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Title page

Optimized Methods for Targeted Peptide-based Quantification of Human Uridine 5´-diphosphate-glucuronosyltransferases in Biological Specimens using Liquid Chromatography Tandem Mass Spectrometry

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Words in Discussion: 1235

Abbreviations used in this paper: BSA, bovine serum albumin; CV, coefficient of variation; CYP, cytochrome P450; HIM, human intestinal microsomes; HKM, human kidney microsomes; HLM, human liver microsomes; IS, internal standard; IVIVE, *in vitro-in vivo* extrapolation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; MMTS, methyl methanethiol sulfonate; MRM, multiple reaction monitoring; OATP, organic anion-transporting polypeptide; OG, octyl glucoside; PAR, peak area ratio; rhUGTs, recombinant human UGT-expressing insect cell membranes; SDC, sodium deoxycholate; TCEP, tris(2-carboxyethyl)phosphine; UGT, uridine 5’-diphosphate-glucuronosyltransferase

Abstract

The aim of this study was to optimize methods for quantifying 13 uridine 5´-diphosphate-glucuronosyltransferase (UGT) isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, and 2B17) in human liver, intestinal, kidney microsomes, and recombinant human UGT-expressing insect cell membranes (rhUGTs) by targeted peptide-based quantification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Production of targeted peptides was compared by combining three denaturing agents (urea, sodium deoxycholate (SDC), and octyl glucoside (OG)) and three denaturing temperatures (37°C, 60°C, and 95°C) followed by tryptic digestion for 2–20 h. Denaturing conditions and digestion times yielding high production efficiency varied markedly among isoforms and specimens, indicating the importance of specific optimization. Each UGT isoform was quantified using the methods found to be optimal. The expression of ten (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15, and 2B17), six (1A1, 1A3, 1A4, 1A10, 2B7, and 2B17), and three (1A6, 1A9, and 2B7) isoforms was detected in human liver, intestinal, and kidney microsomes, respectively, and levels were reproducible using multiple protocols. All isoforms were quantified in rhUGTs. Determining the levels of UGTs in human tissue specimens and those in rhUGTs is important for estimating the contribution of glucuronidation to body clearance based on *in vitro-in vivo* extrapolation (IVIVE).

Introduction

Uridine 5´-diphosphate-glucuronosyltransferases (UGTs) catalyze glucuronidation, the transfer of glucuronic acid from uridine 5´-diphosphate glucuronic acid to substrates (Dutton, 1980). In addition to cytochrome P450 (CYP)-mediated oxidation, glucuronidation is an important biotransformation for various xenobiotics as well as endogenous compounds (Tukey and Strassburg, 2000; Wells et al., 2004). Glucuronidation accounts for approximately 35% of Phase II drug metabolism (Evans and Relling, 1999) and represents the primary clearance pathway for 10% of the 200 most prescribed drugs (Williams et al., 2004). Evaluation of the contribution of glucuronidation to body clearance is therefore an important issue in drug development.

The contribution of metabolic pathways to drug metabolism *in vivo* can be estimated from the activity of enzymes determined *in vitro* (Iwatsubo et al., 1997a; Iwatsubo et al., 1997b). In principle, *in vitro* metabolic activity (activity per enzyme molecule) determined using a recombinant enzyme is multiplied by the amount of enzyme in human tissues to calculate the *in vivo* metabolic rate. To extrapolate *in vitro* glucuronidation activities, information must be acquired regarding the amounts of UGTs expressed in recombinant systems as well as in human tissues.

Although immunochemical approaches such as western blotting or enzyme-linked immunosorbent assays are standard methods for protein quantification, preparing UGT isoform-specific antibodies is generally difficult because of their high degree of sequence similarity (Milne et al., 2011). An alternative approach is targeted peptide-based protein quantification using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Gerber et al., 2003). In this approach, the protein of interest is enzymatically digested after denaturation, and the proteotypic peptide

fragment is quantified using LC-MS/MS. Typically, a peptide of the same sequence labeled with stable isotopes is used as the internal standard (IS). Major advantages of LC-MS/MS-based quantification include its specificity, large quantification range ($>10^3$), and ability to analyze multiple analytes simultaneously.

