A preliminary investigation on the influence of flavonoids on ethyl glucuronide formation

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Abstract

Aims: A large variation of the formation of ethyl glucuronide (EtG) which is a minor metabolite of ethanol has been observed in man. At present there is only a single investigation on glucuronosyltransferases (UGT) responsible for catalyzing EtG formation whereas a possible influence of nutritional components on EtG formation has not been addressed at all.

Methods: Following optimization of the substrate concentration, incubation conditions such as buffer and time, as well as isolation of EtG from the incubation mixture, recombinant UGT enzymes (UGT 1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, 2B15) were screened for their activity towards ethanol. Subsequently, quercetin and kaempferol were chosen to study their possible influence on the glucuronidation of ethanol for UGT1A1, 1A3 and UGT1A9. Analysis of EtG formation was performed by LC-MS/MS following solid phase extraction. EtG-d_5 was used as the internal standard. For inhibition experiments, EtG formation was determined after addition of kaempferol and quercetin in different concentrations to the incubation mixture to determine the half maximum inhibitory constants (IC_{50}).

Results: Optimization of both, the incubation and isolation procedures resulted in a significant decrease of matrix effects. EtG was formed by all enzymes under investigation at variable rates ranging from 0.79 – 10.41 pmol/min/mg at 50 mM ethanol and 0.95-12.9 pmol/mg/min at 200 mM ethanol. Respective kinetics followed the Michaelis-Menten model so that the Michaelis-Menten constant K_m and the maximum velocity V_{max} values could be calculated. Both flavonoids reduced formation of EtG, irrespective of the enzyme involved. The IC_{50}-values ranged from 3.04 µM quercetin for UGT1A1 to 38.47 µM kaempferol for UGT1A9.

Conclusions: Formation of EtG from ethanol is catalyzed by multiple UGT isoforms. In addition, co-incubation affected the glucuronidation rate, irrespective of the particular enzyme. It seems that nutritional components will influence conversion of ethanol to EtG which may partly explain its variable formation in man.

1. Introduction

Ethyl glucuronide (EtG) has been established as a sensitive and specific marker of recent drinking [1]. Formation of EtG is characterized by net addition of glucuronic acid to ethanol. This clearance pathway is catalyzed by the gene superfamily of UDP-glucuronosyltransferases (UGT). UGTs catalyze the glucuronidation of a wide range of endogenous compounds, such as flavonoids, which are ubiquitary in nearly all vegetables and fruits or other foodstuff (e.g. grapes, onions and broccoli). The UGT isoforms UGT1A1, 1A9 and 2B7 are reportedly involved in EtG formation [2,3] whereas a possible influence of nutritional compounds has not been addressed at all. The purpose of this study was to identify the specific UGTs which are responsible for EtG formation and to determine their kinetic parameters. In addition, the impact of the co administration of nutritional components, such as the flavonoids kaempferol and quercetin, was investigated.

2. Material and Methods

EtG and EtG-d_5 were purchased from Medichem (Steinenbronn, Germany). 3-[(Cholamino-propyl)dimethyl-ammonio]-1-propanesulfonate) (CHAPS), magnesium chloride, kaempferol and quercetin were obtained from Sigma Aldrich (Taufkirchen, Germany). Uridine 5'-diphospho-glucuronic acid (UDPGA), K_3PO_4-buffer and recombinant UGT enzymes were purchased from Becton-Dickinson Biosciences (Heidelberg, Germany). HPLC-grade acetonitrile and HPLC-grade methanol were obtained from Roth (Karlsruhe, Germany). Ethanol (99.8%) was purchased from Merck (Darmstadt, Germany). Water was obtained from Fresenius Kabi AG (Bad Homburg, Germany). Isolute NH_2-SPE cartridges (100 mg, 3 mL) were received from Biotage (Uppsala, Sweden).

2.1. Formation Rate and Kinetics

Incubation experiments to assess the formation rates and kinetics of EtG in recombinant UGT enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, 2B15) were conducted using 0.5 mg/mL microsomal protein. Recombinant UGTs and CHAPS (2 mg CHAPS/mg protein) were placed on ice for 5 min to circumvent latency in order to increase UGT activity. K_3PO_4-buffer (50 mM; pH 7.4), magnesium chloride (4 mM), EtG-d_5 as internal standard (50 ng/mL), ethanol (50 mM and 200 mM to determine formation rates and 0 mM-300 mM to determine kinetic parameters) and water were added to the incubation mixture and placed in a 37° C water bath for 3 min. Incubations (60 min at 37°C) were initiated by the addition of UDPGA (3 mM) and stopped by 200 µL ice-cold water. All incubations were conducted in duplicate at a total volume of 400 µL. Kinetic parameters were estimated by using the nonlinear regression analysis program SigmaPlot 9.0 (Systat, Erkrath, Germany).

