

In vitro UGT assay for inhibition studies of benzodiazepines and opiates during phase II-metabolism

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Introduction

Hydroxy-benzodiazepines and opiates are metabolized through glucuronidation which is the predominant pathway in the clearance mechanism of exogenous and endogenous substances during phase II-metabolism. The reaction is catalyzed by uridine-diphosphoglucuronyltransferases (UGTs) [1]. The presented work is motivated by the fact that the combination of benzodiazepines and opiates is common both in clinical practice and as combined abuse. Inhibition of the glucuronidation can lead to a decelerated elimination of the phase I-metabolites, respectively of substances which are subjects of the phase II-metabolism. In the case of pharmacological and toxicological active substances, like the hydroxy-benzodiazepines and opiates, this can result in a prolonged pharmacological activity and in a higher risk of side effects of these substances [2, 3]. Therefore an in vitro UGT-assay for inhibition studies between hydroxy-benzodiazepines and opiates has been developed and optimized using pooled human liver microsomes (HLMs).

Methods

For the investigation of the interaction capabilities of some benzodiazepines and opiates during phase II-metabolism an in vitro assay was developed for oxazepam and temazepam (10-1000 μmol), morphine and codeine (2.5, 5 and 10 mM) using pooled human liver microsomes (HLMs, BD Bioscience) with a total protein concentration of 1.0 mg/ml. As shown in Fig. 1, the maximum of the enzymatic activity of the UGTs was achieved at a pH of 9 using 50 mM Tris buffer, 40 °C, 8 mM UDPGA, and an incubation time of 240 min. It is striking, that the pH- and temperature-values do not reflect physiological conditions. Therefore, the incubations were performed at 37 °C in 50 mM Tris buffer (pH 7.4) with 5 mM MgCl_2 , 8 mM UDPGA, and alamethicin (50 $\mu\text{g}/\text{mg}$ microsomal proteins) in a total volume of 100 μl for 240 min. After quenching of the enzymatic activity and centrifugation, the supernatant was analyzed by HPLC/DAD.