The results of studies on LC-MS/MS-based UGT quantification in human tissue specimens are not always consistent (Fallon et al., 2008; Harbourt et al., 2012; Ohtsuki et al., 2012; Sato et al., 2012b; Schaefer et al., 2012). This may be explained by differences in the proteotypic peptides employed as the calibrants or experimental procedures, such as denaturation and proteolytic digestion. Certain studies indicate that the accuracy of targeted peptide-based protein quantification varies according to the method used as well as the protein, and that optimizing proteolysis is important. For example, Proc et al. (2010) compared 14 methods for tryptic digestion of human plasma proteins using various denaturing agents and found that sodium dodecyl sulfate and sodium deoxycholate (SDC) facilitated the generation of digested peptides. Balogh et al. (2012) investigated targeted peptide-based quantification of organic anion-transporting polypeptide (OATP) in human liver membranes using various denaturing methods and found that the production of OATP-proteotypic peptides varied markedly among denaturing agents, and that the highest yields were obtained using SDC.

Here, we compared 36 methods for their ability to efficiently produce proteotypic peptides of 13 UGT isoforms using three denaturing agents, three denaturing temperatures, and four digestion times. Protein quantification in human tissue specimens and recombinant human UGT-expressing insect cell membranes

(rhUGTs) was then performed utilizing methods that were optimized for each isoform and biological specimen.

Materials and methods

Chemicals and Reagents. Bovine serum albumin (BSA), urea, ammonium bicarbonate, and methyl methanethiol sulfonate (MMTS) were purchased from Wako Pure Chemicals (Osaka, Japan), and tris(2-carboxyethyl)phosphine (TCEP) was from Tokyo Chemical Industry (Tokyo, Japan). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). SDC, octyl glucoside (OG), unlabeled standard peptides (purity ≥95%), and AQUA® peptides (peptides whose sequences were identical to the standard peptides and labeled at their C-termini with ^{13}C and ^{15}N) were obtained from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLM, 20 mg/mL), human intestinal microsomes (HIM, 10 mg/mL), and human kidney microsomes (HKM, 10 mg/mL) were obtained from XenoTech LLC (Lenexa, KS). Solutions of rhUGTs (5 mg/mL) were purchased from BD Biosciences (San Jose, CA). The protein concentrations of the biological specimens were provided by the suppliers. All other chemicals and reagents used were commercially available with guaranteed purity. Appropriate containment and personal protective equipment were used for handling potentially biologically hazardous materials.

Equipment. An LC-MS/MS system comprising a Prominence HPLC (Shimadzu, Kyoto, Japan) and QTRAP®5500 (AB SCIEX, Foster City, CA) was used.

Denaturing and digestion methods. Samples were prepared in duplicate. A 2-μL aliquot of human tissue specimens (pooled HLM, HIM, or HKM) or a mixture of 13 rhUGT solutions was added to a 14-μL aliquot of 100 mM ammonium bicarbonate. A denaturing solution (2 μL) and 50 mM TCEP (2 μL) were added and incubated to

denature and reduce the proteins. Denaturing solutions were 10 M urea, 10% SDC, and 10% OG. Incubation conditions were 37°C for 1 h, 60°C for 1 h, and 95°C for 15 min. MMTS (2 μL, 200 mM, isopropanol solution) was added to the preparations at room temperature for 10 min to alkylate thiol residues. A 5-μL aliquot of trypsin aqueous solution (0.5 mg/mL) was added and incubated at 37°C for 2, 4, 8, and 20 h. Tryptic digestion was terminated by adding a 4-fold volume of 2% formic acid. A 2-μL aliquot of an IS solution (mixture of 13 AQUA® peptide solutions) was added and mixed. After filtration using centrifugal filters, a 12-μL aliquot of supernatant was injected into the LC-MS/MS system. LC was performed using a Synergi Fusion-RP 100A 100 × 2.00 mm, 2.5-micron column (Phenomenex, Torrance, CA) at a flow rate of 0.5 mL/min. The HPLC mobile phases, 0.1% formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B), were delivered with a gradient program of 5–16% B for 4 min, 16–50% B for 9 min, 50–100% B for 9.01 min, 100% B for 10 min, 100–5% B for 10.01 min, and 5% B for 12 min. The sample rack and column temperatures were maintained at 10°C and 45°C, respectively. Analysis was performed in positive multiple reaction monitoring (MRM) mode and data were processed using the Analyst 1.5.2 software package (AB SCIEX). The peak area ratio (PAR) was calculated to determine the efficiency of denaturing and digestion methods for producing UGT-proteotypic peptides.