2.2. Inhibition Experiments

Recombinant UGT1A1, 1A3, 1A9 were selected for preliminary inhibition studies at ethanol concentrations of respective K_m-values with co-addition of kaempferol and quercetin at different concentrations (½* K_m, K_m, 2*K_m) [4,5]. Incubation procedure followed the protocol identical to that described in section 2.1. If glucuronidation rate of ethanol was reduced by > 60%, further experiments were performed at seven different inhibitor concentrations ranging from ½*Km – 2*Km. All analyses were performed in duplicate for each condition. Data analysis was performed by SigmaPlot 9.0.

2.3. Analytical Methods

EtG extraction was performed by SPE using Isolute NH_2- cartridges. The extraction method was similar to that previously published by Foti and Fisher [3]. After reconstitution in 50 µL of 4 mM ammonium acetate-buffer pH 3.2/methanol/acetonitril (10/18/72 vol %) 20 µL were injected into the LC-MS/MS system.

Analysis was performed on a API 4000 triple quadrupole (AB Sciex, Darmstadt, Germany) with a TurboIon™ ionization source (negative mode), interfaced to a HPLC pump and an autosampler (HP 1100 series, Agilent, Waldbronn, Germany). Separation was achieved on a Synergi-Polar RP column (250 x 2.0 mm; 4µm; Phenomenex, Aschaffenburg, Germany) with 4 mM ammonium acetate buffer pH 3.2/methanol/acetonitrile (10/18/72 vol%) as the mobile phase. Transitions monitored for quantification were *m/z* 221.0→75.0, *m/z* 221.0→85.0 and *m/z* 221.0→113.0 for EtG as well as *m/z* 226.0→75.0 and *m/z* 226.0→85.0for EtG-d_5. Transition *m/z* 221.0→75.0 was used for EtG quantification.

3. Results and Discussion

3.1. Formation Rates and Kinetic Parameters

All recombinant UGTs under investigation (UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B10, 2B15) were able to produce EtG in significant amounts; UGT1A9 and UGT2B7 exhibited the highest EtG formation rates at both concentrations (50 mM and 200 mM) with 10.41 pmol/min/mg and 12.96 pmol/min/mg as well as 7.38 pmol/min/mg and 10.37 pmol/min/mg, respectively. Formation rates for all tested UGT isoforms are shown in Fig. 1, expressed as a percentage of the maximum observed formation rates from UGT1A9. These findings are in accordance with data published by Al Saabi et al. [2] . In contrast to Foti et al.[3] , who found the highest glucuronidation rate with UGT1A1 and to Al Saabi et al.[2], who could not determine any activity from UGT1A1 towards ethanol, our experiments showed a low but detectable glucuronidation rate of ethanol with UGT1A1 (0.95 pmol/min/mg). These discrepancies could be due to experimental conditions such as the type of reagent used to circumvent latency in order to increase UGT activity (alamethicine versus CHAPS), the buffers used for the incubation mixture (TRIS buffer pH 7.4. versus K_3PO_4-buffer pH 7.4) and especially to SPE thus avoiding severe ion suppression.

The kinetic parameters of EtG formation were determined for all UGT isoforms. For all tested UGTs, EtG production is best described by the classic Michaelis-Menten equation. The calculated kinetic parameters (K_m, V_{max} and $Cl_{intr.}$) for each isoform are shown in table 1.

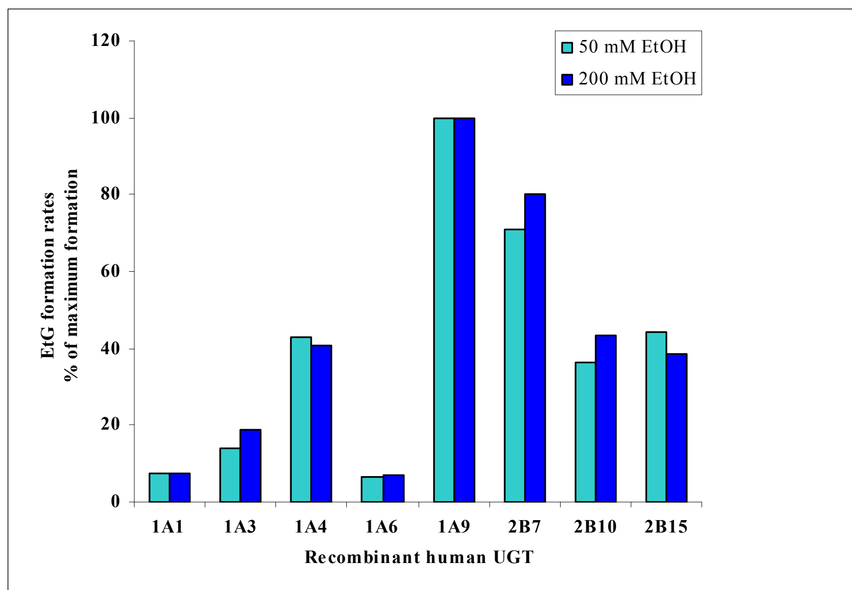

Fig. 1. EtG formation rates of all tested recombinant UGT isoforms. Rates were normalized to the maximum observed formation rate from UGT1A9 for two different ethanol concentrations.

