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HT-2 Toxin 4-Glucuronide as New T-2 Toxin Metabolite: Enzymatic Synthesis, Analysis, and Species Specific Formation of T-2 and HT-2 Toxin Glucuronides by Rat, Mouse, Pig, and Human Liver Microsomes

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ABSTRACT: Glucuronides of the mycotoxin T-2 toxin and its phase I metabolite HT-2 toxin are important phase II metabolites under *in vivo* and *in vitro* conditions. Since standard substances are essential for the direct quantitation of these glucuronides, a method for the enzymatic synthesis of T-2 and HT-2 toxin glucuronides employing liver microsomes was optimized. Structure elucidation by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry revealed that besides T-2 toxin glucuronide and HT-2 toxin 3-glucuronide also the newly identified isomer HT-2 toxin 4-glucuronide was formed. Glucuronidation of T-2 and HT-2 toxin in liver microsomes of rat, mouse, pig, and human was compared and metabolites were analyzed directly by liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS). A distinct, species specific pattern of glucuronidation of T-2 and HT-2 toxin was observed with interesting interindividual differences. Until recently, glucuronides have frequently been analyzed indirectly by quantitation of the aglycone after enzymatic cleavage of the glucuronides by β-glucuronidase. Therefore, the hydrolysis efficiencies of T-2 and HT-2 toxin glucuronides using β-glucuronidases from *Helix pomatia*, bovine liver, and *Escherichia coli* were compared.

KEYWORDS: *mycotoxin, Fusarium, trichothecene, T-2 toxin, HT-2 toxin, liver microsomes, glucuronidation, β-glucuronidase, enzymatic synthesis, glucuronide, LC−MS/MS, NMR, rat, mouse, pig, human*

INTRODUCTION

The mycotoxins T-2 toxin and HT-2 toxin are toxic secondary fungal metabolites, produced by several *Fusarium* species like *Fusarium sporotrichioides*, *Fusarium poae*, or *Fusarium langsethiae*. These fungi infect crops in the field or during storage. Therefore, T-2 toxin and HT-2 toxin are common contaminants in cereals like wheat, barley, maize and especially oat and products thereof. T-2 and HT-2 toxin are prominent members of the group of trichothecene mycotoxins, which are characterized by a tetracyclic sesquiterpenoid 12,13-epoxy-trichothec-9-ene structure (Figure 1).[1,2] Among the toxic effects observed for T-2 toxin are inhibition of protein and DNA synthesis, induction of apoptosis, and immuno- and hematotoxicity. HT-2 toxin is not only a natural contaminant in cereals, but also the main metabolite of T-2 toxin. Toxic effects can partly be ascribed to HT-2 toxin, and for this reason, a tolerable daily intake of 100 ng/kg b.w. was recently established for the sum of T-2 and HT-2 toxin.[2,3]

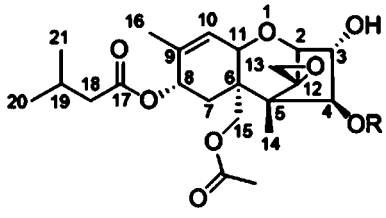

Figure 1. Chemical structure of T-2 toxin and HT-2 toxin.

T-2 toxin is rapidly metabolized and depending on the test system and the animal species a variety of metabolites is formed. Main reactions involved in T-2 metabolism are hydrolysis, hydroxylation, de-epoxidation, acetylation and conjugation to polar moieties. Deacetylation of T-2 toxin at position 4 forming HT-2 toxin is one of the most important phase I metabolic reactions, shown for example in microsomes,[4] human cells in primary culture[5,6] or animals.[3,7,8] Additionally, phase II reactions, especially glucuronidation of T-2 toxin, HT-2 toxin and further phase I metabolites, are essential for the metabolism and excretion. The incubation of T-2 toxin with rat liver microsomes in the presence of uridine 5′-diphosphoglucuronic acid (UDPGA) leads to the formation of HT-2 toxin 3-glucuronide.[9]

After administration of tritium labeled T-2 toxin and analysis of the metabolites by thin layer chromatography (TLC) combined with radioactivity detection it was observed in several *in vitro* and *in vivo* studies that a considerable percentage of the radioactivity remained at the initial spotting sites of the TLC plates. These polar substances were supposed to be glucuronides. When these samples were subsequently treated with β-glucuronidase, mainly HT-2 toxin was liberated. After perfusion of rat liver for 1 h with tritium labeled T-2 toxin, for

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example, analysis of the bile showed that polar conjugates remaining at the initial spotting site of the TLC plate accounted for 88% of the radioactivity in bile. After hydrolysis of these polar compounds with β-glucuronidase HT-2 toxin accounted for 80% of the radioactivity, 3′-hydroxy HT-2 toxin for 11% and T-2 tetraol for 1%.[10] In another study, about half of the 34% of radioactivity that appeared at the initial spotting site of TLC plates after 24 h of incubation of rat hepatocytes with T-2 toxin was associated with HT-2 toxin.[11]

When rats were administered tritium labeled T-2 toxin intravascularly or intraduodenally, roughly half of the radioactivity in bile was linked to 3′-hydroxy HT-2 toxin and 25% to polar compounds remaining at the initial spotting site of the TLC plate. Enzymatic treatment of these polar fractions resulted in 59% HT-2 toxin and only 17% 3′-hydroxy HT-2 toxin, although this was the main unconjugated metabolite in bile.[12]

Mainly T-2 toxin, HT-2 toxin, 3′-hydroxy T-2 toxin and 3′-hydroxy HT-2 toxin were detected in bile and urine of pigs 4 h after intravascular dosing with T-2 toxin. Enzymatic treatment revealed that only about half of the 3′-hydroxy HT-2 toxin was present in the form of its glucuronide, whereas T-2 and HT-2 toxin were almost completely conjugated to glucuronic acid, thus emphasizing the importance of HT-2 toxin glucuronide as a metabolite of T-2 toxin. Overall, glucuronides represented about 77% of the radioactivity excreted in the bile and 63% of the radioactivity excreted in the urine of these pigs.[13]

In the studies cited above, analysis of glucuronides was done indirectly by hydrolysis with β-glucuronidase. This widely employed method for the quantitation of glucuronides compares the amount of a metabolite before and after enzymatic cleavage of its glucuronides, assuming the difference to be the glucuronide content of the sample. This method, however, suffers from several drawbacks, for instance loss of structural information, time-consuming sample preparation or risk of underestimation of glucuronide content due to incomplete cleavage by β-glucuronidase treatment. Consequently, during recent years, efforts have been made to directly quantitate glucuronides.[14−16]

