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Quantification of Human Uridine-Diphosphate Glucuronosyl Transferase (UGT) 1A Isoforms in Liver, Intestine and Kidney using nanoLC-MS/MS

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Abstract

Uridine-disphosphate glucuronosyl transferase (UGT) enzymes catalyze the formation of glucuronide conjugates of Phase II metabolism. Methods for absolute quantification of UGT1A1 and UGT1A6 were previously established utilizing stable isotope peptide internal standards with LC-MS/MS. The current method expands upon this by quantifying eight UGT1A isoforms by nanobore HPLC coupled with a linear ion trap-time of flight mass spectrometer platform. Recombinant enzyme digests of each of the isoforms were used to determine assay linearity and detection limits. Enzyme expression level in human liver, kidney and intestinal microsomal protein was determined by extrapolation from spiked stable isotope standards. Intraday and Interday variability was <25% for each of the enzyme isoforms. Enzyme expression varied from 3 pmol/mg protein to 96 pmol/mg protein in liver and intestinal microsomal protein digests. Expression levels of UGT1A7, 1A8 and 1A10 were below detection limits (<1 pmol/mg protein) in HLMs. In kidney microsomes the expression of UGT1A3 was below detection limits, but levels of UGT1A4, 1A7, 1A9 and 1A10 protein were higher relative to liver, suggesting that renal glucuronidation could be a significant factor in renal elimination of glucuronide conjugates. This novel method allows quantification of all nine UGT1A isoforms, many previously not amenable to measurement with traditional methods such as immunologically based assays. Quantitative measurement of proteins involved in drug disposition, such as the UGTs, significantly improves the ability to evaluate and interpret in vitro and in vivo studies in drug development.

Keywords

UGT; nanoLC-MS/MS; quantitative proteomics; human microsomes

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Introduction

UGT enzymes catalyze the formation of glucuronide conjugates of Phase II metabolism. Through the actions of the cofactor uridine-disphosphate glucuronide acid (UDPGa) and UGT enzymes, glucuronidation encompasses the most common and clinically important of the Phase II metabolic pathways[1] . Large numbers of endogenous and exogenous compounds are converted to more hydrophilic conjugates, which are eliminated by excretion. In humans, the UGT genome consists of two families and three subfamilies. The UGT1A locus is found on chromosome 2q37 and encodes for nine functional enzymes located throughout the body[2] . Traditional quantification methods that have been used to evaluate relative UGT levels include Western blots, ELISAs and RT-PCR studies, but these methods lack the sensitivity, reproducibility and dynamic range to examine expression in liver tissues. Using RT-PCR the correlation between mRNA levels measured and protein expression is often poor, making this method unreliable for evaluation of expression in biological systems[3] . In addition, the high degree of sequence homology between UGT enzymes (as high as 94% in some isoforms) prevents the raising of antibodies against specific UGT isoforms, with few exceptions[1,4] .

Other traditional approaches to relative protein quantification include differential gel electrophoresis (DIGE) which involves separation through 2D-gel electrophoresis followed by fluorescent tagging of lysine side chains[5]. While multiple proteins may be monitored through the DIGE technique using alternate fluorescent tags, it is often difficult to distinguish high and low molecular weight proteins, and DIGE often lacks the sensitivity to detect low abundant proteins[6]. In recent years, advances in electrospray ionization (ESI), coupled with sensitive tandem mass spectrometry (MS/MS), has allowed for the development of several approaches to relative protein quantification. Methods measuring proteins within cells include *in vivo* metabolic labeling in enriched media (C^{13}, N^{15}, O^{18}), followed by digestion and quantification of labeled peptides to evaluate changing expression of proteins of interest[7-10]. These methods have been used to determine expression changes in myoglobins, immune response, HIV levels, glycoproteins and mitochondrial proteins. Recently, label-free techniques have also been used that can evaluate global expression changes, but these methods are not commonly employed for targeted protein quantification due to variability across instrumentation platforms and ion suppression from complex matrices[5].