Quantification of UGTs in biological specimens. Biological specimens (HLM, HIM, HKM, and rhUGTs) were denatured under optimum conditions and then alkylated as described above. These samples were prepared in triplicate. After adding the IS solution, tryptic digestion was performed for 2, 4, and 8 h. For calibration, a reference

standard solution of UGT-proteotypic peptides and IS solution were added to the BSA solution (20 mg/mL) digested as described above. Quantification was performed based on the calibration curves. The lower limit of quantification (LLOQ) was set as follows: 1 pmol/mg protein for HLM (3 pmol/mg protein for UGT2B17); 2 pmol/mg protein for HIM and HKM; and 50 pmol/mg protein for rhUGTs.

Results

LC-MRM. A high-throughput LC-MRM method for simultaneous determination of 13 UGT-proteotypic peptides was developed. To select proteotypic peptides that could be digested efficiently, we preliminarily analyzed tryptic digests of rhUGTs using enhanced mass spectrometry scanning, and identified peptide fragments by ProteinPilot^{TM} (AB SCIEX) according to a published method (data not shown) (Sato et al., 2012a). Proteptypic peptides identified with highest confidence values here or reported to yield highly reproducible quantities by others were selected as analytes (Supplemental Table 1) **(**Fallon et al., 2008; Harbourt et al., 2012; Sato et al., 2012b; Fallon et al., 2013b). Each peptide sequence was confirmed as unique among all sequences in the database of the Universal Protein Resource Knowledge Base. All 13 analytes were determined in a single 12-min run (Supplemental Figure 1).

Characterization of denaturing and digestion methods. The efficiencies of urea, SDC, and OG for generating high yields of proteotypic peptides of 13 UGT isoforms in four biological specimens were compared by combining three denaturing temperatures (37°C, 60°C, and 95°C) and four tryptic digestion times (2, 4, 8, and 20 h). The results of generating UGT2B7-proteotypic peptide in HLM are shown in Fig. 1. The highest PAR was achieved when the sample of HLM was denatured in the presence of SDC at 37°C for 1 h followed by tryptic digestion for 4 h. High yields of peptides (≥75% of the highest PAR) were also achieved using three other denaturing conditions as follows: SDC at 60°C for 1 h, OG at 37°C for 1 h, and OG at 60°C for 1 h.

Similar experiments were performed using other isoforms and biological specimens (Supplemental Figure 2). Denaturing conditions that generated high yields of targeted peptides are summarized in Fig. 2. Proteotypic peptides of 10 isoforms (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15, and 2B17) were detected in HLM. For each peptide, a PAR ≥75% was achieved using multiple conditions, and denaturation with SDC or OG at 37°C generated high yields of these 10 peptides (Fig. 2A). In HIM, proteotypic peptides of six isoforms (1A1, 1A3, 1A4, 1A10, 2B7, and 2B17) were detected, and high yields of these peptides were generated when the sample of HIM was denatured in the presence of SDC at 60°C (Fig. 2B). Proteotypic peptides of three isoforms (1A6, 1A9, and 2B7) were detected in HKM. UGT1A6-proteotypic peptide was efficiently produced when the sample of HKM was denatured with SDC or OG at 95°C. Specific peptides of the other two isoforms (UGT1A9 and 2B7) were produced at high yields when denatured with SDC or OG at 60°C (Fig. 2C). In rhUGTs, all 13 proteotypic peptides were produced in high yield when specimens were denatured with SDC at 37°C (Fig. 2D).

Quantification of UGTs in biological specimens. Quantification methods of UGTs in HLM, HIM, HKM, and rhUGTs were optimized based on the results presented above. Denaturing conditions were as follows: HLM – SDC, 37°C, 1 h; HIM – SDC, 60°C, 1 h; HKM – SDC, 60°C, 1 h and SDC, 95°C, 15 min; rhUGTs – SDC, 37°C, 1 h. Samples were digested for 2, 4, and 8 h to ensure that reactions reached a plateau. The quantities of targeted peptides were quantified according to calibration curves with correlation coefficients of ≥0.98. The highest values from three time points were defined as the final result.