For direct quantitative analysis, glucuronide standards are necessary, but they are not commercially available. Therefore, one aim of this work was to synthesize glucuronides of T-2 toxin and HT-2 toxin enzymatically using liver microsomes. There are only limited data on glucuronidation of T-2 and HT-2 toxin by humans. Only one report exists about the occurrence of T-2 toxin glucuronide and HT-2 toxin 3-glucuronide in a human colon carcinoma derived cell line (HT-29) as well as in human primary renal proximal tubule epithelial cells (RPTEC).[6] Additionally, information on species specific differences in glucuronidation of T-2 and HT-2 toxin is scarce. Thus, glucuronidation of T-2 and HT-2 toxin by liver microsomes of rat, mouse, pig and human was compared and glucuronides were analyzed directly by LC−MS/MS. Moreover, the degree of hydrolysis of T-2 and HT-2 toxin glucuronides by different β-glucuronidases was examined.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals were purchased from Sigma-Aldrich GmbH (Seelze, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), VWR International GmbH (Darmstadt, Germany) or AppliChem GmbH (Darmstadt, Germany). Water was purified by a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany). Uridine 5′-diphosphoglucuronic acid, β-glucuronidases type B1 from bovine liver, type H5 from *Helix pomatia* and type IX-A from *Escherichia coli* were bought from Sigma-Aldrich GmbH (Seelze, Germany). Livers of female pigs (Angler/Sattel x Pietrain) were obtained from Gut Wewel (Senden, Germany). *In vitro* CYP M human liver microsomes (50 donors pooled), female Göttingen minipig liver microsomes, female ICR/CD mouse liver microsomes and female Wistar rat liver microsomes were purchased from Celsis In Vitro Technologies (Brussels, Belgium). T-2 and HT-2 toxin were isolated from cultures of *F. sporotrichioides* in our laboratory[17] and d_3-T-2 toxin was synthesized in our laboratory according to Beyer et al.[17]

Preparation of Microsomes. Pig livers were removed shortly after the death of the animals, frozen in liquid nitrogen and stored at −80 °C until further usage. Microsomes were prepared according to Lake.[18] Protein content was determined by Bradford-assay[19] using bovine serum albumin as reference material.

Incubation with Liver Microsomes. For preparative isolation of glucuronides, pig liver microsomes were first mixed with 10 μg of alamethicin and kept on ice for 10 min before the other substances were added. Incubations were carried out in a total volume of 250 μL in 25 mM TRIS buffer pH 7.4 containing 5 mM $MgCl_2$, 3.8 mM UDPGA, 0.8 mM T-2 toxin and 1 mg of microsomal protein. After incubation for 2 h at 37 °C, the reaction was stopped by addition of 500 μL of acetonitrile. The precipitate was removed by centrifugation (16 000*g* for 10 min) and several assays were combined for further purification. For comparison of glucuronidation with different liver microsomes, incubation was slightly modified; 50 mM TRIS buffer, containing 10 mM $MgCl_2$, 0.34 mM UDPGA, 0.086 mM T-2 toxin and 500 μg of microsomal protein was used. No alamethicin was added. Incubations were carried out in triplicate.

Isolation and Purification of Glucuronides. For the isolation of glucuronides, acetonitrile in the combined reaction mixtures was evaporated using a BA-VC-300H vacuum concentrator (H. Saur, Reutlingen, Germany). The aqueous residue was then extracted three times with equal volumes of chloroform. The aqueous phase was acidified with formic acid to pH = 3 and loaded on Bond Elut Plexa SPE columns (200 mg) (Agilent, Waldbronn, Germany) which had been conditioned with 5 mL of methanol and equilibrated with 5 mL of water. Columns were washed ten times with 3 mL of water and five times with 3 mL of 5% (v/v) methanol. Substances were eluted with 60% (v/v) methanol. After concentration in a vacuum concentrator, glucuronides were purified by preparative HPLC on a Varian Pro Star system (Varian, Waldbronn, Germany) using a 250 × 9.4 mm i.d.; 5 μm, Eclipse XDB C18 column (Agilent, Waldbronn, Germany). Mobile phase was water (A) and a mixture of acetonitrile and water in a ratio of 99.9%:0.1% (v/v) (B), both containing 5 mM ammonium acetate. The gradient was as follows: 22% B for 22 min, then a change to 30% B in 1 min and an isocratic step at 30% B for 9 min. The column was washed for 1 min with 90% B and equilibrated for 8 min at starting conditions. Flow rate was set to 4.5 mL/min. Fractions (30 s) were collected, checked for glucuronides using LC−MS/MS, combined and concentrated in a vacuum concentrator. To remove salts, a final purification was performed on a Bond Elut Plexa SPE cartridge as described above.

Purity Determination by HPLC-ELSD. Purity of isolated glucuronides was determined on a Shimadzu LC 20AT HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV (220 nm, 254 nm) and an evaporative light scattering detector (ELSD) ELSD LT (Shimadzu, Kyoto, Japan). ELSD parameters were: nebulizer temperature 40 °C, air pressure 2.5 bar. Separation was performed on a 250 mm × 4.6 mm i.d., 5 μm, Gemini C18 column (Phenomenex, Aschaffenburg, Germany), using the mobile phase as described above for preparative HPLC at a flow rate of 1 mL/min. The gradient started at 25% B, which was held for 3 min, and afterward increased linearly in 27 min to 100% B, which was held for 2 min. For calculating the purity, the peak area of the peak corresponding to the glucuronide was compared to the sum of all peak areas.

Exact Mass Measurements and Fragmentation. Measurements of the exact mass and the fragmentation patterns of glucuronides were done by Fourier transformation mass spectrometry (FTMS) on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher

Scientific, Bremen, Germany) using heated electrospray ionization (HESI). Data were processed using Xcalibur software version 2.0.7 SP1 (Thermo Fisher Scientific). Solutions of the purified glucuronides were introduced by a syringe pump (5 μL/min). The mass spectrometer was set to positive mode and a resolution of 100 000. Parameters were as follows: capillary temperature 225 °C, vaporizer temperature 50 °C, sheath gas flow 8, aux gas flow 5, source voltage 3 kV, capillary voltage 12 V, tube lens voltage 102 V. Product ion spectra were acquired using collision induced dissociation (CID) at 20%. Exact masses measured by FTMS were as follows: HT-2 toxin 3-glucuronide, found m/z 618.2749, calculated for $[C_{28}H_{40}O_{14} + NH_4]^+$ 618.2756; HT-2 toxin 4-glucuronide, found m/z 618.2754, calculated for $[C_{28}H_{40}O_{14} + NH_4]^+$ 618.2756; T-2 toxin glucuronide, found m/z 660.2853, calculated for $[C_{30}H_{42}O_{15} + NH_4]^+$ 660.2862.

