Selective Conjugation of 7-Hydroxycoumarin by Recombinant Human Uridine 5'-diphospho-glucuronosyltransferase (UGT)

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ABSTRACT

7-Hydroxycoumarin (7-HC) has been used as a probe substrate for phase II enzymes uridine 5'-diphospho-glucuronosyltransferase (UGT) and sulfotransferase (ST) in both subcellular fractions and in cellular systems. It may be used directly as a substrate to measure phase II activities or may be the intermediate metabolite from the phase I metabolism of the parent substrates 7-ethoxycoumarin or couarmin. In humans, 7-HC glucuronide is the predominant phase II metabolite. However, the specificity of 7-HC as a phase II substrate for the UGT isozymes has not been reported. To investigate 7-HC specificity as a substrate for UGT isozymes, we used a panel of recombinant human enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17). Rank order of the activity showed a stratification amongst the UGTs tested: (1A6, 1A9) > (1A1, 1A10, 2B7, 2B15) > (1A3, 2B4, 2B17). These groups spanned a wide range of activities from UGT1A6 at 3808 pmol/min/mg, to UGT1A1 at 289 pmol/min/mg to UGT1A3 at 31 pmol/min/mg with UGT1A4 not showing any significant activity. Km and Vmax values ranged from 200 µM to 1620 µM and 108 pmol/min/mg to 6945 pmol/min/mg, respectively. The results showed that 7-HC is not metabolized equally between the UGT isozymes but is a substrate for 9 of the 10 rUGTs tested. This data may aid in interpreting metabolism differences between tissues or donors due to differential distribution of UGT isozymes.

INTRODUCTION

7-Hydroxycoumarin (7-HC), also known as umbelliferone, is the intermediate metabolite of coumarin and 7-ethoxycoumarin, and is quickly conjugated by phase II enzymes to 7-hydroxycoumarin glucuronide (7-HCG) and 7-hydroxycoumarin sulfate (7-HCS). To bypass phase I kinetics, 7-HC may be used as a substrate to directly measure UGT and ST activities[1]. However, there are no reports in literature addressing the specificity of 7-HC for the phase II isozymes. Herein, we will focus on UGTs.

UGTs catalyze the conjugation of uridine 5'-diphosphoglucuronic acid (UDPGA) to lipophilic substrates, a process that increases the metabolite's water solubility and excretion. To date, there are 19 genes encoding for human UGT1A and UGT2B families[2], of which 10 are found in the liver: UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15 and 2B17[3]. UGT enzymes are transmembrane proteins located in the endoplasmic reticulum along with cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) enzymes. Liver UGT activities may be observed in hepatocytes, liver slices and microsomes.

UGTs show both specific and overlapping substrate binding. For example, ß-estradiol 3-glucuronide has been used to specifically characterize UGT1A1 metabolism of estradiol[4]. Other substrates are conjugated by multiple UGTs as in the case of ibuprofen. Ibuprofen shows reactivity with UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 2B4, 2B7, 2B15 and 2B17 with the majority of the conjugation observed with UGT1A3, 1A9, 2B4 and 2B7[5]. Therefore, understanding the isozymes responsible for conjugation is critical to understand the utility of a substrate as a probe.

Reaction phenotyping is the elucidation of enzymes involved in the biotransformation of a test article. Traditional methods utilize antibody, chemical inhibitors and recombinant UGT isozymes to determine the enzyme(s) responsible for the metabolism. Antibodies and specific inhibitors of UGT isoforms are not complete and therefore not sufficient to discover the key enzymes involved in the reaction. Recombinant UGTs are available for the 10 known human UGTs found in the liver. The recombinant UGTs do have disadvantages, such as varying expression levels and no procedure to directly measure enzyme content like cytochrome P450 enzymes in order to normalize the activity between isozymes; however, they do represent the best format for reaction phenotyping for UGTs, to date.

Herein, we utilize recombinant UGTs to determine the reaction phenotype of 7-HC in human liver UGTs. Activity was tested using a single concentration of 7-HC at a single time point and normalized for protein. Where possible, Km and Vmax determinations were derived from Michaelis-Menten kinetics.

MATERIALS & METHODS

Reagents

Recombinant UGT enzymes were purchased from BD Biosciences (Bedford, MA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Stock Solutions

A 40X stock of 7-HC (3 mM) solution in acetonitrile was prepared to achieve final concentration of 75 µM for single concentration reactions. For Michaelis-Menten kinetics reactions, 50X stocks of 7-HC in acetonitrile (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.098 mM) to achieve final 10-point concentration response curve from 1000 to 2 µM. A 20X stock of UDPGA (30mM) solution in 0.1 M Tris buffer (pH 7.4) was used to achieve a final concentration of 1.5 mM.

Single Concentration Reactions

rUGTs were thawed and stored on ice. A reaction solution of common reagents (0.1M Tris buffer, 7-HC and alamethicin) were mixed together and 150 µL transferred to 48-well plate for each isozyme in triplicate. For each rUGT, 40 µL was transferred to the 48-well plate. The plate was warmed to 37°C. UDPGA (10 µL) was added to each well to initiate the reaction and incubated at 37°C for 20 minutes. The reaction was terminated by adding equal volume (200 µL) of methanol. Samples were transferred to cryovials and stored at – 20°C prior to bioanalysis.