Along with the use of capillary LC-MS/MS for relative quantification for global proteomics, stable isotope internal standards also have been employed for targeted absolute quantitative proteomics. Labeling techniques such as ICAT and iTRAQ can be utilized for relative quantification or for absolute quantification if a known amount of labeled targeted peptide is added during sample preparation[11-14]. However, because ICAT reagents only target peptides with cysteine groups, the number of potential targeted peptides is limited[6]. Furthermore, the selection of precursor ions in complex matrices can be difficult using iTRAQ methods[6]. Although both ICAT and iTRAQ methods have greatly increased sensitivity and selectivity of peptide/protein analysis, when compared with gel based methods, other more general approaches are being developed[6,14].

In recent years stable isotope labeled peptides as internal standards have successfully been used for the absolute quantification of proteins and peptides. Barr *et al*. [15] were able to accurately quantify apolipoprotein A-1 using unique heavy-labeled peptides. Following these initial experiments, both C-reactive protein (CRP) and prostate specific antigen (PSA) were quantified within human plasma by preselecting heavy isotope-labeled peptide internal standards added prior to a tryptic protein digestion[16,17]. Using sensitive capillary LC coupled to MS/MS, it was found that the concentrations of PSA and CRP measured were

comparable to those determined by immunoassay, with lower detection limits[16,17]. These methods fostered the development of stable isotope labeling with amino acids in cell culture (SILAC), and absolute quantification (AQUA) and Quantitative Concatamer (QCAT) stable isotope standards, and have been applied more recently for determining gonadotropin releasing hormone (GnRH)[18]. While QCAT standards require development of recombinant protein expression in cell lines to produce heavy labeled internal standard proteins for quantification, heavy isotope-labeled peptides can be readily prepared following preselection using standard peptide synthesis techniques.

While LC connected to triple quadrupole tandem MS has been used for MRM quantification, other mass spectrometry platforms have also been successfully implemented for these purposes. LC-MS/MS has been applied using quadrupole time of flight (Q-TOF) mass spectrometry for both metabolite detection and peptide quantification[19, 20]. In addition, a new MS platform that couples capillary (nano)LC to a linear ion trap time of flight (LIT-TOF) mass spectrometer from Hitachi High-Technologies has been utilized for both qualitative and quantitative mass spectrometry[21-24]. Recently, Ito *et al*.[24] were able to successfully quantify a novel glycosylated phospholipid and demonstrate that the LIT-TOF platform was not only very sensitive, but also has a dynamic range approaching three orders of magnitude.

We have previously demonstrated the utility of quantifying UGTs using stable isotope standards and tandem LC-MS[25]. While this method was successfully applied for quantification of two of the nine active human UGT isoforms, it has been necessary to extend the method for the remaining UGT1A isoforms to not only better examine the relationship within and across species, but also, in a single chromatographic run, to be able to quantify other UGT1A isoforms that lack specific antibodies. By taking advantage of the increased sensitivity offered by nanoLC compared to standard bore HPLC and by extending the LC gradient for better separation of peptides, we demonstrate here the ability to selectively quantify eight of the nine active UGT1As (recombinant UGT1A5 was unavailable for this work) in a single chromatographic run. Here we present our method employing nanoLC coupled to a LIT-TOF mass spectrometer operated in the extracted MRM mode.

Experimental Section

Materials

Analytical grade acetonitrile and methyl alcohol (anhydrous) were purchased from Fisher Scientific Co. (Pittsburg, PA). Ammonium bicarbonate, dithiothreitol, iodoacetamide, ammonium hydroxide, formic acid and TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) treated trypsin from bovine pancreas (≥ 10,000 BAEE units/mg protein) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Bond Elut solid phase extraction (SPE) cartridges (C18 100mg, 1mL) were purchased from Varian, Inc. Recombinant UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10 and control supersomes were purchased from BD Biosciences (San Jose, CA). Human liver microsomes (HLMs) (20 mg/mL) were purchased from BD Biosciences (pool of 33; 15 female, 18 male) and Xenotech LLC (Lenexa, KA) (pool of 50; 26 female, 24 male). A human liver microsome library of individual donors (n=9, 3 female, 6 male, 20 mg/mL) was purchased from Human Biologics International (Scottsdale, AZ). Human kidney microsomes were purchased from BD Biosciences (pool of 33, 14 Female, 19 Male, 20 mg/mL) and pooled human intestinal microsomes (HIMs) were obtained from Xenotech LLC (Lenexa, KA) (8 donors, 5 Female, 3 Male). Human intestinal microsome samples from individual donors (n=3, 1 Female, 2 Male) was graciously donated by Dr. Mary Paine (UNC-Chapel Hill, Chapel Hill, NC)[26]. Protein concentrations were