NMR Measurements. NMR spectra were recorded on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany) or on a Varian 600 unity plus (Varian, Palo Alto, CA) NMR spectrometer. Samples were dissolved in d_4-methanol and signals are reported referenced to methanol. For structure elucidation and assignment of NMR signals, ^{1}H, ^{13}C, COSY, HMBC, HSQC, TOCSY and NOE measurements were conducted.

HT-2 toxin 3-β-D-glucuronide: ^{1}H NMR (400 MHz, d_4-MeOD) δ: 5.75 (d, 1 H, J = 5.8 Hz, H-10), 5.31 (d, 1 H, J = 5.4 Hz, H-8), 4.69 (d, 1 H, J = 7.8 Hz, H-1′), 4.60 (d, 1 H, J = 3.0 Hz, H-4), 4.38 (dd, 1 H, J = 5.0 Hz, 3.0 Hz, H-3), 4.31 (d, 1 H, J = 12.5 Hz, H-15), 4.23 (d, 1 H, J = 5.9 Hz, H-11), 3.99 (d, 1 H, J = 12.5 Hz, H-15), 3.69 (d, 1 H, J = 9.5 Hz, H-5′), 3.65 (d, 1 H, J = 5.0 Hz, H-2), 3.49 (1 H, H-4′), 3.43 (t, 1 H, J = 8.9 Hz, H-3′), 3.33 (H-2′), 2.97 (d, 1 H, J = 4.0 Hz, H-13), 2.78 (d, 1 H, J = 4.0 Hz, H-13), 2.37 (dd, 1 H, J = 15.2 Hz, 5.8 Hz, H-7), 2.16 (m, 2 H, H-18), 2.11−2.06 (m, H-19), 2.05 (s, 3 H, acetyl-CH_3), 1.97 (d, 1 H, J = 15.2 Hz, H-7), 1.73 (s, 3 H, H-16), 0.97 (d, 3 H, J = 3.2 Hz, H-20/21), 0.96 (d, 3 H, J = 3.2 Hz, H-20/21), 0.80 (s, 3 H, H-14).

^{13}C NMR (100 MHz, d_4-MeOD) δ: 174.1 (C-17), 172.2 (acetyl-C═O), 137.0 (C-9), 125.5 (C-10), 103.4 (C-1′), 86.1 (C-3), 80.3 (C-2), 80.0 (C-4), 78.1 (C-3′), 74.4 (C-2′), 73.5 (C-4′), 69.6 (C-8), 68.6 (C-11), 65.9 (C-15), 65.3 (C-12), 50.0 (C-5), 47.3 (C-13), 44.6 (C-18), 44.0 (C-6), 28.7 (C-7), 26.8 (C-19), 22.8 (C-20/21), 22.7 (C-20/21), 21.1 (acetyl-CH_3), 20.5 (C-16), 7.4 (C-14).

HT-2 toxin 4-β-D-glucuronide: ^{1}H NMR (400 MHz, d_4-MeOD) δ: 5.74 (d, 1 H, J = 5.9 Hz, H-10), 5.32 (d, 1 H, J = 5.5 Hz, H-8), 4.74 (d, 1 H, J = 2.6 Hz, H-4), 4.65 (d, 1 H, J = 7.8 Hz, H-1′), 4.32 (d, 1 H, J = 12.5 Hz, H-15), 4.29−4.25 (m, 2 H, H-3, H-11), 4.03 (d, 1 H, J = 12.5 Hz, H-15), 3.69 (d, 1 H, J = 9.0 Hz, H-5′), 3.51 (d, 1 H, J = 4.9 Hz, H-2), 3.48 (1 H, H-4′), 3.42 (t, 1 H, J = 8.9 Hz, H-3′), 3.29 (H-2′), 3.04 (d, 1 H, J = 3.9 Hz, H-13), 2.87 (d, 1 H, J = 3.9 Hz, H-13), 2.38 (dd, 1 H, J = 15.2 Hz, 5.9 Hz, H-7), 2.17 (m, 2 H, H-18), 2.13−2.08 (m, H-19), 2.06 (s, 3 H, acetyl-CH_3), 1.90 (d, 1 H, J = 15.2 Hz, H-7), 1.73 (s, 3 H, H-16), 0.97 (d, 3 H, J = 3.3 Hz, H-20/21), 0.96 (d, 3 H, J = 3.3 Hz, H-20/21), 0.84 (s, 3 H, H-14).

^{13}C NMR (100 MHz, d_4-MeOD) δ: 174.2 (C-17), 172.3 (acetyl-C═O), 137.2 (C-9), 125.3 (C-10), 102.5 (C-1′), 86.4 (C-4), 80.8 (C-2), 79.8 (C-3), 77.6 (C-3′), 74.0 (C-2′), 73.6 (C-4′), 69.5 (C-8), 68.5 (C-11), 66.0 (C-15), 65.9 (C-12), 50.1 (C-5), 47.7 (C-13), 44.5 (C-18), 44.3 (C-6), 29.1 (C-7), 26.9 (C-19), 22.8 (C-20/21), 22.7 (C-20/21), 21.5 (acetyl-CH_3), 20.4 (C-16), 7.7 (C-14).

T-2 toxin 3-β-D-glucuronide: ^{1}H NMR (600 MHz, d_4-MeOD) δ: 6.03 (d, 1 H, J = 3.0 Hz, H-4), 5.78 (d, 1 H, J = 5.9 Hz, H-10), 5.32 (d, 1 H, J = 5.8 Hz, H-8), 4.66 (dd, 1 H, J = 5.0 Hz, 3.0 Hz, H-3), 4.47 (d, 1 H, J = 7.7 Hz, H-1′), 4.40 (d, 1 H, J = 5.9 Hz, H-11), 4.38 (d, 1 H, J = 12.5 Hz, H-15), 4.09 (d, 1 H, J = 12.5 Hz, H-15), 3.79 (d, 1 H, J = 5.0 Hz, H-2), 3.60−3.30 (sugar), 3.03 (d, 1 H, J = 4.0 Hz, H-13), 2.83 (d, 1 H, J = 4.0 Hz, H-13), 2.39 (dd, 1 H, J = 15.1 Hz, 5.9 Hz, H-7), 2.16 (m, 2 H, H-18), 2.09 (s, 3 H, acetyl-CH_3), 2.08 (s, 3 H, acetyl-CH_3), 1.91 (d, 1 H, J = 17.3 Hz, H-7), 1.73 (s, 3 H, H-16), 0.97 (d, 3 H, J = 4.5 Hz, H-20/21), 0.96 (d, 3 H, J = 4.4, H-20/21), 0.73 (s, 3 H, H-14). H-19 is overlapping with the signals of acetyl-CH_3.