The results for quantifying UGT2B7 in 16 individual HLM are presented in Fig. 3, showing that the level of UGT2B7-proteotypic peptide plateaued with low intra-assay variability. Similar experiments were performed with the other isoforms and biological specimens (Supplemental Figures 3 and 4), and the protein levels are summarized in Table 1. The level of UGT2B7 in 16 individual HLM was highest (average 200 pmol/mg protein) among 10 isoforms, followed in order by 1A1, 2B4, 2B15, 1A4, 2B10, 1A9, 2B17, 1A6, and 1A3. The level of UGT2B17 was <3 pmol/mg protein in 5 of 16 individual HLM. In six individual HIM, the level of UGT2B17 was highest (average 112 pmol/mg protein) among 6 isoforms, followed in order by 1A1, 1A10, 2B7, 1A3, and 1A4. In pooled HKM of four individuals, the levels of UGT1A6, 1A9, and 2B7 were 13.9, 171, and 95.3 pmol/mg protein respectively. The levels of 13 UGTs in rhUGTs ranged from 292 to 4500 pmol/mg protein.

Discussion

Targeted peptide-based quantification of UGTs in human tissue specimens using LC-MS/MS has been reported (Fallon et al., 2008; Harbourt et al., 2012; Ohtsuki et al., 2012; Sato et al., 2012b; Schaefer et al., 2012). These studies employed various denaturing and digestion methods but did not clearly state how the method was optimized. Here, we compared the efficiencies of urea, SDC, and OG for generating high yields of proteotypic peptides of 13 UGT isoforms in four biological specimens. The efficiencies of proteolysis by trypsin in the presence of these denaturing agents are generally higher than that in the absence of the denaturant (Katayama et al., 2001; Katayama et al., 2004; Zhang and Li, 2004; Zhou et al., 2006; Chen et al., 2007; Masuda et al., 2008; Proc et al., 2010; Balogh et al., 2012). As done by others, the best practice would be investigating multiple peptides per protein when available (Harbourt et al., 2012; Ohtsuki et al., 2012; Schaefer et al., 2012; Fallon et al., 2013b; Achour et al., 2014). However, the properties of each peptide might respond differently according to the denaturing conditions. Therefore, our approach was to ensure that the results were reliable by optimizing the denaturing method as well as using one peptide yielding a good outcome.

Urea is easily separated from the analyte using LC-MS/MS (Chen et al., 2007). SDC aids high-efficiency digestion of membrane proteins (Zhou et al., 2006; Masuda et al., 2008; Proc et al., 2010; Balogh et al., 2012), and OG increases the efficiency of tryptic digestion and does not interfere with MS analysis (Katayama et al., 2001; Katayama et al., 2004; Zhang and Li, 2004). Because the denaturing temperature and tryptic digestion time vary among these studies, we tested three temperatures (37°C, 60°C, and 95°C) and four digestion times (2, 4, 8, and 20 h).

The results of generating UGT2B7-proteotypic peptide in HLM varied markedly depending on the conditions. Interestingly, the amount of targeted peptide decreased after longer digestion (20 h) for most conditions (Fig. 1). These results suggest that this UGT2B7-proteotypic peptide may have been adsorbed or degraded during the reaction and that the yields reflect its loss relative to production.

Proteotypic peptides of 10 isoforms (1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15, and 2B17) were detected in HLM, which was consistent with the results reported very recently by another study (Fallon et al., 2013b). In HIM, proteotypic peptides of six isoforms (1A1, 1A3, 1A4, 1A10, 2B7, and 2B17) were detected. Peptides of five of these isoforms (1A1, 1A3, 1A4, 2B7, and 2B17) were also detected in HLM, but denaturing conditions generating high yields of these peptides were not completely consistent between HLM and HIM (Fig. 2B). Proteotypic peptides of three isoforms (1A6, 1A9, and 2B7) were detected in HKM. While these three peptides were also detected in HLM, efficient denaturing conditions in HLM did not necessarily generate high yields of these peptides in HKM (Fig. 2C). In rhUGTs, all 13 proteotypic peptides were detected. It is noteworthy that 10 peptides were produced at high yields using urea which was not efficient for generating any peptides in human microsomal samples (Fig. 2D). Results of the production efficiencies suggest that the effective denaturing conditions varied according to the nature of the biological specimen, even for the same targeted peptide. Therefore, to quantify UGTs, it is important to optimize conditions for a particular peptide and its source.