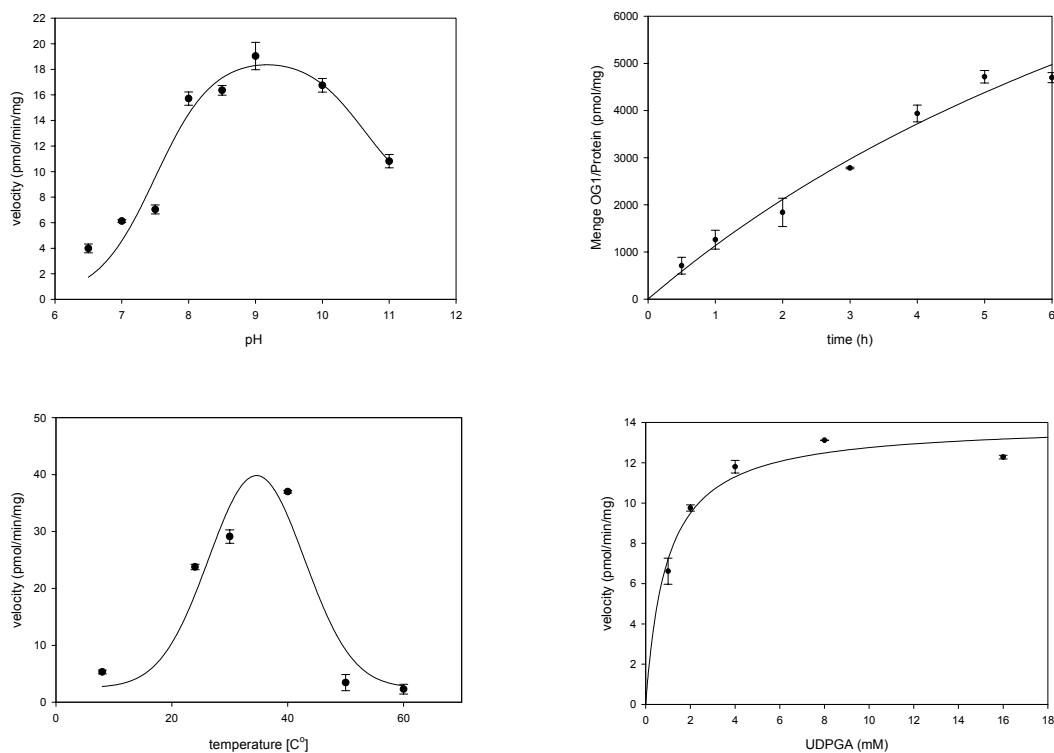


Fig. 1: The maximum of enzymatic activity of the UGT-enzyme superfamily could be archived at pH 9 in 50 mM Tris buffer, incubation time of 4 h, 40 °C and 8 mM and UDPGA.

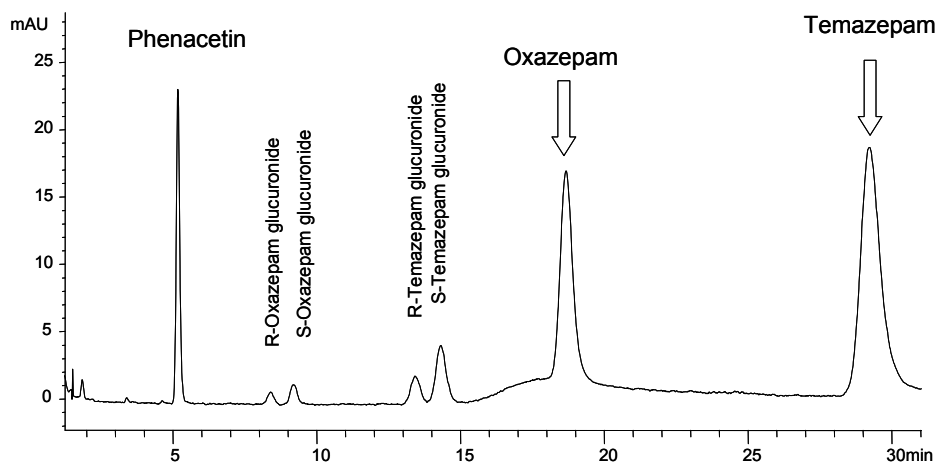


Fig. 2: Chromatographic separation of the diastereomeric benzodiazepine-glucuronides and its aglycons. Phenacetin was used as internal standard.

Chromatographic separation (Fig. 2) of the diastereomeric benzodiazepine glucuronides was possible using a RP-C₁₈ column (Spherisorb ODS2, 5 μm, 250 x 4 mm, Trentec), an column oven temperature of 40 °C, and an isocratic mobile phase (flow rate 1.7 mL/min.) consisting of 0.3 % phosphoric acid (78 %),

acetonitrile (16 %), and isopropanol (6 %). For morphine-3- and morphine-6-glucuronide a mobile phase consisting of 98 % 10 mM phosphoric acid (pH 2.7) and 2 % acetonitrile was used and for codeine-6-glucuronide the concentration was 93 % 10 mM phosphoric acid (pH 2.7) and 7 % acetonitrile.

Results

K_m and V_{max} values have been evaluated for both enantiomers of temazepam and oxazepam with two batches of HLMs (Fig. 3).