LC−MS/MS Measurement. For the comparison of the metabolism of T-2 toxin in liver microsomes of rat, mouse, pig and human, T-2 toxin, HT-2 toxin and HT-2 toxin glucuronides were quantified using LC−MS/MS. After incubation and centrifugation, supernatants were diluted 1:10 with water, and 25 ng of d_3-T-2 toxin was added as internal standard. To calculate calibration curves for each analyte, the ratios of the peak area of the analyte to the peak area of d_3-T-2 toxin were plotted against analyte concentration. Analysis was done on an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) linked to an API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany), using Analyst 1.4.2 software (Applied Biosystems) for data acquisition.

HPLC Parameters. Separation was performed on a 50 mm × 2.0 mm i.d., 2.4 μm, Pursuit UPS C18 column (Agilent, Waldbronn, Germany) equipped with a 4 × 2.0 mm i.d. C18 guard column (Phenomenex, Aschaffenburg, Germany) using the mobile phase as described above for preparative HPLC. The gradient started at 20% B and increased to 100% B in 8 min. The column was washed with 100% B for 1 min and equilibrated at starting conditions for 6 min. Flow rate was 300 μL/min, column oven temperature was set to 40 °C and injection volume to 50 μL.

MS/MS Parameters. The mass spectrometer was operated in the positive multiple reaction monitoring (MRM) mode, quadrupoles were set at unit resolution. For electrospray ionization, the ion voltage was set to 5500 V, zero grade air served as nebulizer gas (35 psi) and heated to 350 °C as turbo gas (45 psi). Nitrogen was employed as curtain gas (20 psi) and as collision gas for fragmentation of the $[M + NH_4]^+$ adducts (3.5×10^{-5} Torr). Parameters for each mass transition were optimized by direct infusion of the analytes. Values for declustering potential (DP), collision energy (CE) and cell exit potential (CXP) are given in brackets. T-2 toxin, 484−305 (DP 65 V, CE 19 V, CXP 19 V), 484−215 (DP 60 V, CE 24 V, CXP 14 V); d_3-T-2 toxin, 487−308 (DP 65 V, CE 19 V, CXP 19 V); HT-2 toxin, 442−263 (DP 54 V, CE 22 V, CXP 18 V), 442−215 (DP 54 V, CE 18 V, CXP 16 V); HT-2 toxin glucuronide, 618−215 (DP 61 V, CE 28 V, CXP 17 V), 618−245 (DP 61 V, CE 24 V, CXP 19 V), 618−263 (DP 61 V, CE 26 V, CXP 17 V); T-2 toxin glucuronide, 660−305 (DP 65 V, CE 25 V, CXP 15 V), 660−215 (DP 65 V, CE 28 V, CXP 21 V). Entrance potential (EP) was 10 V. Each mass transition was monitored for 150 ms. The following transitions served as quantifiers: 484−305 (T-2 toxin), 442−215 (HT-2 toxin), 618−215 (HT-2 toxin 4-glucuronide) and 618−263 (HT-2 toxin 3-glucuronide).

Animal Study. The animal study with pigs was performed at the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany. Experiments and procedures were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the Regional Council of Braunschweig, Lower Saxony, Germany (File Number 33.9.42502-04-054/09). The experimental details of this study will be published elsewhere by Dänicke et al. and will only be briefly summarized here. Female pigs were dosed with a single dose of T-2 toxin either iv with 80 μg/kg b.w. (n = 2) or orally with 4 mg/animal (n = 4) resulting in approximately 89−100 μg/kg b.w. Urine was collected after 3, 6, 12, 24, and 48 h and selected samples were analyzed after syringe filtration (0.45 μm, regenerated cellulose) employing LC−MS/MS as described above.

Incubation with β-Glucuronidase. Hydrolysis of T-2 and HT-2 toxin glucuronides was carried out in a total volume of 250 μL. To cleave the glucuronides, 200 μL of a glucuronide solution in water or in spiked pig urine was mixed with 50 μL of β-glucuronidase suspended in 750 mM buffer and incubated for definite times at 37 °C. For incubation with β-glucuronidase type B1 from bovine liver and type H5 from *H. pomatia*, sodium acetate buffer (pH 4.5) was used in contrast to incubation with β-glucuronidase type IX-A from *E. coli* where KH_2PO_4/Na_2HPO_4 buffer (pH 6.5) was employed. Incubation was terminated by addition of 250 μL of acetonitrile and the precipitate was removed by centrifugation (16 000g for 5 min). After 1:10 dilution of the samples and addition of 25 ng of d_3-T-2 toxin, analysis was carried out as described above. To check for their stability, T-2 and HT-2 toxin were incubated under the same conditions and glucuronides were incubated without addition of β-glucuronidase to the buffer. T-2 and HT-2 toxin as well as HT-2 toxin glucuronides were used in a concentration of 10 mM in the 250 μL incubation