determined using the Pierce BCA Protein Assay Kit, in which bovine serum albumin is used as the standard.

Instrumentation

Samples were analyzed using nanoLC (NanoFrontier L series; Hitachi High-Technologies, Tokyo, Japan) attached to a LIT-TOF mass spectrometer (Hitachi High-Technologies, Tokyo, Japan). The nanoLC was coupled to an AT10PV nano-flow gradient generator and connected to the LIT-TOF instrument with an electrospray ion source[24, 27]. The LC column was a monolithic C-18 column (GL Sciences, Tokyo, Japan, 150 mm, 0.075 mm ID). The instrument was operated in positive ion mode for MRM analysis. Data analysis was performed using NanoFrontier LD-ECD Data Processing software (Hitachi High-Technologies, Tokyo, Japan).

Stable isotope labeled internal standards

Synthetic peptide standards (most were 8-14 mer; Table 1), each containing one amino acid heavy labeled with ^{13}C [98 %] and ^{15}N [95 %], were purchased from Thermo Electron (Thermo Scientific, Waltham, MA) using previously reported selection criteria, including manufacturer recommendations[7, 25, 28]. For each UGT isoform, two unique peptides were purchased for analysis and validation. Amino acid sequences for the nine active human UGT1A isoforms (including for UGT1A5, which was unavailable in recombinant form) were obtained using the Universal Protein Resource Knowledge Base (UniProtKB). Peptide uniqueness of tryptic fragments was verified by NCBI Blast (National Center for Biotechnology Information Basic Local Alignment Search Tool). Amino acid analysis on each peptide to determine sample purity was conducted as previously described[25]. Known variable single nucleotide polymorphisms (SNPs) were also considered during peptide selection. While at the time of purchase there were no known interferences with SNPs along the targeted protein sequence, reports indicate that there are potential conflicts due to polymorphisms for UGT1A1 (Peptide 2, frequency <1 %), and UGT1A6 (Peptide 4, frequency ~4 % in Caucasians)[29-31]. Furthermore, while it was initially intended to obtain two unique peptides per isoform, the dearth of unique peptides for UGT1A7, UGT1A8 and UGT1A9 that are amendable to the LC-MS approach only provided one unique peptide for these isoforms along with one peptide that is shared between the two isoforms. Upon application, several selected peptides failed to provide useful data for quantitative measurement, thus UGT1A3, UGT1A4 and UGT1A10 were also only measured based on one heavy labeled peptide. The high sequence homology of the UGTs and exon sharing with identical C-terminus for the UGT1As made peptide selection challenging.

Sample preparation

Aliquots of 50 μg of microsomal protein, recombinant enzyme or UGT control supersomes were denatured and digested as previously reported with some modifications[25]. Samples were denatured and reduced by heating at 95 °C for 10 min in 5 mM dithiothreitol (sample volume 90 μL; buffer 50 mM ammonium bicarbonate). This was followed by alkylation with 100 mM iodoacetamide stock for 20 min in the dark. Heavy labeled peptides were then added as internal standards (10 pmol for each peptide, excluding peptides 16 and 17 for which signal intensities were too low; Table 1) and the residual acetonitrile (<5 μL) was removed by evaporation under nitrogen for ~5 min. Samples were then digested with trypsin in 50 mM ammonium bicarbonate; enzyme/protein ratio = 1:50; for 4 h at 37 °C (sample volume was brought to 150 μL by addition of HPLC water). The reaction was quenched by addition of 50 μL acetonitrile. Following centrifugation at 1000*g* for 10 min, the organic content was removed by evaporation under nitrogen for ~10 min. An aliquot of 0.9 mL of 50 mM ammonium bicarbonate was then added in preparation for solid phase extraction. SPE cartridges were conditioned with methanol and distilled water. Samples were added and the

