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Evaluation of mutation effects on UGT1A1 activity toward 17β-estradiol using liquid chromatography–tandem mass spectrometry☆,☆☆

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Abstract

Mutations in the gene encoding UDP-glucuronosyltransferase 1A1 (UGT1A1) may reduce the glucuronidation of estradiol, bilirubin, etc. In the present study, we used a liquid chromatography–tandem mass spectrometry (LC/MS/MS) method to assay the activities of recombinant mutated UGT1A1 toward 17β-estradiol (E2), by determining its glucuronide (E2G) content. Direct evidence for glucuronide formation was provided by E2G-specific ion peaks. The UGT1A1 activities of G71R (exon 1), F83L (exon 1), I322V (exon 2) and G493R (exon 5) mutants were 24, 30, 18 and 0.6% of the normal UGT1A1 activity, respectively. In conclusion, our study showed that LC/MS/MS enabled accurate evaluation of the effects of mutations on recombinant UGT1A1 activity towards E2.

Keywords: UGT1A1; Mutation; 17β-Estradiol

1. Introduction

UDP-glucuronosyltransferases (UGTs) are membrane-bound enzymes that are localized inside the endoplasmic reticulum and catalyze the conjugation of compounds with uridine diphosphoglucuronic acid (UDPGA), i.e. glucuronidation. Glucuronidation is a major process for the detoxification and excretion of endo- and xenobiotics in humans and other mammalian species [1]. Consistent with their broad substrate profiles, UGTs are known to exist as a superfamily of independently regulated enzymes, that can be divided into two subfamilies, namely UGT1A and UGT2B [2]. The UGT1A subfamily, derived from a single gene locus at chromosome 2q37, comprises at least 13 promoters and first exons that are separately spliced to common exons 2–5, resulting in transcripts that encode enzymes with unique amino termini preceding an identical carboxyl terminus [3,4]. The UGT2B subfamily consists of five similar genes located in a cassette on chromosome 4q13 [5].

UGT1A1, a member of UGT1A family, is expressed mainly in the liver and works as a main glucuronidation enzyme for bilirubin and 17β-estradiol (E2) [6–8]. More than 60 genetic variants, mutations or polymorphisms, in the *UGT1A1* gene have been reported (see "UDP glucuronosyltransferase home page" on the Internet), and many of them were found in the patients

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☆☆ Electronic-Database Information: 1. UDP Glucuronosyltransferase home page: http://som.flinders.edu.au/FUSA/ClinPharm/UGT/index.html.
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with Crigler-Najjar syndrome and Gilbert syndrome which hamper bilirubin-glucuronide formation [9–11]. The observation of Crigler-Najjar patients presenting a significant decrease in the E2 glucuronide (E2G) formation has proved that UGT1A1 is also a main glucuronidation enzyme for E2 [7].

One of the most common genetic variants is a dinucleotide repeat polymorphism in the atypical TATA box region of the *UGT1A1* promoter. The variant allele consists of seven TA repeats in the A(TA)nTAA motif, whereas six TA repeats characterize the common allele. Homozygous individuals carrying the $A(TA)_7TAA$ allele (1–19% of the population) show significantly higher plasma levels of unconjugated bilirubin caused by a 30% reduction in *UGT1A1* gene transcription [12–15].

The expression of UGT1A1 has been observed in human breast cancer cell lines and postulated that genetic variants of *UGT1A1* leading to reduction in E2-glucuronidation is associated with the development of breast cancer [16]. In the African-American population, four *UGT1A1* variant alleles characterized by a variation in the number of TA from five through eight repeats in the TATA box have been described and the variant alleles with seven and eight repeats were reportedly associated with the development of breast cancer.

Although the TATA box polymorphism is infrequent in the East Asian populations, but the G71R (glycine to arginine at codon 71) mutation is the most common variant there. Thus, it is necessary to determine the relationship between G71R mutation and breast cancer when we study the genetic risk factor for breast cancer in the East Asian populations. However, effects of the G71R mutation on the E2-glucuronidation have never been studied so far.

In the present study, we used a liquid chromatography–tandem mass spectrometry (LC/MS/MS) method to assay the activities of the UGT1A1 enzymes expressed in the cultured cells. Recently, glucuronides have been determined by LC/MS/MS in order to clarify the metabolism of chemicals, including drugs, pollutants, etc., in the body, and this method can provide direct evidence for glucuronide formation from the observation of specific ion peaks [17–19]. Here, we determined the levels of the E2G product in reaction mixtures after incubating E2 with the recombinant mutated UGT1A1 enzymes and UDPGA, and calculated the remaining activities of them.