Tab. 1. Kinetic parameters of EtG formation by recombinant UGTs.

rUGT	K_m [mM]	V_{max} [pmol/min/mg]	Cl_{intr} [nl/min/mg]
UGT1A1	10.32 ± 1.28	0.96 ± 0.01	0.093
UGT1A3	39.87 ± 3.51	2.88 ± 0.06	0.072
UGT1A4	12.16 ± 1.42	5.45 ± 0.08	0.448
UGT1A6	21.49 ± 3.90	0.99 ± 0.03	0.046
UGT1A9	26.17 ± 2.49	15.26 ± 0.29	0.584
UGT2B7	32.41 ± 2.68	12.06 ± 0.22	0.372
UGT2B10	35.06 ± 2.43	6.58 ± 0.10	0.188
UGT2B15	8.05 ± 1.98	5.14 ± 0.12	0.639

3.2. Inhibition by Kaempferol and Quercetin

UGT1A1, 1A3, 1A9 were chosen to determine whether co-administration of kaempferol and quercetin has inhibitory effects on EtG formation. Preliminary inhibition experiments at three different kaempferol and quercetin concentrations (½*K_m, K_m, 2*K_m) showed that at 2*K_m inhibitor concentration both flavonoids reduce formation of EtG by >60% of the formation rate observed without inhibitor, irrespective of the UGT isoform used (Fig 2).

Further inhibition experiments to determine the half maximal inhibitory concentration (IC_{50}) showed values that ranged from 3.04 µM quercetin for UGT1A1 to 38.47 µM kaempferol for UGT1A9, which means that EtG formation of all UGTs tested is strongly inhibited by the flavonoids kaempferol and quercetin. All IC_{50}-values are listed in table 2.

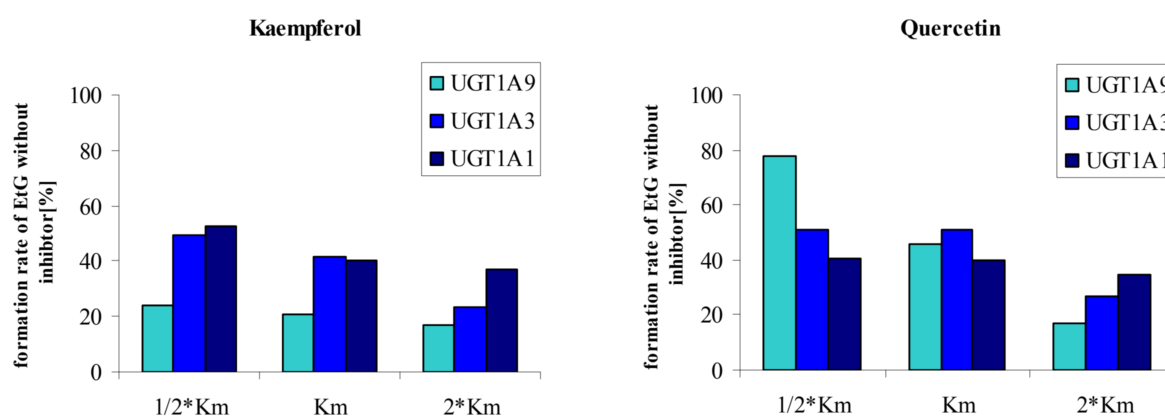

Fig 2. Inhibition of UGT1A1, UGT1A3 and UGT1A9 mediated EtG formation by kaempferol and quercetin at three different concentrations (½*K_m, K_m, 2*K_m).

Tab. 2. IC_{50}-values of kaempferol and quercetin with UGT1A1, 1A3, 1A9.

Kaempferol		**Quercetin**	
UGT isoform	**IC_{50} [µM]**	**UGT isoform**	**IC_{50} [µM]**
UGT1A1	24.39	**UGT1A1**	3.04
UGT1A3	25.23	**UGT1A3**	17.82
UGT1A9	38.47	**UGT1A9**	37.94

4. Conclusions

Ethanol elimination is catalyzed by all UGT isoforms under investigation whereas UGT1A9 and UGT2B7 exhibited the highest formation rates of EtG. The glucuronidation of ethanol could be best described by the classical Michaelis-Menten equation for all UGTs.

Inhibition experiments showed that the glucuronidation of ethanol is strongly affected by the flavonoids kaempferol and quercetin, which appear to be strong competitive inhibitors for the UGTs tested. It seems that nutritional components will influence the elimination of ethanol via UGTs to EtG which may partly explain its variable formation in man. Further investigation will be necessary to determine the type and strength of the inhibition by those flavonoids.

5. References

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