Enzyme-assisted synthesis and characterization of benzodiazepine glucuronides

T. Pallmann, M. Wagner, M. Thevis, U. Jonas, M. A. Rothschild, H. Käferstein, K. Bender

Introduction

Glucuronidation is the predominant pathway in the clearance mechanism of exogenous and endogenous substances during phase II-metabolism, catalyzed by uridine-diphosphoglucuronyltransferases (UGTs). Not all phase I-metabolites are pharmacological or toxicological inactive. Phase I-metabolites which undergo phase II-metabolism can accumulate or their elimination is delayed if the enzymes of the phase II-metabolism are inhibited. In case of pharmacological active phase I-metabolites this can cause elongated drug activities and a higher risk of side effects. Interactions during metabolism are expected when substances compete against the same metabolizing enzymes. Lorazepam and the diazepam phase I-metabolites oxazepam and temazepam, as well as the opiates morphine and codeine are extensively glucuronidated. Therefore, interactions during phase II-metabolism can be expected. Because of the fact, that opiates and benzodiazepines are intensely medically prescribed and on the other hand are highly abusive substances; these substances are of particular pharmacological and forensic interest.

The identification and quantification of the metabolites in body fluids is indispensable to study the metabolic pathways of exogenous substances. The aim of this study was the synthesis of O-glucuronide conjugates of the benzodiazepines temazepam, oxazepam and lorazepam as analytical reference substances. Since the synthetic coupling of the hydroxy-benzodiazepines with glucuronic acid was not successful, an enzyme-assisted synthesis strategy was developed. The obtained pure R- and S-enantiomers were fully characterized by ^{1}H-NMR- and MS/MS-spectroscopy. Enzyme-assisted synthesis of other glucuronides, except of the benzodiazepine-glucuronides, are described in literature [1,2].

Methods

Liver microsomes have been obtained from fresh swine liver by differential ultra-centrifugation [3]. The total protein concentration of the microsomes was determined by using the bicinchonic acid (BCA)-method (BCA-kit obtained from Interchim). The synthesis assay contained 36 mg of microsomal protein, 1 mM of

oxazepam, temazepam or lorazepam dissolved in 3 ml methanol, 5 mM $MgCl_2$, 5 mM UDPGA, and 0.12 mg Brij in 50 mM Tris-buffer (pH 7.4) at 37° C in a final volume of 30 ml. After the incubation time of 24 h the reaction was stopped by using ice cold dichloromethane. Preparative HPLC (RP-C_{18} column: Spherisorb ODS2, 5 µm, 250 x 10 mm, Trentec, at room temperature, and an isocratic mobile phase (flow rate 7.5 ml/min.) consisting of 0.3 % phosphoric acid (78 %), acetonitrile (16 %) and isopropanol (6 %) and solid phase extraction have been used to gain the diastereomeric pure forms of the glucuronides. The glucuronides were characterized by HRMS, MS/MS- and ^{1}H-NMR-spectroscopy.

Results

It was possible to obtain the benzodiazepine glucuronides with a yield of 10-28 % (table 1).

Table 1: Yields of the enzymatic synthesis products.

Substance	Product	Yield [%]
Oxazepam	R-oxazepam-glucuronide	16
	S- oxazepam-glucuronide	10
Temazepam	R-temazepam-glucuronide	25
	S- temazepam-glucuronide	28
Lorazepam	R-lorazepam-glucuronide	26
	S-lorazepam-glucuronide	10

Employing ESI-MS on the LTQ Orbitrap mass analyzer it was possible to determine the elemental compositions of the benzodiazepine glucuronides with a mass accuracy < 1 ppm in comparison to the theoretical values. MS/MS experiments showed the expected loss of the glucuronic acid moiety generating the ion $(M+H)^+$ generally followed by consecutive eliminations of water and carbon monoxide, as observed.

^{1}H-NMR-spectroscopy supports the MS/MS results. ^{1}H-NMR experiments showed the typical 8-carbon coupling constant of the benzene ring. Furthermore the β-D-configuration of the sugar was confirmed by the determined vicinal coupling constant $^3J_{G1, G2}$. The determined coupling constants of G1 of the S-glucuronides were 7.5 Hz and for the R-glucuronides 7.8 Hz. Differneces in the stereochemistry of the R- and S-glucuronides could be shown in the different chemical shifts of the proton on position 3 and position G1. The proton of the 3-carbon and the proton on the position G1 showed a higher chemical shift for all S-enantiomers than for the R-enantiomers as shown in Fig. 1.

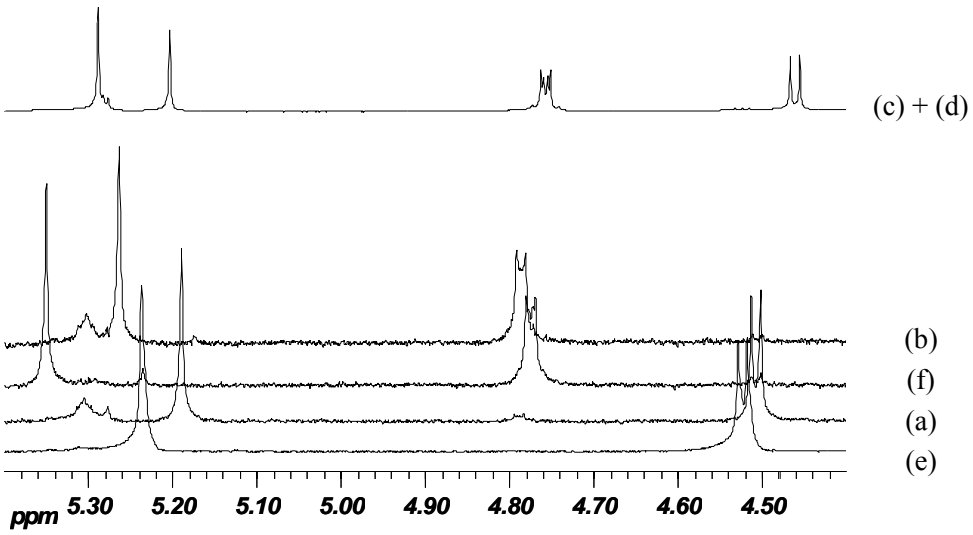

Fig. 1. ^{1}H-NMR measurements were carried out at 306K in CD_3CN/D_2O on the 500 and 700 MHz Spectrometer: (a): R-oxazepam-glucuronide, (b): S-oxazepam-glucuronide, (c+d): R-temazepam-glucuronide and S-temazepam-glucuronide, (e): R-lorazepam-glucuronide, (f): S-lorazepam-glucuronide.

Conclusion

By using the presented enzyme assisted synthesis it was possible to synthesize glucuronides of temazepam, oxazepam and lorazepam of mg-amounts. The glucuronides have been fully characterized by HRMS, MS/MS- and NMR-spectroscopy. As a result they can be used as analytical reference material.

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Corresponding author:
Dr. Katja Bender
Institut für Rechtsmedizin
Klinikum der Universität zu Köln
E-Mail: katja.bender@uk-koeln.de