Michaelis-Menten Kinetics Reactions

rUGTs were thawed and stored on ice. A reaction solution of common reagents (0.1M Tris buffer, rUGT isozyme and alamethicin) were mixed together. The total protein per reaction was 0.05 mg (UGT1A1, 1A4, 1A6, 1A9 and 1A10), 0.1 mg (UGT2B7 and 2B15), 0.15 mg (UGT1A3) or 0.2 mg (UGT2B4 and 2B17). A 186 µL aliquot was transferred to 48-well plate for each 7-HC concentration in triplicate. A volume of 4 µL from each of the 10-point 7-HC curve stock was transferred to the 48-well plate. The plate was warmed to 37°C. UDPGA (10 µL) was added to each well to initiate the reaction and incubated at 37°C for 30 minutes (UGT1A4, 1A6 and 1A9) or 60 minutes (UGT1A1, 1A3, 1A10, 2B4, 2B7, 2B15 and 2B17). The reaction was terminated by adding equal volume (200 µL) of methanol. Samples were transferred to cryovials and stored at – 20°C prior to bioanalysis.

Bioanalysis

Concentrations of 7-HCG were determined using HPLC methods.

Data Analysis

For single concentration reactions, activity rates were derived from the following equation:

(pmol of 7-HCG) / (minutes of reaction time)/ (mg of protein per reaction)

For Michaelis-Menten kinetics reactions, activity rates were derived from the above equation. Activities were plotted for each of the 10-point concentration response curve and analyzed by non-linear regression method by Prism 5.0.

REFERENCES

1. Li, AP *et al.* Chem Biol Interact. 21: 17-35 (1999)
2. Bock KW. Biochem Pharmacol. 80: 771-7 (2010)
3. Izukawa T et al. Drug Metab Dispos. 37: 1759-68 (2009)
4. Donato MT *et al.* Anal Bioanal Chem. 396: 2251-2263 (2010)
5. Keuhl GE *et al.* Drug Metab Dispos. 33: 1027-1035 (2005)

Single Concentration Reactions

A panel of rUGTs were used to assess the specific activity of 7-HCG formation. A single concentration and time point was used to provide a relative rate of the reaction. From this data, the UGTs were ranked into 4 categories. The high activity group consisted of UGT 1A6 and 1A9. The moderate activity group was approximately 1/10th of the high rates and consisted of UGT1A1, 1A10, 2B7 and 2B15. The low activity group was approximate 1/10th of the moderate activity group and consisted of UGT1A3, 2B4 and 2B17. The final group showed no activity and consisted of UGT1A4.

There is currently no method to quantify the UGT enzyme content as can be done with CYP enzymes. Therefore, normalizing to pmol of enzyme cannot be performed as with CYPs. As well, the rUGT expression levels cannot be assumed to be equal between isozymes or from batch to batch of the same isozyme. A direct comparison between isozymes cannot be made. The only absolute conclusion that can be made is that 7-HC is or is not a substrate for a given UGT isozyme. As such, UGT1A4 does not appear to metabolize 7-HC to 7-HCG. The other 9 human liver UGTs do metabolize 7-HC to 7-HCG.

Table 1 (*Right*). Specific activity of 10 human liver rUGTs to metabolize 7-HC to 7-HCG at the indicated protein content and incubation time.