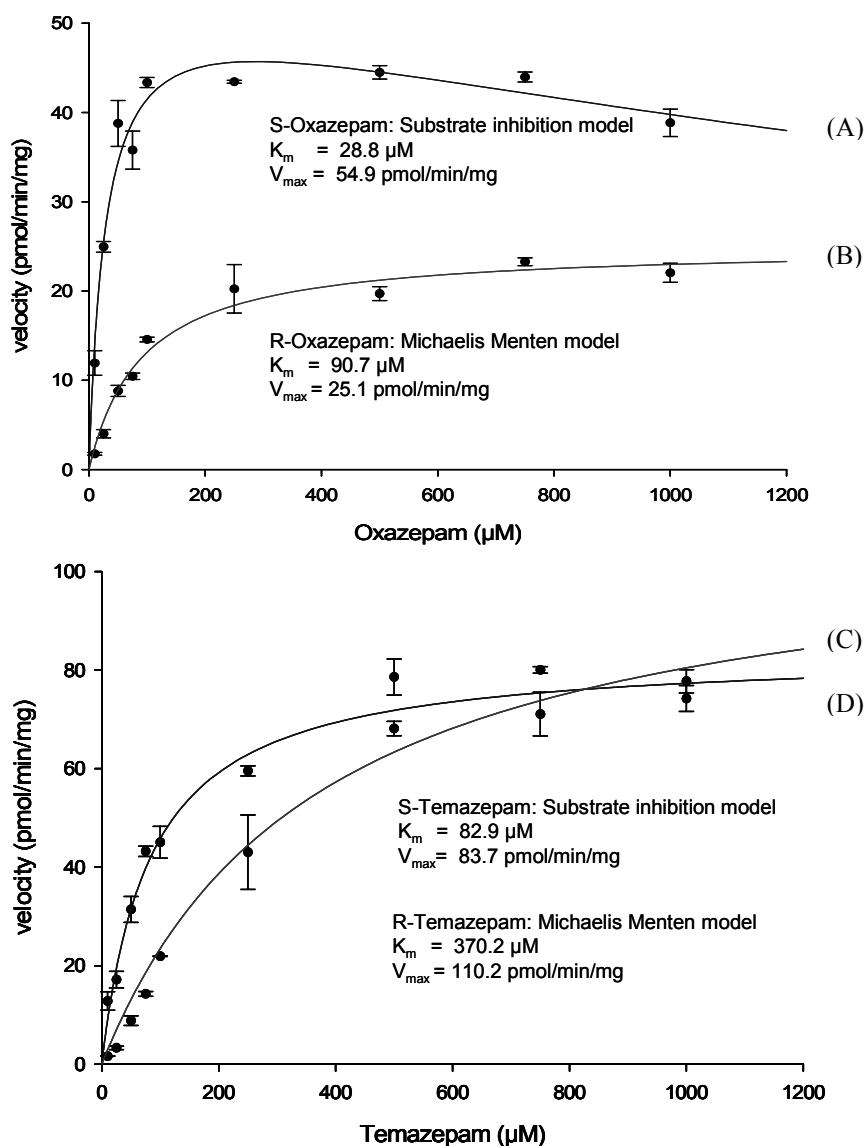


Fig. 3: Enzyme kinetic data of S-oxazepam (A), R-oxazepam (B), R-temazepam (C), and S-temazepam using HLMs.

The results show that the K_m values for S-oxazepam (28.8 \pm 5.3 and 37.1 \pm 9.6 μmol) and S-temazepam (77.4 \pm 12.6 and 82.9 \pm 7.7 μmol) were lower than for R-enantiomers (R-oxazepam (90.7 \pm 12.6 and 104.9 \pm 10.6 μmol), R-temazepam (336.4 \pm 37.6 and 370.2 \pm 94.6 μmol)). The determined K_m -values of R- and S-oxazepam indicating a higher affinity to the UGT-enzyme superfamily as the enantiomers of temazepam. Furthermore the measured K_m -values are indicating a higher UGT-affinity of the S-enantiomers unlike the R-enantiomers of the benzodiazepines oxazepam and temazepam. Inhibition studies between the benzodiazepines oxazepam, temazepam and the opiates morphine and codeine as inhibitors showed that the K_i -values for S-enantiomers are smaller than for R-enantiomers, which is exemplarily shown in Fig. 4.