Quantification methods of UGTs in HLM, HIM, HKM, and rhUGTs were optimized based on the results of the production efficiency. The amount of targeted peptides did not consistently depend on digestion time, and the highest yields were

obtained after incubation for 2–8 h (Fig. 1 and Supplemental Figure 2). Thus, our strategy for quantification was to add IS solution before starting the tryptic digestion. This allowed normalizing any loss of the targeted peptide caused by adsorption or degradation throughout proteolysis. Further, quantification was performed at 2, 4, and 8 h after digestion to ensure that reactions reached a plateau. For most isoforms, high yields of targeted peptides were obtained with multiple denaturing conditions including those adopted, indicating that the levels determined by the adopted methods were reproducible using multiple protocols.

The level of UGT2B7 was the highest in HLM. Average levels in 16 individual HLM were comparable to the levels in pooled HLM of 50 individuals for all isoforms except UGT1A3 and 2B17, which showed large inter-individual variability with coefficient of variation (CV) values of 99.9% and 112%, respectively. UGT2B17 was present in undetectable or minimally detectable levels in five individuals (approximately 30%), which is consistent with results published recently (Fallon et al., 2013b). We previously demonstrated that the levels of UGT1A1 and 2B7 correlate significantly with corresponding marker activities (estradiol 3β-glucuronidation and morphine 3-*O*-glucuronidation) with coefficient of determination (r^2) values of 0.9434 and 0.5184, respectively (Sato et al., 2012b). Although the levels of UGT1A1 and 2B7 determined here were higher than those in our previous study using urea, the correlation between protein levels and marker activities was similar ($r^2 = 0.9256$ and $p < 0.0001$ for UGT1A1, $r^2 = 0.6408$ and $p = 0.0002$ for UGT2B7). These results suggest that relative protein levels can be investigated without optimizing the quantification method.

The level of UGT2B7 was also the highest in HIM. Average levels in 6 individual HIM were comparable to the levels in pooled HIM of 18 individuals. Five other isoforms (1A1, 1A3, 1A4, 1A10, and 2B17) were measurable and levels of 1A3 and 1A4 were very low, which is similar to the results reported by another study (Fallon et al., 2013a). Low levels of UGT1A6, 1A7, 1A8, and 1A9 in HIM were reported by the other study (< 8.4 pmol/mg protein) (Harbourt et al., 2012). The difference between the results of this study and those presented here may be due to inter-individual variability, because the number of specimens was low in both studies.

The level of UGT1A9 was the highest in HKM. UGT2B7 was also detected at a high level. Although the number of specimens was small, these results suggest that UGT1A9 is the major UGT isoform present in human kidney and that UGT2B7 is highly expressed in all three tissues. Isoforms detected in HKM (1A6, 1A9, and 2B7) are consistent with the results reported by Fallon et al (2013a). Detection of UGT1A1, 1A4, 1A7, 1A8, and 1A10 in pooled HKM was reported by Harbourt et al (2012). Resolving this discrepancy will require further research.

In rhUGTs, although the total protein concentration of each membrane solution was equal (5 mg/mL), the levels of UGTs were markedly different among isoforms. As shown in a previous study using CYPs (Iwatsubo et al., 1997b), determining UGT levels in rhUGTs is useful for estimating the glucuronidation activity per UGT isoform. This value can be further multiplied by protein levels in human tissue specimens to assess the contribution of glucuronidation to body clearance based on *in vitro-in vivo* extrapolation (IVIVE).

In conclusion, optimum denaturing and digestion methods for targeted peptide-based quantification of UGTs in biological specimens were established. Yields

of targeted peptides varied greatly among methods. The expression of 10, 6, and 3 isoforms was detected in human liver, intestine, and kidney, respectively. The data were reproducible, because different protocols yielded similar results. This report demonstrates the quantities of 13 UGT isoforms in HLM, HIM, HKM as well as in rhUGTs, as determined by optimizing proteolysis conditions followed by analysis using LC-MS/MS.

Authorship contributions

Participated in research design: Sato, Nagata, Tetsuka

Conducted experiments: Sato

Contributed new reagents or analytic tools: Sato, Nagata, Tetsuka

Performed data analysis: Sato, Nagata

Wrote or contributed to the writing of the manuscript: Sato, Nagata, Tetsuka, Tamura, Miyashita, Kawamura, Usui

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Footnotes

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Figure Legends

Fig. 1. Comparison of the efficiency of generating UGT2B7-proteotypic peptide in HLM. Pooled HLM were denatured at 37°C for 1 h, 60°C for 1 h, or 95°C for 15 min in the presence of 1 M urea, 1% SDC, or 1% OG followed by tryptic digestion. Digestion was terminated after 2, 4, 8, and 20 h followed by addition of IS. The relative PAR was determined by defining the highest PAR as 100%. Denaturing conditions that yielded relative PARs ≥75% of the maximum and the corresponding data are shown in red.