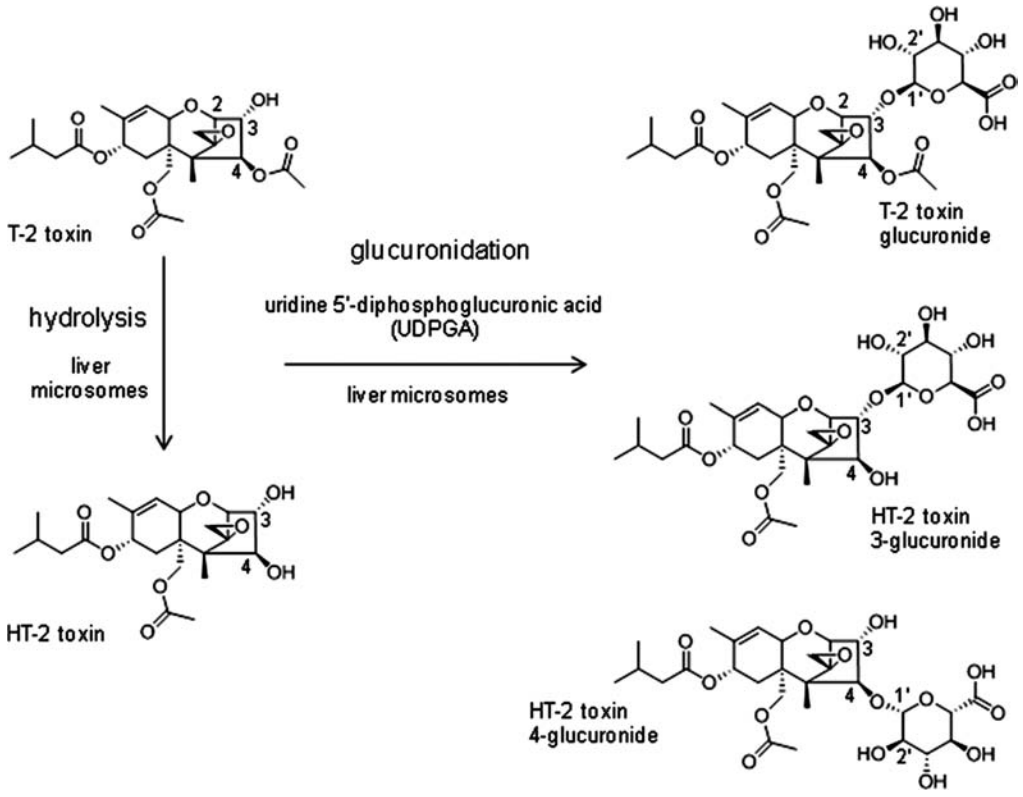

Figure 2. Overview of the formed metabolites when incubating T-2 toxin with pig liver microsomes in the presence of the cofactor UDPGA.

mixture. The initial amount of T-2 toxin glucuronide could not be quantified exactly; hydrolysis efficiencies were examined by comparing the amount of T-2 toxin glucuronide before and after incubation. All incubations were done in triplicate

RESULTS AND DISCUSSION

Glucuronide Synthesis and Isolation. Although glucuronides of T-2 toxin and its phase I metabolite HT-2 toxin are key metabolites in *in vitro* and *in vivo* studies, these glucuronides have until now usually been analyzed indirectly after hydrolysis with β-glucuronidase.[10−13] For direct analysis of glucuronides using sensitive and selective LC−MS/MS techniques, standard substances are essential. Since they are not commercially available, a method for the enzymatic synthesis and purification of T-2 and HT-2 toxin glucuronides using pig liver microsomes was developed and glucuronides were characterized by MS and NMR.

To synthesize T-2 and HT-2 toxin glucuronides, T-2 toxin was incubated in a buffered system with pig liver microsomes in the presence of the cofactor uridine 5′-diphosphoglucuronic acid (UDPGA). Liver microsomes served as a source for the uridine 5′-diphospho-glucuronosyltransferases, which transfer the glucuronic acid to a substrate like T-2 or HT-2 toxin (Figure 2). Besides the Königs-Knorr synthesis, this enzymatic approach is widely described in literature to obtain glucuronides.[20−22] For instance, Roush et al.[9] were able to isolate HT-2 toxin 3-glucuronide after incubation of T-2 toxin with rat liver microsomes and UDPGA. Figure 3 depicts a typical LC−MS/MS chromatogram of an incubation mixture of T-2 toxin with pig liver microsomes after 2 h of incubation. In accordance to literature data,[4,23] T-2 toxin was hydrolyzed quickly by certain carboxylesterases[24] present in pig liver microsomes forming HT-2 toxin. Moreover, it was shown that T-2 toxin as

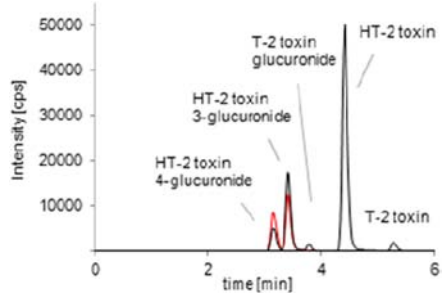

Figure 3. LC−MS/MS chromatogram of a mixture obtained after 2 h incubation of T-2 toxin with pig liver microsomes and UDPGA. The following mass transitions are shown: T-2 toxin 484−305; HT-2 toxin 442−215; T-2 toxin glucuronide 660−305; HT-2 toxin glucuronides 618−215 (red), 618 − 263 (black).

well as HT-2 toxin were glucuronidated, since one peak with mass transitions expected for the T-2 toxin glucuronide and surprisingly two peaks with mass transitions expected for the HT-2 toxin glucuronide were observed. No glucuronide peaks were present when microsomes, T-2 toxin, or UDPGA was omitted from the reaction mixture. Different incubation conditions were tested to optimize the glucuronide formation. A ratio of about 4 to 1 of molar amounts of UDPGA to T-2 toxin was chosen as a compromise between glucuronide yield and high cost for UDPGA. Addition of alamethicin, a protein forming pores in the microsomal vesicles, increased the glucuronide yield, as described in literature for estradiol glucuronidation.[25]

After incubation, the supernatant was extracted with chloroform thereby removing most of the T-2 and HT-2 toxin, while the glucuronides remained in the aqueous phase. During the following purification step by solid phase extraction,

the amount of buffer salts was reduced. Separation of the glucuronide isomers was then achieved by preparative HPLC. The yield was about 5−6% for each HT-2 toxin glucuronide isomer and below 1% for the minor component T-2 toxin glucuronide. Similar yields were obtained by Roush et al.[9] who isolated about 10% of HT-2 toxin 3-glucuronide when incubating T-2 toxin with rat liver microsomes. However, the relatively low yield could be increased in our study by recovery of T-2 and HT-2 toxin from the incubation mixture by solvent extraction as described above and reuse in further incubation experiments. Purity of the HT-2 toxin glucuronides, measured with an evaporative light scattering detector (ELSD), was about 97%. HPLC-UV, LC−MS/MS and NMR measurements only showed traces of impurities. For T-2 toxin glucuronide ELSD measurements resulted in a purity of about 40%. Impurities in the case of the T-2 toxin glucuronide are supposed to be caused by salts as one larger injection peak occurred in the ELSD chromatogram and NMR measurements of T-2 toxin showed only some minor impurities.

