectrometry analogous to a previously published method (Schänzle et al., 1999) with minor modifications. The mobile phases used for HPLC were: A) 1% acetic acid in water and B) 1% acetic acid in acetonitrile. HPLC separation was achieved on a LiChrospher 100 RP-18 end-capped analytical column (125 x 3-mm i.d., 5 μm particle size) at a flow rate of 0.5 ml/min using a linear gradient from 8% B to 40% B in 8 min. The mass spectrometer (HP 1100 MSD, Hewlett-Packard, Waldbronn, Germany) was operated in the selected ion monitoring mode using the respective MH⁺ ions, m/z 476 for codeine-6-glucuronide and m/z 479 for codeine-6-glucuronide-d₃. Calibration points ranged from 1 to 1000 pmol of codeine-6-glucuronide. Coefficient of variation (CV) of the interassay variability ranged between 0.7 and 3.2%.

Data Analysis. The Michaelis-Menten constant Kₘ and the maximum velocity Vₘₐₓ for codeine glucuronidation and the inhibition constant Kᵢ for diclofenac were calculated using the GRAPHER 4.04 software (Erithacus Software Ltd., Surrey, UK, 1998). The untransformed kinetic data (mean of duplicates) were fitted to a one-enzyme Michaelis-Menten equation. The intrinsic clearance (Clᵢ) was calculated as Vₘₐₓ/Kₘ. To determine diclofenac-Kᵢ, the model of competitive inhibition and noncompetitive inhibition, using the equations \( V = V_{max}S(K_m(1 + (S/K_i)) + S) \) and \( V = V_{max}S(K_m(1 + (S/K_i)) + (S(1 + (S/K_i))) \), were investigated. Goodness of fit was assessed using the \( \chi^2 \) test.

Results and Discussion

The formation of codeine-6-glucuronide in homogenate of human liver and enterocyte homogenate of exhibited Michaelis-Menten kinetics are shown in Table 1 and Fig. 1. \( V_{max} \) of codeine-6-glucuronidation in liver homogenate was on average 93.6 ± 35.3 pmol/mg/min; in enterocyte homogenate, \( V_{max} \) was less than 10% compared to liver (8.5 ± 3.8 pmol/mg/min). The Kᵢ values were in the milli-molar range (Table 1) and not different between liver (1488 ± 253 μM) and enterocytes (1857 ± 413 μM). The Kᵢ values were similar to those in human liver microsomes (Kᵢ = 2.1 mM) reported by Yue et al. (1990). The calculated average intrinsic clearance of codeine-6-glucuronidation in liver homogenate was 0.062 μl/min/mg. The intrinsic clearance in enterocyte homogenates was only 7.3% of the intrinsic clearance in corresponding liver homogenate. We therefore conclude that the small intestine contributes only to a minor extent to total codeine glucuronidation.

**TABLE 1**

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Kₘ</th>
<th>S.E.</th>
<th>Vₘₐₓ</th>
<th>S.E.</th>
<th>Kᵢ</th>
<th>χ²</th>
<th>Clᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver donor no. 1</td>
<td>1547</td>
<td>55.8</td>
<td>89.0</td>
<td>1.23</td>
<td>9.1</td>
<td>1.91</td>
<td>0.058</td>
</tr>
<tr>
<td>Liver donor no. 2</td>
<td>1707</td>
<td>64.6</td>
<td>131</td>
<td>1.86</td>
<td>6.8</td>
<td>2.12</td>
<td>0.050</td>
</tr>
<tr>
<td>Liver donor no. 3</td>
<td>1211</td>
<td>40.8</td>
<td>60.8</td>
<td>0.68</td>
<td>0.68</td>
<td>0.68</td>
<td>0.005</td>
</tr>
<tr>
<td>Small intestine donor no. 2</td>
<td>2149</td>
<td>311</td>
<td>11.1</td>
<td>0.51</td>
<td>0.01</td>
<td>0.01</td>
<td>0.004</td>
</tr>
<tr>
<td>Small intestine donor no. 3</td>
<td>1565</td>
<td>219</td>
<td>5.80</td>
<td>0.28</td>
<td>0.02</td>
<td>0.02</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Fig. 1.** Codeine-6-glucuronide formation from codeine in liver homogenate from three different donors (mean of duplicates ± S.D.). A, codeine-6-glucuronide formation from codeine in liver homogenate from three different donors (mean of duplicates ± S.D.). B, codeine-6-glucuronide formation from codeine in small intestine homogenate from two different donors (donors 2 and 3) (mean of duplicates ± S.D.).
DICLOFENAC INHIBITS GLUCURONIDATION OF CODEINE

In two of the liver homogenates inhibition studies were performed with five different concentrations (0.5, 5, 10, 50, and 100 μM) of diclofenac and three different concentrations of codeine (0.1, 1, and 10 mM). Diclofenac produced noncompetitive inhibition of codeine glucuronidation with Ki values of 9.1 μM (χ² 1.91) and 6.8 μM (χ² 2.12) (Fig. 2).

In our studies, diclofenac was a potent noncompetitive inhibitor of codeine glucuronidation in the given concentration range with an average Ki value of 7.9 μM. Recently, it was shown that diclofenac at a concentration of 50 μM produced in vitro an inhibition >50% of dihydrocodeine glucuronidation, which is a structural analog of codeine (Kirkwood et al., 1998). In contrast, diclofenac and various other NSAIDs do not alter the formation of morphine from codeine (Dayer et al., 1992), which is mediated by the polymorphic CYP2D6. We therefore performed only glucuronidation experiments.

Interestingly, morphine, amitriptyline, diazepam, probenecid, and chloramphenicol inhibited codeine glucuronidation in human liver microsomes (Yue et al., 1990). Metabolic interactions have also been described with morphine and oxazepam at the conjugation level, as oxazepam inhibits glucuronidation of morphine in human liver microsomes (Säwe et al., 1982).

Expressed UGT2B7 has recently been shown to catalyze codeine-6-glucuronidation. In this system the rates of codeine-6-glucuronidation are about 1% of the rate of morphine glucuronidation (Coffmann et al., 1997). UGTs are expressed primarily in the liver but also in kidney, brain, skin, and the luminal surface of the intestinal epithelium. The presence of UGTs in extrahepatic tissues might be due to tissue-specific variations in UGT expression (Burchell and Coughtrie, 1989). Screening for RNA expression by reverse transcription-polymerase chain reaction recently exhibited the expression of UGT2B7 in all intestinal segments (jejunum, ileum, and colon) from six human subjects (Randominska-Pandya et al., 1998).

The inhibition of codeine glucuronidation might have different clinical implications: glucuronidation is the major metabolic pathway of codeine, whereas only a small amount of codeine (less than 10%) is O-demethylated to the pharmacologically active metabolite morphine. Because there is no inhibition of codeine O-demethylation to morphine by diclofenac (Dayer et al., 1992), it can be speculated that due to inhibition of codeine glucuronidation the amount of codeine available for other pathways, especially O-demethylation to morphine, is increased, resulting in higher morphine serum levels and therefore higher analgesic efficacy. This has to be verified in further pharmacokinetic and pharmacodynamic in vivo studies.

In summary, these data suggest a potential involvement of a pharmacokinetic interaction in the diclofenac codeine combination therapy of moderate pain suggested by the World Health Organization.

Acknowledgments. We appreciate the excellent technical assistance of S. Seefried and M. Pecia.

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