2. Experimental

2.1. Mutations

A G-to-A missense mutation at nucleotide (nt) 211 in exon 1 (glycine to arginine at codon 71, G71R) is common in the East Asian population [20]. The other three mutations in the *UGT1A1* gene, T-to-C at nt 247 in exon 1 (phenylalanine to leucine at codon 83, F83L), A-to-G at nt 964 in exon 2 (isoleucine to valine at codon 322, I322V) and G-to-C at nt 1477 in exon 5 (glycine to arginine at codon 493, G493R), were identified in a series of study on neonatal jaundice in Thailand and Malaysia [21–23] (Fig. 1).

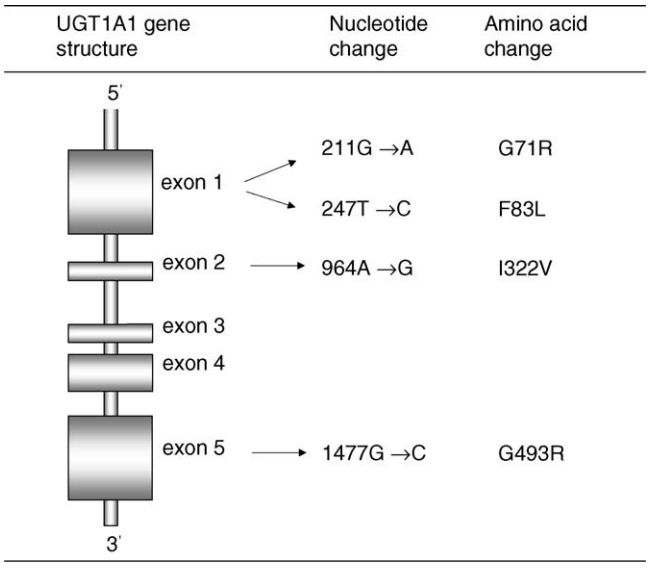

Fig. 1. Mutations of the *UGT1A1* gene.

2.2. Materials

A human liver cDNA library was purchased from Cosmo Bio (Tokyo, Japan). A directional TOPO Cloning Kit containing the pENTR™/D-TOPO vector, the pcDNA-DEST40 Gateway™ vector and Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA, USA). A luciferase reporter vector (pGL3-vector) was purchased from Promega (Madison, WI, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin–streptomycin, glacial acetic acid (>99% purity), E2, 17α-ethynylestradiol (EE2) and E2G standard were purchased from Sigma (St. Louis, MO, USA). The enzyme reaction reagents, UGT Reaction Mix Solution A (including 25 mM UDP-glucuronic acid in water) and UGT Reaction Mix Solution B (including 250 mM Tris–HCl, 40 mM $MgCl_2$ and 0.125 mg/ml alamethicin in water) were purchased from BD Gentest (Franklin Lakes, NJ, USA). Perchloric acid (>99% purity), methanol and acetonitrile were of HPLC grade, and purchased from Wako Pure Chemicals (Osaka, Japan). Deionized water was obtained using a Milli-Q water system (Millipore, Milford, MA, USA).

2.3. Construction of UGT1A1 cDNA clones

Human *UGT1A1* cDNA was obtained from a human liver cDNA library by PCR-amplification using the primer set 5′-CAC CAT GGC TGT GGA GTC CC-3′ and 5′-CTT ATT TCC CAC CCA CTT CTC AAT G-3′, and inserted into a pENTR™/D-TOPO vector to construct an entry clone containing the wild-type *UGT1A1* cDNA sequence.

Mutations were introduced to the entry clone using a site-directed mutagenesis method [24]. The primers used to introduce the mutations were: G71R: 5′-TGT AAA ATG CTC TGT CTC TGA TG-3′; F83L: 5′-CTC CCT TTG GAG TGG CAC AGG G-3′; I322V: 5′-GCA TCA GCA ACT GCC ATA GC-3′; and G493R: 5′-CAA GAG GAA ACG AAT CAC GTC C-3′.

Subsequently, the wild-type and mutant cDNA sequences in the entry clones were transferred into an expression vector (or a destination vector), the pcDNA-DEST40 Gateway™ vector, by recombination. The wild-type and mutant *UGT1A1* cDNA sequences were confirmed by nucleotide sequencing.

2.4. Transfection and expression of UGT1A1 cDNA in COS-7 cells

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. At 24 h prior to transfection, the cells were seeded in the antibiotic-free medium.