Fig. 2. Optimum denaturing conditions for generating UGT-proteotypic peptides in HLM (A), HIM (B), HKM (C), and rhUGTs (D). Pooled HLM, HIM, HKM, and a mixture of 13 rhUGTs were denatured using nine conditions and incubated with trypsin for 2, 4, 8, and 20 h. Denaturing conditions that yielded relative PARs ≥75% of the maximum are highlighted.

Fig. 3. Quantification of UGT2B7-proteotypic peptide in 16 individual HLM. HLM were denatured at 37°C for 1 h in the presence of 1% SDC and incubated with trypsin for 2, 4, and 8 h after addition of IS.

Table 1. Protein levels of UGT isoforms in biological specimens.

Isoform	HLM				HIM				HKM[c]	rhUGT
	individual			pool[a]	individual			pool[b]		
	ave[d]	SD	%CV		ave[e]	SD	%CV			
1A1	124	63.1	50.9	135	39.6	21.4	54.1	30.6	ND	3740
1A3	20.6	20.6	99.9	11.7	1.93[f]	1.10	56.9	1.30	ND	1280
1A4	84.0	30.4	36.2	80.0	1.60[f]	0.90	56.5	1.40	ND	826
1A6	22.6	12.7	55.9	26.4	ND			ND	13.9	1980
1A7	ND			ND	ND			ND	ND	1250
1A8	ND			ND	ND			ND	ND	4500
1A9	61.1	22.2	36.4	57.5	ND			ND	171	1900
1A10	ND			ND	17.9	6.95	38.7	13.1	ND	1960
2B4	102	47.6	46.9	98.8	ND			ND	ND	883
2B7	200	67.8	33.9	152	15.7	9.81	62.6	12.5	95.3	2720
2B10	69.3	31.7	45.7	49.5	ND			ND	ND	292
2B15	99.7	42.6	42.7	90.8	ND			ND	ND	878
2B17	54.3	60.8	112	25.5	112	36.6	32.7	72.8	ND	2860

ND: Not determined because protein levels were below the LLOQ (1 pmol/mg protein for HLM, and 2 pmol/mg protein for HIM and HKM). [a]Pooled microsomes of 50 individuals (31 males and 19 females). [b]Pooled microsomes of 18 individuals (11

males and 7 females). [c]Pooled microsomes of 4 individuals (2 males and 2 females). [d]Average value of 16 individuals (9 males and 7 females). The levels of UGT1A6 and UGT2B17 were below the LLOQ in one and five samples, respectively. Values below the LLOQ were treated as zero for calculation. [e]Average value of 6 individuals (6 males). [f]Protein levels in four individual samples were below the LLOQ and were extrapolated, because the analyte peak was detected clearly (the signal to noise ratio >5). The average was calculated using extrapolated values.