cartridges were washed with 1 mL 10 mM ammonium acetate (pH 3). Samples were then eluted with acetonitrile/25 mM formic acid (40:60). The eluate was evaporated to dryness under nitrogen at 42°C in a water bath, and samples were reconstituted with 250 μL (80:20) acetonitrile/25 mM formic acid at pH 3 (pH was adjusted to 3 by drop wise addition of ammonium hydroxide), a high volume to minimize sample loss on glass tubes. Following initial reconstitution, samples were transferred to injection vials and dried again under nitrogen at 42 °C. They were then reconstituted in 50 μL (15:85) acetonitrile/25 mM formic acid at pH 3. Samples were stored at −20 °C until analyzed by nanoLC-MS/MS.

MRM selection

All peptide heavy labeled standards were directly infused into the LIT-TOF mass spectrometer at a rate of 2 μL/min for MRM optimization. Upon infusion, most peptide standards were doubly charged. Peptides 5, 16 and 17, were found to be triply charged or greater and were ultimately excluded because of inadequate MRM selection (Table 1). For each peptide, two MRMs above 500 *m/z* were selected for use in quantification (Figure 1). MRM selections were confirmed on the LIT-TOF following optimization of the nanoLC gradient using a solution containing 10 pmol of each peptide.

NanoLC-MS/MS conditions

The mobile phase consisted of 25 mM formic acid in water (solvent A) and acetonitrile (solvent B). A 1 μL injection (approximately 1 μg of digested protein) was loaded onto a 5 cm trap column (C18, 2.1 mm ID, 5 μm particle size) at a rate of 10 μL/min for 4 min. The sample was then transferred onto the analytical column and analyzed under the following linear gradient conditions, 0 min, 5 % B; 30 min, 55 % B; 30.1 min, 100 % B; 40 min, 100 % B; 40.1 min, 5 % B and then equilibrated until 65 min. The gradient pump maintained a 100 μL/min flow rate while the nanoflow pump was run at 200 nL/min during analysis. MS conditions were similar to those previously reported by Ito *et al*[24] except the AP1 temperature was set to 120 °C, with an isolation time of 50 ms.

Calibration curves

Recombinant enzymes of all UGT1A isoforms, excluding UGT1A5 (not commercially available in recombinant form), were used to establish recombinant enzyme calibration curves that demonstrated linearity. Tryptic digestion volumes were maintained by dilution with tris buffer at pH 7.4. Prior to the digestion, 10 pmol of each heavy labeled peptide standard was added and the response ratio between the labeled and unlabeled peptides was used to establish a calibration curve and to verify linearity. Equality of response between the labeled and unlabeled peptides along with complete tryptic digestion was assumed in the creation of the calibration curves. Calibration curves were constructed using peak area ratios of unlabeled to labeled for each MRM selected. Enzyme concentrations, in units of pmol/mg (microsomal) protein, were extrapolated from the curves for unknown samples.

Inter- and intraday assay variability

To examine intraday variability, five replicate calibration curves for human liver, intestinal and kidney microsomes were prepared and analyzed on the same day. Where available, measurements for two peptides per UGT1A isoform were averaged to obtain final enzyme expression levels. Interday variability experiments were carried out in a similar manner with standard curves being prepared and analyzed on five different days for digests of human liver and intestinal microsomes.

Human liver and intestinal microsomal library

To compare the newly established method to our initial method[25] samples from a library of nine individual liver microsomal donors were analyzed in duplicate. Intestinal microsomes from three individual donors were also analyzed in duplicate. For the human liver microsomal library, enzyme expression levels of UGT1A1 and UGT1A6 were compared to results obtained using the previous assay[25].