Structure Elucidation by NMR and MS. Detailed structure elucidation of the glucuronides was done using ^{1}H, ^{13}C, COSY, HSQC, HMBC and TOCSY NMR data. The HT-2 toxin glucuronide isomer eluting first in LC−MS/MS measurements (Figure 3) was identified as HT-2 toxin 4-glucuronide, a new metabolite which has not been described so far. The second of the HT-2 toxin glucuronide peaks (Figure 3) was found to be HT-2 toxin 3-glucuronide. ^{1}H NMR data were in accordance to literature data[9] although some signals were assigned differently based on the 2D-NMR experiments. First hints for the identification of the glucuronidation sites of the two HT-2 toxin glucuronide isomers were derived from comparison of the ^{1}H NMR data of the HT-2 toxin glucuronides and those of HT-2 toxin (Table 1). Compared

Table 1. Comparison of ^{1}H and ^{13}C NMR Signals of Atoms Near the Glucuronidation Site for HT-2 Toxin, HT-2 Toxin 3-Glucuronide, and HT-2 Toxin 4-Glucuronide

	^{1}H δ (ppm)		
H-atom	HT-2 toxin	HT-2 toxin 3-glucuronide	HT-2 toxin 4-glucuronide
4	4.38	4.60	4.74
3	4.09	4.38	4.29−4.25
2	3.44	3.65	3.51
	^{13}C δ (ppm)		
C-atom	HT-2 toxin	HT-2 toxin 3-glucuronide	HT-2 toxin 4-glucuronide
4	81.8	80.0	86.4
3	80.8	86.1	79.8
2	80.6	80.3	80.8

to HT-2 toxin, the resonance of H-4 is shifted downfield in HT-2 toxin 3-glucuronide and even further downfield in HT-2 toxin 4-glucuronide. H-2 and H-3 on the other hand are more deshielded in HT-2 toxin 3-glucuronide than in HT-2 toxin 4-glucuronide and HT-2 toxin. Similar effects were observed in the ^{13}C NMR spectra, where the signals for C-3 or C-4 were shifted downfield for HT-2 toxin 3-glucuronide or HT-2 toxin 4-glucuronide respectively (Table 1). The downfield shifts of the ^{1}H and ^{13}C NMR signals of the atoms at the glucuronidation site in relation to the signals of the parent compound were comparable to those of other substances.[22,26] The linkage of the glucuronic acid moiety to position 4 in the newly described metabolite HT-2 toxin 4-glucuronide was confirmed by HMBC data in which a correlation between the anomeric H-1′ of the glucuronic acid moiety and C-4 of HT-2 toxin as well as a correlation between H-4 and C-1′ was detected. The proton signal of the anomeric H-1′ of the glucuronic acid residue appeared as a doublet. From the vicinal coupling constant J = 7.8 Hz between H-1′ and H-2′ the presence of two axial protons, and therefore, β-configuration of the glucuronic acid moiety was inferred. ^{1}H NMR signals could be assigned for T-2 toxin glucuronide by comparing the signals for T-2 toxin glucuronide to NMR data of T-2 toxin, although only small amounts of T-2 toxin glucuronide were obtained and the ^{1}H NMR spectrum showed some impurities.

The exact masses measured for the ammonium adducts of T-2 and the HT-2 toxin glucuronides were in accordance to the calculated ones. In the product ion spectra of the ammonium adducts of the glucuronides, fragment ions that are also typical for unconjugated T-2 or HT-2 toxin were observed like m/z 215, 245, 305, or 365 for T-2 toxin glucuronide and m/z 215, 245, or 263 for the HT-2 toxin glucuronides (Figure 4).

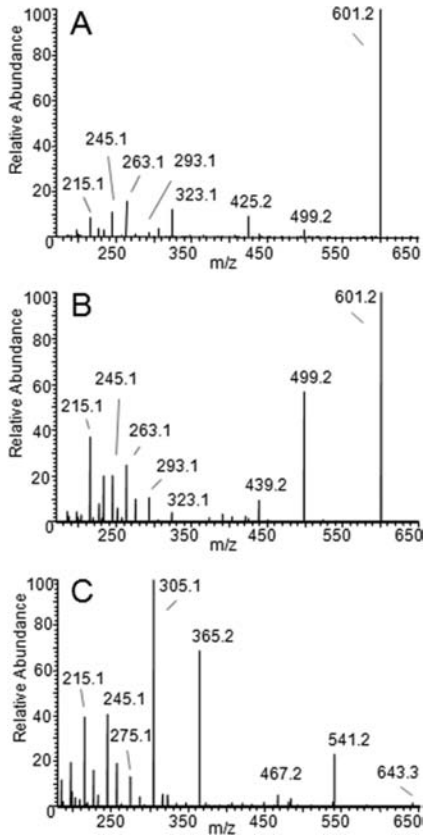

Figure 4. Product ion mass spectra of the $[M + NH_4]^+$ ions measured by FTMS at collision induced dissociation (CID) of 20%. (A) HT-2 toxin 3-glucuronide, (B) HT-2 toxin 4-glucuronide, (C) T-2 toxin glucuronide.

Although these mass spectra of HT-2 toxin 3-glucuronide and HT-2 toxin 4-glucuronide show similar fragment ions there are distinct differences in their intensity. In the mass spectrum of HT-2 toxin 4-glucuronide, signals resulting from the loss of the isovaleric acid side chain (m/z 499) or the loss of the isovaleric acid and acetic acid side chains (m/z 439) have a higher intensity than the signal for HT-2 toxin (m/z 425) resulting from the loss of the glucuronic acid moiety. For HT-2 toxin 3-

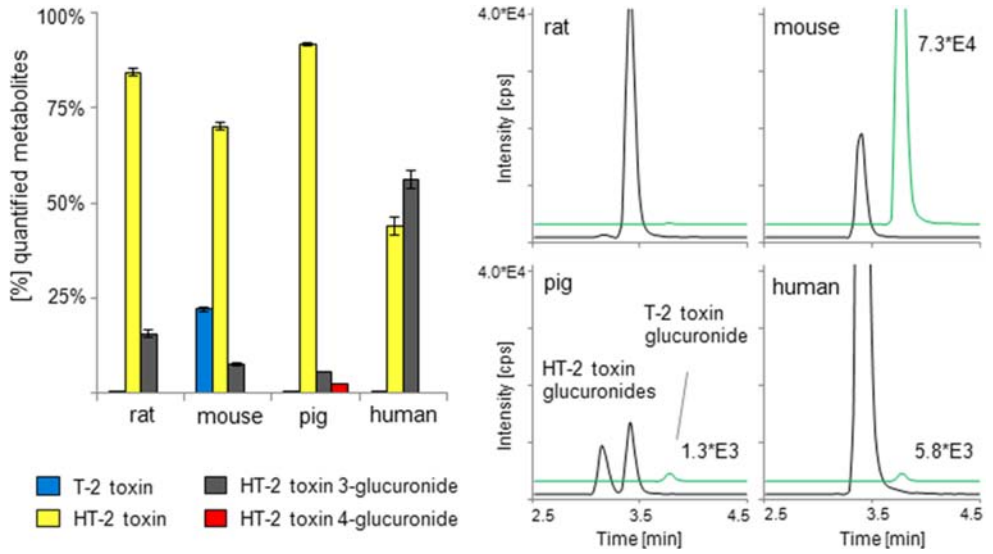

Figure 5. Metabolites formed after 2 h of incubation of T-2 toxin with liver microsomes of rat, mouse, pig and human. (Left) HT-2 toxin, HT-2 toxin glucuronides, T-2 toxin shown as percent of these quantified metabolites. (Right) Details of the LC−MS/MS chromatograms depicting HT-2 toxin glucuronides (black) and T-2 toxin glucuronide (green) and their peak intensities.