Fig. 1

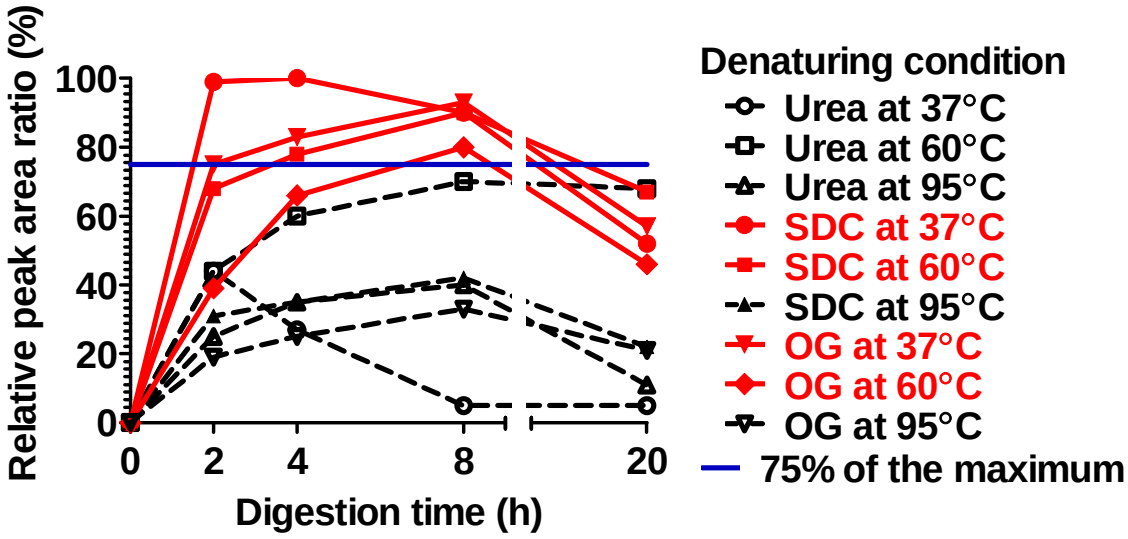

Relative peak area ratio (%)
100
80
60
40
20
0
0
2
4
8
20
Digestion time (h)
Denaturing condition
Urea at 37°C
Urea at 60°C
Urea at 95°C
SDC at 37°C
SDC at 60°C
SDC at 95°C
OG at 37°C
OG at 60°C
OG at 95°C
75% of the maximum

Fig. 2

A

Isoform	Denaturing agents								
	Urea			SDC			OG		
	Denaturing temperature (°C)								
	37	60	95	37	60	95	37	60	95
1A1									
1A3									
1A4									
1A6									
1A9									
2B4									
2B7									
2B10									
2B15									
2B17									

B

Isoform	Denaturing agents								
	Urea			SDC			OG		
	Denaturing temperature (°C)								
	37	60	95	37	60	95	37	60	95
1A1									
1A3									
1A4									
1A10									
2B7									
2B17									

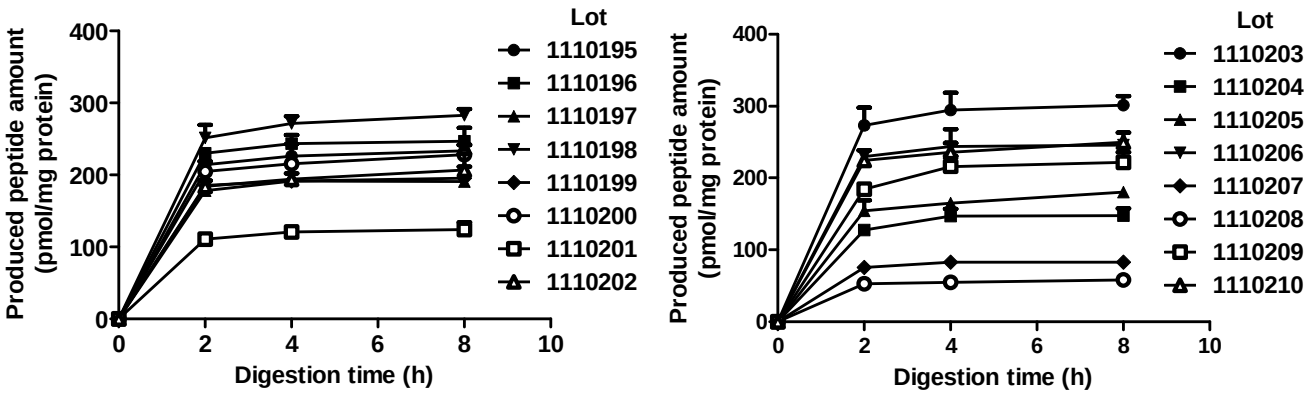
C

Isoform	Denaturing agents								
	Urea			SDC			OG		
	Denaturing temperature (°C)								
	37	60	95	37	60	95	37	60	95
1A6									
1A9									
2B7									

D

Isoform	Denaturing agents								
	Urea			SDC			OG		
	Denaturing temperature (°C)								
	37	60	95	37	60	95	37	60	95
1A1									
1A3									
1A4									
1A6									
1A7									
1A8									
1A9									
1A10									
2B4									
2B7									
2B10									
2B15									
2B17									

Fig. 3


Produced peptide amount (pmol/mg protein)
400
300
200
100
0
0
2
4
6
8
10
Digestion time (h)
Lot
1110195
1110196
1110197
1110198
1110199
1110200
1110201
1110202
Produced peptide amount (pmol/mg protein)
400
300
200
100
0
0
2
4
6
8
10
Digestion time (h)
Lot
1110203
1110204
1110205
1110206
1110207
1110208
1110209
1110210