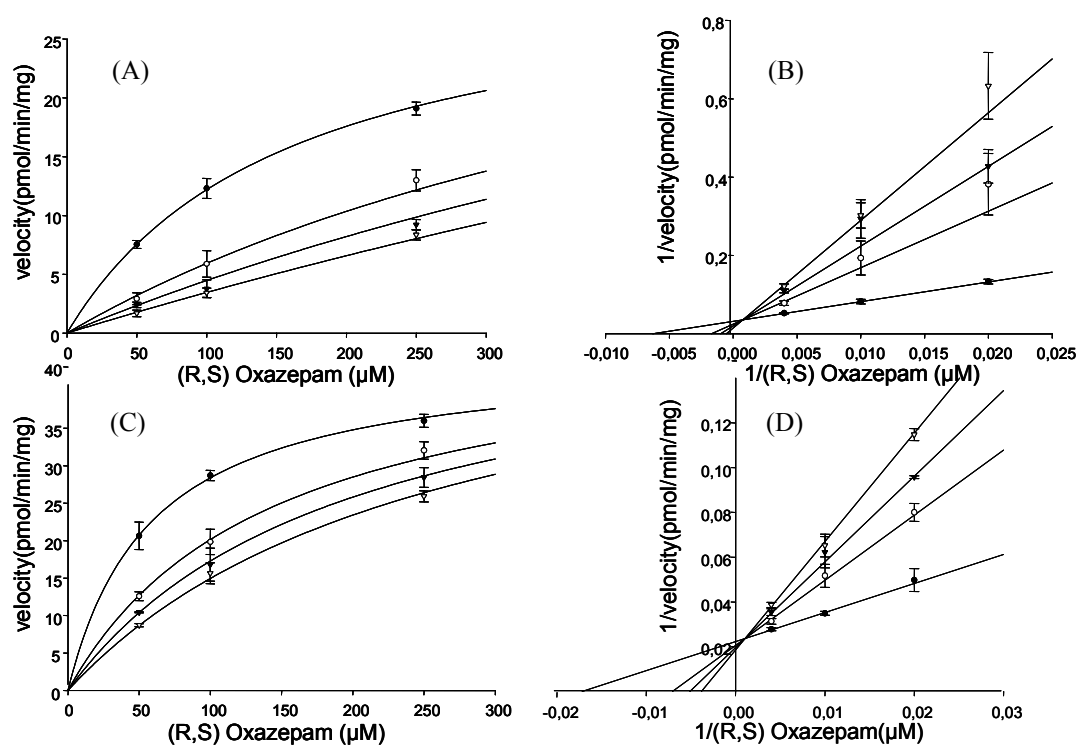


Fig. 4: Michaelis-Menten plot (left) and Lineweaver-Burk plot (right) of R-oxazepam (A/B) and S-oxazepam (C/D) inhibited by 0 μM (-●-), 2.5 μM (-○-), 5.0 μM (-▼-), and 10.0 μM (-▽-) morphine.

By using the benzodiazepines as inhibitors, temazepam showed the highest inhibitory effect on the glucuronidation of morphine, whereas oxazepam inhibited the codeine glucuronidation most. In addition, the glucuronidation of codeine was much more affected by the benzodiazepines than it was observable for morphine.

Conclusion

With the developed and optimized in vitro assay, consisting of pooled human liver microsomes, it was possible to analyze the interaction potentials between the hydroxy-benzodiazepines oxazepam and temazepam and the opiates morphine and codeine. Furthermore it was possible to consider the enantiomer specific differences of the benzodiazepine-glucuronidation. The results show that S-enantiomers of the benzodiazepines exhibit a higher affinity to the UGTs than the R-enantiomers do. This can be deduced from the lower K_m -values of the S-enantiomers compared to the R-enantiomers. The glucuronidation of morphine was more negatively affected by temazepam than by oxazepam. In addition to these findings the benzodiazepines exhibits a higher inhibitory effect on the codeine glucuronidation as on the morphine glucuronidation. Furthermore, the codeine itself is a stronger inhibitor of the benzodiazepine-glucuronidation compared to morphine. In particular, the glucuronidation of the S-enantiomers are more affected by the opiates than the R-enantiomers. This is feasible by the fact that the S-enantiomers exhibits a higher UGT-affinity as mentioned before. It is thought, that S-oxazepam is more active on the GABA_A-receptor than R-oxazepam [4]. Due to this, it can be derived that the inhibition of the S-enantiomer glucuronidation of the benzodiazepines by morphine and codeine can contribute to the side effects of the benzodiazepines.

In fact, UGT-catalyzed metabolic reactions account for about 35 % of all drugs which undergo phase II-metabolism [5]. Assuming, there is a high number of other substances which can at least partly inhibit the glucuronidation, drug interactions based on inhibitory effects during the phase II-metabolism, becomes obvious [2, 3].

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References

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