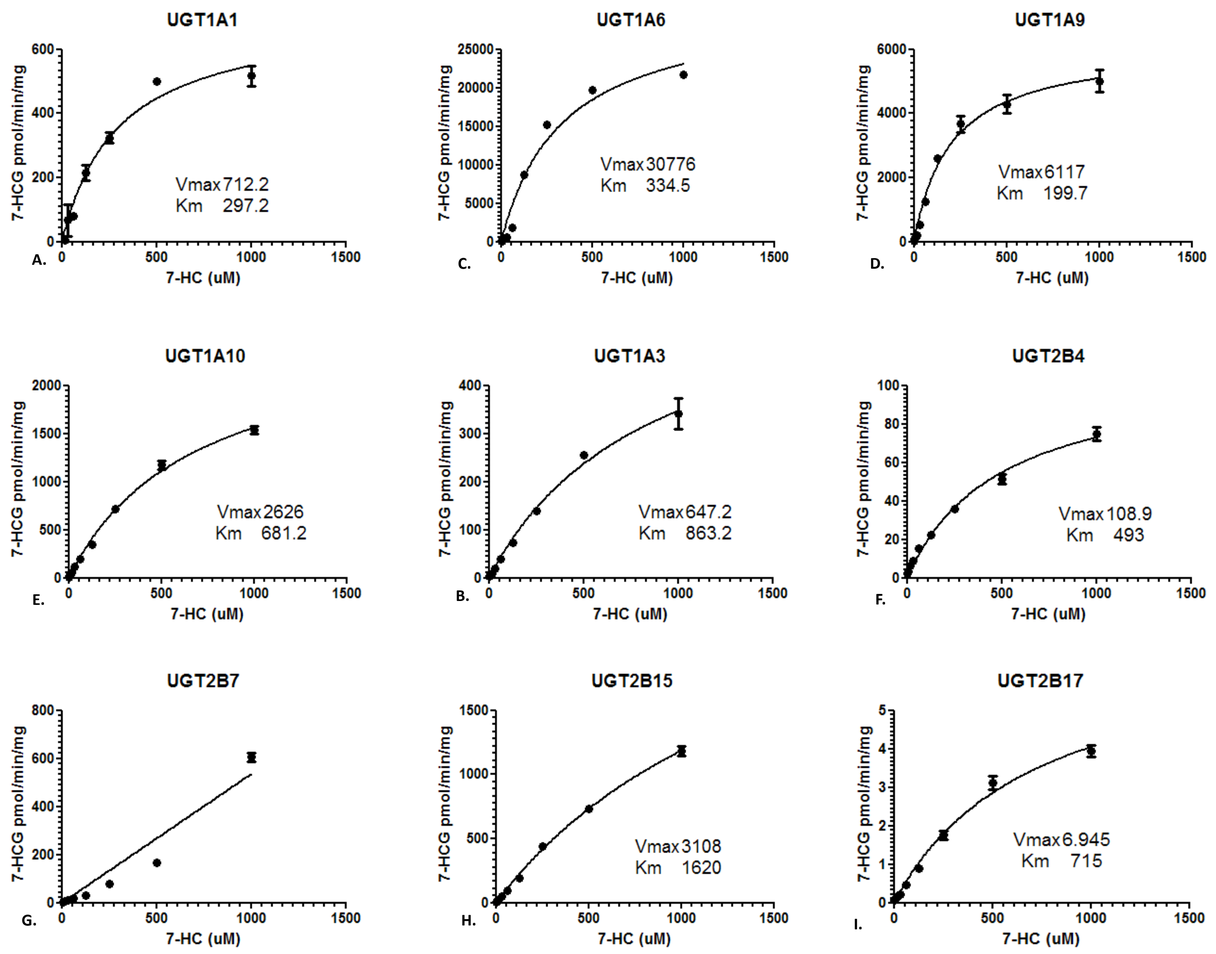
UGT	Specific Activity pmol/min/mg	Protein mg	Time min
1A1	289	0.2	20
1A3	31	0.2	20
1A4	0	0.2	20
1A6	3808	0.2	20
1A9	3610	0.2	20
1A10	140	0.2	20
2B4	8	0.2	20
2B7	196	0.2	20
2B15	154	0.2	20
2B17	6	0.2	20

Michaelis-Menten Kinetics Reactions

Michaelis-Menten kinetics were determined for eight of the UGT isozymes. Km and Vmax values are shown in Table 2. The Km values varied from 199.7 to 1620 µM. The Vmax values ranged from 6.945 to 30776 pmol/min/mg and the Vmax rank order was confirmed with the single concentration activity screen. UGT1A6 and 1A9, which are in the high activity group from the single concentration reaction, showed the highest Vmax of those tested.

UGT1A4 was tested and some metabolism was observed for 7-HC at the highest concentration of 1 mM, deriving an activity of 32 pmol/min/mg. There was insufficient activity to derive Km and Vmax values which confirmed the previous observation that 7-HC is a very poor substrate for UGT1A4.

UGT2B7 rendered a linear plot resulting in ambiguous results. Further investigation is needed to elucidate kinetic values.



UGT	Km µM	Vmax pmol/min/mg
1A1	297.2	712.2
1A3	863.2	647.2
1A4	Insufficient Activity	Insufficient Activity
1A6	334.5	30776
1A9	199.7	6117
1A10	681.2	2626
2B4	493	108.9
2B7	Ambiguous	Ambiguous
2B15	1620	3108
2B17	715	6.945

Table 2 (*Above*). Km and Vmax values derived from Michaelis-Menten kinetics.

Graphs A-I (*Left*). Plots of Michaelis-Menten kinetics for (A.) UGT1A1, (B.) UGT1A3, (C.) UGT1A6, (D.) UGT1A9, (E.) UGT1A10, (F.) UGT2B4, (G.) UGT2B7, (H.) UGT2B15 and (I.) UGT2B17 with apparent Vmax (pmol/min/mg) and Km (µM) values.

CONCLUSIONS

1. 7-HC has been used as a substrate for phase II conjugation as an intermediate metabolite of 7-EC or coumarin, or directly as a non-specific substrate, and yet, the specificity has not been determined.
2. 7-HC is a substrate for 9 out of 10 of the known human liver UGTs with only UGT1A4 providing little to no metabolism.
3. The Km and Vmax varied greatly between the UGTs tested with UGT1A6 and 1A9 providing greatest amount of metabolism as measured by rUGTs.
4. 7-HC is a good non-specific substrate for assessing UGT activities.