The UGT1A1 expression vectors, together with a luciferase reporter vector (pGL3-vector), were transfected into the cells using Lipofectamine™ 2000, according to the manufacturer's instructions. To understand the heterozygous states of the wild-type and each mutant *UGT1A1* allele, a clone mixture (wild-type clone:mutant clone = 1:1) was also transfected into COS-7 cells.

COS-7 cells were harvested at 48 h after transfection and homogenized with 70 μl of 0.1 M Tris–HCl (pH 7.5). The whole cell homogenates were used as enzyme sources for UGT1A1 and luciferase assays. Luciferase activity was measured using a TD-20/20 luminometer (Promega, Madison, WI, USA) and used to normalize the enzyme activities of the UGT1A1 clones based on their transfection efficiencies.

2.5. UGT1A1 enzyme reaction with 17β-estradiol

Glucuronidation of 17β-estradiol was assayed using UGT Reaction Mix, according to the manufacturer's instructions. Briefly, a 99 μl reaction mixture containing 20 μl of Solution A, 8 μl of Solution B, 1 μl of 300 μM E2 in ethanol and 70 μl of cell homogenates, was incubated at 37 °C for 30 min. The reaction was stopped by addition of 50 μl of 94% acetonitrile/6% glacial acetic acid.

After centrifuging the reaction mixture, the supernatant was subjected to an LC/MS/MS assay to determine the amount of E2G. Prior to the LC/MS/MS analysis, we added 1 μl of 300 μM EE2 in ethanol into the reaction mixture.

2.6. HPLC chromatographic and mass spectrometric conditions

Chromatography was performed using a Shimadzu LC10AD system (Shimadzu, Kyoto, Japan) with a mobile phase consisting of 70% (v/v) methanol, 4.5% (v/v) acetonitrile and 0.15 mM perchloric acid in Milli-Q water, at a flow rate of 0.3 ml/min. The column temperature was maintained at 45 °C. A 15 μl aliquot of each sample was injected onto a Shim-pack CLC-ODS column (4.6 mm I.D. × 15 cm; Shimadzu).

An API-3000™ LC/MS/MS system (Applied Biosystems-MDS Sciex, Toronto, Canada) was operated using an electrospray ionization source (ESI; TurboIonSpray interface) coupled with the above-mentioned LC system. Analyst 1.3.1 software was used to control the equipment, as well as for data acquisition and analysis. The MS scanning was performed in a negative ion mode.

2.7. Assay validation

Calibration standards and quality control (QC) samples were prepared in blank reaction mixture by spiking with the working solutions. The blank reaction mixture was the homogenate of the COS-7 cells transfected with pcDNA-DEST40 Gateway™ vector. The calibration standards for E2G were comprised of seven different concentrations (0, 0.00125, 0.0125, 0.125, 0.25, 0.5 and 1.0 μg/125 μl). The QC samples were comprised of two concentrations (0.0125 μg/125 μl, low QC; 1.0 μg/125 μl, high QC). Calibration standards were freshly prepared prior to use. QC samples were prepared and divided into individual tubes and stored frozen until use for the analysis.

To obtain a calibration curve, seven calibration standards were analyzed. The calibration curve was calculated by least-squares linear regression using $1/x$ weighting. Correlation coefficients (r^2) required to be 0.99 or higher.

To determine the intra-assay accuracy and precision, low and high QC samples were analyzed in quintuplicate ($n = 5$) together with calibration standards prepared independently from the QC samples. Accuracy was expressed as the percent difference between the mean of observed concentrations and the theoretical concentration, and was required to be within 20% for the low QC and within 10% for the high QC. The relative standard deviation (RSD) was used to express precision, and the % RSD was required not to exceed 20% for the low QC concentration and 10% for the high QC concentration.

To determine the inter-assay precision, low and high QC samples were analyzed together with calibration standards prepared independently from the QC samples. This procedure was repeated on different 5 days. The % RSD was required not to exceed 20% for the low QC concentration and 10% for the high QC concentration.

To confirm the invariable ionization efficiencies of analyte (E2G) and I.S. (EE2) at different concentrations, response ratios of E2G to EE2 were determined at five sets of different concentrations of both compounds (E2G [μg/125 μl]:EE2 [μg/125 μl] = 1:0.5, 2:1, 4:2, 10:5, 20:10).

2.8. Calculation of UGT1A1 activities

The mutated UGT1A1 activities were calculated as follows: the levels of E2G in whole homogenates of COS-7 cells transfected with the wild-type and mutant *UGT1A1* cDNAs were determined as described above and the amounts of expressed UGT1A1 proteins were normalized based on the activities of luciferase which was co-expressed in the COS-7 cells. Here, wild-type UGT1A1 activity (homozygous model) was designated as normal UGT1A1 activity. The mutated UGT1A1 activities were expressed as a percentage of the normal UGT1A1 activity.