glucuronide, however, the signal for m/z 425 has a higher intensity than the signals for m/z 499 and 439. When fragmenting HT-2 toxin 3-glucuronide the signal for m/z 263 is higher than the signal for m/z 215, in contrast to HT-2 toxin 4-glucuronide (Figure 4). Consequently, monitoring the ratio of the mass transitions 618−215 and 618−263 is a further confirmation of the structure of the respective HT-2 toxin glucuronide besides the retention time in routine measurements (Figure 3 and Figure 6). In the mass spectra of the glucuronides in the negative mode the fragment ions m/z 175 and m/z 113 which are attributable to the glucuronic acid moiety were observed. These fragments were also detected for other glucuronides in the negative mode, further proving the structure of the synthesized standard substances.[21]

Species Specific Glucuronide Formation by Liver Microsomes of Rat, Mouse, Pig, and Human. Using the synthesized glucuronide reference material, the formation of T-2 and HT-2 toxin 3-glucuronide in a human colon carcinoma cell line (HT-29) and primary human renal proximal tubule epithelial cells (RPTEC) could be measured by LC-FTMS.[6] Further information about T-2 toxin glucuronidation in humans is lacking, especially data on glucuronide formation in human liver. Microsomal glucuronidation of T-2 toxin has only been examined using rat liver.[9] To date, the *in vivo* metabolism of T-2 toxin including glucuronidation has only been studied in rats[12] and pigs;[13] however, glucuronides were measured only indirectly by enzymatic cleavage. Therefore, to investigate interspecies differences in glucuronidation, T-2 and HT-2 toxin were incubated with microsomes of rat, mouse, pig, and human livers. A LC−MS/MS method was developed which allowed the direct analysis of T-2 toxin, HT-2 toxin and their respective glucuronides.

A typical chromatogram of an incubation of T-2 toxin with pig liver microsomes is displayed in Figure 3. Figure 5 depicts the formation of metabolites after 120 min of incubation of T-2 toxin with different microsomes. T-2 toxin glucuronide could not be quantified but differences between species could be monitored by comparison of peak areas (see chromatograms in Figure 5). As expected, T-2 toxin was hydrolyzed quickly by all liver microsomes except for mouse liver, where hydrolysis was less pronounced. Hydrolysis of T-2 toxin during incubation with liver microsomes forming mainly HT-2 toxin besides other minor hydrolysis products has frequently been reported in literature.[4,23,27] From experiments with different enzyme inhibitors, it was concluded that certain carboxylesterases were responsible for this hydrolysis.[24] Ohta et al.[27] reported a higher capacity for hydrolysis of T-2 toxin in human liver microsomes compared to mouse liver microsomes. Using rat liver microsomes, even less HT-2 toxin was formed than with mouse liver microsomes. When Kobayashi et al.[23] or Knupp et al.[4] however incubated T-2 toxin with liver microsomes in the presence of a NADPH generating system in order to study hydroxylation, they noticed that T-2 toxin was much better hydrolyzed by rat liver microsomes compared to mouse liver microsomes, which is also in accordance to our results.

Liver microsomes of rat, mouse, pig, and human each exhibited a distinct pattern of glucuronidation (Figure 5). When incubating T-2 toxin with mouse liver microsomes, much more T-2 toxin glucuronide was formed than by incubation with microsomes of the other species. This might be due to the slower hydrolysis of T-2 toxin by mouse liver microsomes. Only trace amounts of T-2 toxin glucuronide were detected in incubation mixtures containing rat liver microsomes (see chromatograms in Figure 5). HT-2 toxin 3-glucuronide accounted for about half of the quantified metabolites in human liver microsomes. Liver microsomes of the other species had a much lower glucuronidation rate (Figure 5). When studying rat liver microsomes, HT-2 toxin 3-glucuronide accounted for about 17% of the metabolites quantified. Roush et al.[9] found 49% of HT-2 toxin 3-glucuronide after 2 h of incubation of small amounts of tritium-labeled T-2 toxin with β-naphthoflavone-induced hepatic microsomes from rats. Differences in the extent of glucuronidation should be considered when evaluating the toxicity of T-2 and HT-2 toxin toward several species. Interestingly, only pig liver microsomes were able to form substantial amounts of HT-2 toxin 4-glucuronide, a new isomer, which has not been identified before (Figure 5). In incubation mixtures with rat liver microsomes, only trace amounts of HT-2 toxin 4-glucuronide were found besides larger amounts of HT-2

toxin 3-glucuronide, the only glucuronide identified by Roush et al.[9] When HT-2 toxin was employed (instead of T-2 toxin) as starting material for incubations with microsomes, HT-2 toxin glucuronide formation was comparable. Stereo- and regioselective formation of glucuronides as well as significant differences in the rate of glucuronidation depending on the animal species used as a source for the liver microsomes has been observed for a variety of other substances.[22,26,28,29] This was supposed to be connected to differences concerning the uridine 5′-diphospho-glucuronosyltransferase isoforms present in each species.

To verify that HT-2 toxin 4-glucuronide was not only formed *in vitro* by pig liver microsomes, urine of female pigs that were given T-2 toxin orally or iv(obtained from S. Dänicke, Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany) was analyzed by LC−MS/MS. Figure 6 illustrates that HT-2 toxin 4-glucuronide, as well as HT-2 toxin 3-glucuronide, was found in pig urine. Consequently, both HT-2 toxin glucuronide isomers are also formed *in vivo* and should be taken into account when studying T-2 toxin metabolism. This information about HT-2 toxin glucuronide isomers was lost when Corley et al.[13] examined the metabolism of T-2 toxin in pigs, since glucuronides in physiological samples were analyzed indirectly after hydrolysis with β-glucuronidase.

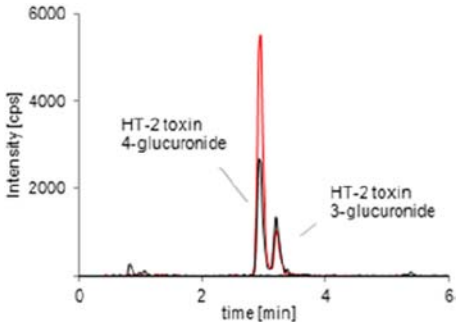

Figure 6. LC−MS/MS chromatogram of urine (collected after 6 h) of a pig administered T-2 toxin (4 mg) orally. The chromatogram shows 2 mass transitions for HT-2 toxin glucuronides, 618−215 (red) and 618−263 (black).