2.9. Statistics

We assayed the recombinant UGT1A1 activities in quadruplicate ($n = 4$) and calculated the mean and S.D. of the activity. The activities of the normal and mutated UGT1A1 enzymes were

Table 1
Parameters of mass spectrometric conditions

Parameters	E2G	EE2
Q1 mass	447.1	299.1
Q3 mass	271.1	145.1
Retention time (min)	8.3	10.8
Electrospray voltage (V)	−4200	−4200
Declustering potential	51.0	91.0
Focusing potential	150.0	330.0
Collision energy	56.0	52.0
Collision cell exit potential	21.0	5.0

compared by *t*-tests. A *p*-value of less than 0.05 was considered to indicate a significant difference.

3. Results and discussions

3.1. LC/MS/MS conditions

Q1 mass spectra of E2G (analyte) and EE2 (I.S.) revealed predominant deprotonated molecules $[M-H]^-$ at *m/z* 447.1 and 295.1, respectively. Q3 mass (product ion) spectra of the two compounds were recorded by allowing the deprotonated molecules at 271.1 and 145.1 respectively, to fragment in the collision cell. The product ion at *m/z* 271.1 was used for analysis of E2G and the ion at *m/z* 145.1 was used for analysis of EE2 and E2G, in combination with their $[M-H]^-$ precursors. Analytical conditions were automatically optimized by analyte infusion and indicated in Table 1. Thus, precursor/product ion pairs at *m/z* 447.1/271.1 and 295.1/145.1 were selected for the analysis of E2G and EE2 in the multiple reaction monitoring (MRM), respectively (Fig. 2).

The MRM chromatography of reaction mixtures guaranteed the peak purity and selectivity of E2G and EE2. As shown in Fig. 3, there were no peaks in the chromatogram which interfered with E2G peak. There were no peaks, either, which interfered with EE2 peak (data not shown). E2G amount was calculated using the peak area ratios of E2G to EE2, on the basis of the calibration curve.

3.2. Assay validation

3.2.1. Linearity

Calibration standards were analyzed in a dynamic range of 0–1.0 μg/125 μl total reaction mixture for E2G. The calibration curve correlates over the concentration range for this study. The equation of the liner calibration curve in the practical quantification range was $y = 499x + 0.0589$ ($R^2 = 0.9976$). Calibration curves showed acceptable linear correlation for E2G.

3.2.2. Accuracy and precision

The intra-assay precision (% RSD) determined at low QC and high QC was 8.5 and 5.5%, respectively. The inter-assay precision (% RSD) determined at low QC and high QC was 3.2 and 8.1%, respectively. Accuracy of high and low QC for intra-assay were −6.9 and 3.7%.

Precision data for the intra- and inter-assays met the requirements at the low QC and high QC concentrations, as did accuracy data. From these results, we confirmed that our method had high precision and accuracy for quantification of E2G.

3.2.3. Response ratios

The response ratios of E2G to EE2 showed acceptable variation at five sets of different concentrations of both compounds (E2G [μg/125 μl]:EE2 [μg/125 μl] = 1:0.5, 2:1, 4:2, 10:5, 20:10). The RSD among them was 5.3%.

3.2.4. Stability of the analytes in the reaction mixture

The analyte, E2G, was stable in the reaction mixture for at least one month at −20 °C, or for 24 h at 4 °C. In addition, E2G was shown to remain stable in the reaction mixture even after three repeat of freeze and thaw procedures.

3.3. Remaining UGT1A1 activities

In the present study, we calculated the remaining UGT1A1 activities of homozygous and heterozygous models (Table 2), representing individuals who carry the same alleles and those who carry the wild-type and mutant alleles, respectively. The UGT1A1 activity of a homozygous model of wild-type alleles was considered to be the normal UGT1A1 activity.

According to our method, the remaining UGT1A1 activities of the homozygous model with the G71R, F83L, I322V and G493R mutations were 24, 30, 18 and 0.6% of the normal UGT1A1 activity, respectively. The remaining UGT1A1 activities of the heterozygous model with the G71R, F83L, I322V and G493R mutations were 80, 79, 68 and 38% of the normal UGT1A1 activity, respectively.

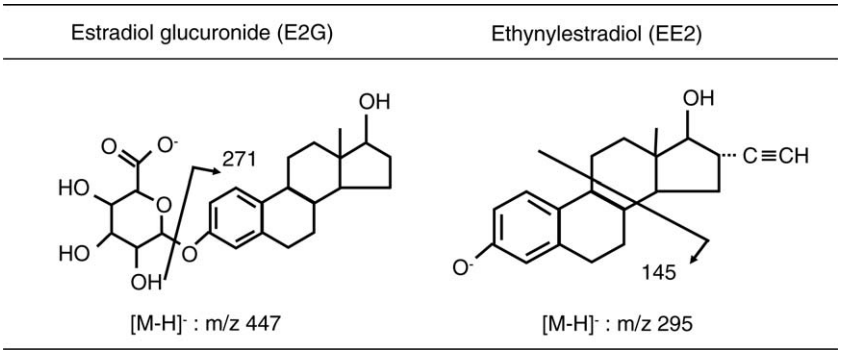

Fig. 2. Structures of 17β-estradiol 3-glucuronide (E2G) and 17α-ethynylestradiol (EE2).

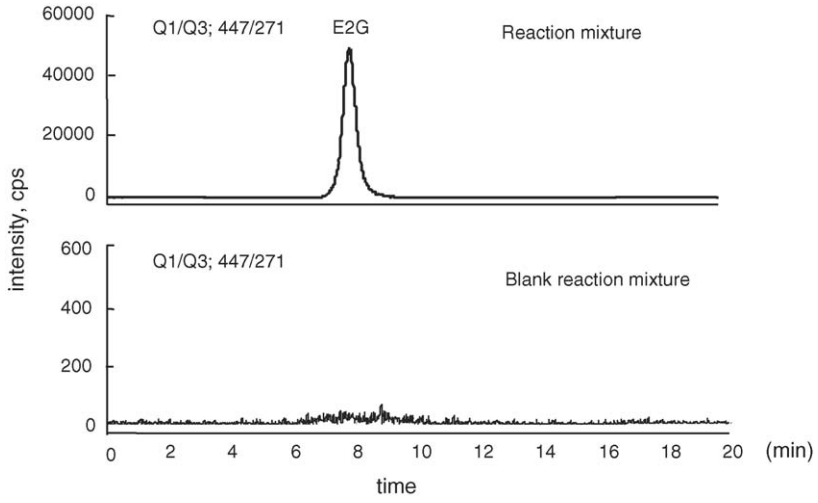

Fig. 3. MRM chromatograms of 17β-estradiol 3-glucuronide (E2G) for reaction mixture with or without UGT1A1 enzyme.

Table 2
Mutated UGT1A1 activities

Mutated UGT1A1	E2G amount[a]	Remaining activity (%)	*P* value
Homozygous models ($n=4$)			
Wild	5.45 ± 0.86	100	–
G71R	1.29 ± 0.40	23.6	0.00088
F83L	1.62 ± 0.40	29.8	0.0012
I322V	0.99 ± 0.02	18.2	0.0019
G493R	0.03 ± 0.17	0.6	0.0011
Heterozygous models (mutant:wild = 1:1) ($n=4$)			
G71R/Wild	4.34 ± 2.87	79.6	0.63
F83L/Wild	4.29 ± 2.19	78.8	0.38
I322V/Wild	3.72 ± 1.31	68.2	0.15
G493R/Wild	2.09 ± 1.78	38.4	0.055

Note: To make sure of releasing all UGT1A1 enzymes from the transfected COS-7 cells, we added a channel forming antibiotic, alamethicin, to the whole cell homogenate. Alamethicin may lead to unlimited penetration of the enzymes through the membrane [26].

[a] μg/1000 unit luciferase activity on TD-20/20 luminometer.

In the present study, we measured relative UGT1A1 activities with mutations toward E2. The remaining relative activities of UGT1A1 with G71R toward E2 in the homozygous and heterozygous models were consistent with those toward bilirubin [25]. However, to clarify whether or not the low activities of mutated UGT1A1s towards E2 are due to the same functional defect of those towards bilirubin, it requires further studies on the binding site, catalytic site and oligomerization of the mutated UGT1A1s.

3.4. Conclusion

In the present study, recombinant UGT1A1 enzymes were expressed in COS-7 cells and their activities toward E2 were assayed using LC/MS/MS. LC/MS/MS detected a small amount of E2G with great accuracy, confirming reduced E2-glucuronidation activities of the mutated UGT1A1 enzymes. Elucidating the discrepancy between the UGT1A1 activity and the clinical features will lead to full understanding of the glucuronidation systems for endo- and xenobiotics.

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