Hydrolysis of Glucuronides with Different β-Glucuronidases. The enzymatic hydrolysis using β-glucuronidases is routinely employed for indirect quantitation of glucuronide metabolites. Various hydrolysis protocols exist and differences in deconjugation efficiencies have been observed for several substances depending e.g. on type and amount of β-glucuronidase, matrix, incubation time or temperature.[30,31] For T-2 and HT-2 toxin glucuronides this kind of information is scarce. Therefore, to examine enzymatic hydrolysis of T-2 and HT-2 toxin glucuronides, buffered aqueous solutions or spiked pig urine samples were treated with different amounts of three commonly used types of β-glucuronidases (type H5 from *H. pomatia*, type B1 from bovine liver, type IX-A from *E. coli*). The degree of deconjugation over time was monitored by comparing amounts of glucuronides before and after hydrolysis as well as by quantitating formed T-2 toxin and HT-2 toxin. T-2 and HT-2 toxin glucuronides were stable for 24 h at 37 °C in buffered aqueous solutions or pig urine in the absence of β-glucuronidase. The stability of the hydrolysis products T-2 toxin and HT-2 toxin in the incubation mixture was also tested. While HT-2 toxin was stable for 24 h in the presence of any of the three β-glucuronidases, T-2 toxin was only stable in mixtures containing β-glucuronidase from bovine liver or *E. coli*. When treated with β-glucuronidase from *H. pomatia*, T-2 toxin was degraded and HT-2 toxin was formed. After 30 or 60 min of incubation, only 76% or 37% of the originally applied T-2 toxin concentration remained. Similarly, after treatment of T-2 toxin glucuronide with β-glucuronidase from *H. pomatia*, not only T-2 toxin was formed, but also HT-2 toxin 3-glucuronide and HT-2 toxin. Consequently, β-glucuronidase from *H. pomatia* is not suitable for the analysis of samples containing T-2 toxin glucuronide or T-2 toxin.

Table 2 summarizes the hydrolysis efficiencies for 10 mM solutions of HT-2 toxin glucuronides under various incubation conditions. Results are given as percentage of HT-2 toxin formed by enzymatic cleavage compared to the amount of HT-2 toxin that would be formed after complete hydrolysis of the applied glucuronide. In general it was observed, that longer incubation times and higher amounts of enzyme improved hydrolysis to a certain extent, which was also proven for other glucuronides.[30,32] Moreover, when glucuronides were dissolved in urine instead of water, harsher reaction conditions were

Table 2. Hydrolysis of HT-2 Toxin 3-Glucuronide and HT-2 Toxin 4-Glucuronide with β-Glucuronidases under Different Incubation Conditions

substance incubated	incubation time [h]	type (amount) of β-glucuronidase	matrix	% HT-2 toxin formed ± SD[a]
HT-2 toxin 4-glucuronide	2	BL[b] (250 U)	buffer	39.8 ± 3.8
	2	HP[c] (250 U)	buffer	89.2 ± 6.0
	2	EC[d] (250 U)	buffer	98.1 ± 1.8
	4	EC (500 U)	urine	73.3 ± 3.8
	4	EC (1000 U)	urine	87.8 ± 1.3
HT-2 toxin 3-glucuronide	4	BL (500 U)	buffer	2.3 ± 0.1
	24	BL (1000 U)	buffer	10.4 ± 0.3
	4	HP (500 U)	buffer	49.4 ± 3.5
	24	HP (1000 U)	buffer	92.3 ± 5.7
	2	EC (250 U)	buffer	104.7 ± 7.1
	24	EC (2000 U)	urine	70.6 ± 3.3
	24	EC (4000 U)	urine	79.0 ± 3.4

[a]The amount of HT-2 toxin formed after hydrolysis ± standard deviation is given in percent of the amount of HT-2 toxin expected after complete hydrolysis of the glucuronides. [b]Bovine liver. [c]*H. pomatia*. [d]*E. coli*.

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necessary to achieve deconjugation probably due to interferences with the matrix. HT-2 toxin 4-glucuronide was hydrolyzed more easily, whereas the hydrolysis of the more prevalent HT-2 toxin 3-glucuronide required higher amounts of enzyme and longer incubation times. Achieving the hydrolysis of T-2 toxin glucuronide was even more difficult. When incubated with 4000 U of *E. coli* β-glucuronidase for 24 h, about half of the glucuronide was still not hydrolyzed. Distinct differences were observed between the deconjugation efficiencies of the three enzymes. Bovine liver β-glucuronidase poorly hydrolyzed HT-2 toxin 3-glucuronide. Similar problems were observed by Pace[10] when hydrolyzing the polar substances at the initial spotting site that were left after analysis of metabolites of T-2 toxin in bile by thin layer radiochromatography. The polar compounds proved to be poor substrates for bovine liver β-glucuronidase, whereas after hydrolysis with β-glucuronidase from limpets, about 92% of the radiolabel was associated with free metabolites, mainly HT-2 toxin. With enzymes from *H. pomatia*, a better hydrolysis for HT-2 toxin glucuronides was obtained, but as discussed above, this enzyme is not suitable for samples containing T-2 toxin. Applying *E. coli* β-glucuronidase, the highest hydrolysis efficiency was achieved. Nevertheless, for hydrolysis of HT-2 toxin 3-glucuronide in urine, high amounts of enzyme and 24 h of incubation were necessary. Although these were the only conditions tested under which no more HT-2 toxin 3-glucuronide could be detected in urine, only about 80% of the expected amount of HT-2 toxin was recovered.

These examples demonstrate that not every deconjugation protocol and enzyme is suitable for the hydrolysis of certain glucuronides. Incomplete hydrolysis of glucuronides or side reactions might lead to errors in the quantitation of these metabolites. Moreover, sample preparation is time-consuming and structural information is lost. These limitations emphasize the need for glucuronide reference material for the direct analysis of T-2 and HT-2 toxin glucuronides in physiological samples. Direct analysis of glucuronides revealed species specific differences in the formation of T-2 and HT-2 toxin glucuronides by liver microsomes of rat, mouse, pig, and human. The new metabolite HT-2 toxin 4-glucuronide was detectable by LC−MS/MS not only in pig liver microsomes, but also *in vivo* in pig urine.

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Notes

The authors declare no competing financial interest.

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