Results and Discussion

MRM Scheduling

Following initial tests on the instrumentation, it was found that peptides of interest eluted between 17 and 32 min. Further increases in the % B at the beginning of the nanoLC gradient or increases in slope of the gradient to decrease run times resulted in band broadening or loss of signal. Peptides eluted consistently within five separate time windows ($T_2 – T_6$) (T_1 0-17 min, T_2 17-19.5 min, T_3 19.5-22.0 min, T_4 22.1-26.4 min and T_5 26.5-28 min, T_6 28.1-32 min) and MRM scheduling was optimized with the NanoFrontier LD-ECD software. Selected MRM extractions from a representative single chromatographic run are shown in Figure 2. MRMs for peptides 5, 14, 16 and 17 were not consistently detectable, and were excluded from sample analysis.

Calibration curves

One calibration curve was constructed for each MRM, two per peptide when available, resulting in a maximum of six curves per UGT isoform (three peptides were used for UGT1A6). Concentrations for each isoform, in units of pmol/mg protein, were expressed as an average of concentrations determined from all curves. Calibration curves produced a linear range (Figure S-1) for recombinant microsomal protein with an LOD of approximately one third of the lowest concentration on the calibration curves (the lowest concentration on the curves was ~1 pmol enzyme/mg protein). Calibration curves were similar between MRMs for each peptide. Peptides that did not generate quality product ion scans, stable and reproducible LC retention times, sufficient sensitivity and precise quantification (% C.V. <25 % with repeat sampling) were excluded from all UGT enzymatic analyses. All R^2 values for calibration curves were between 0.96 and 0.99.

Intraday and interday variability

Intraday variability measurements (n=5) for the UGTs provided reproducible results with minimal variation (<25 %) between samples. UGT1A1 enzyme levels were 22.7 pmol/mg protein in human liver microsomes (Table S-1) which is similar to what we reported earlier[15]. UGT1A6 levels were 8.5 pmol/mg protein in the same matrix (Table S-1). These concentrations were more reproducible than those of UGT1A1 and less reproducible than those of the other liver isoforms (Table S-1). Peptides for isoforms UGT1A7, UGT1A8 and UGT1A10 were below detection limits in the liver, which is in agreement with previous reports of the liver expression of these isoforms as determined by mRNA analysis[1, 32, 33]. In addition, UGT1A3 and UGT1A4 were below detection limits in the intestine[1, 32]. In contrast, UGT1A3 was the only isoform assayed that was below detection limits in the kidney (1A5 was not assayed). With few exceptions, interday C.V. values were below 25 % for each of the UGT enzymes (Table S-1). Intraday variability studies produced similar results with regard to both variability and expression (Table S-2).

Human liver, intestine and kidney UGT expression

The data from liver microsomes indicated that UGT1A1 and UGT1A9 consistently had the highest enzyme expression levels among the UGTs (Figure 3). While UGT1A3, UGT1A4

and UGT1A6 were readily detectable, their expression levels (3.2 for 1A3, 7.4 for 1A4, and 8.5 pmol/mg protein for 1A6) were generally lower when compared to the other two enzymes. UGT1A1 expression was highest in the liver along with UGT1A3 and UGT1A6 relative to the two other tissues examined. UGT1A8 was found at its highest levels within the intestine while UGT1A7, UGT1A9 and UGT1A10 were more extensively expressed in the kidney.

Human liver and intestinal libraries

Results of analysis of the human liver and intestinal libraries are shown in Table 2. UGT1A1 expression varied five fold (7.0 pmol/mg protein to 32.6 pmol/mg protein) in the liver microsomes which was the general trend for variation in liver expression for the other measurable isoforms. An exception was UGT1A9, where variation was more than ten fold in the liver (9.0 pmol/mg protein to 96.4 pmol/mg protein in liver). Unlike the liver library, the intestinal enzyme expression data suggested less variability between individuals though the sample size was only three. Most isoforms demonstrated between 1.5 to 3 fold variability between the lowest and highest concentration in intestine. While UGT1A4 was detected in two of the three individual intestinal specimens, it was not detected in the pooled human intestinal microsomal digests. Another exception was UGT1A8 which demonstrated five fold variability (1.9 pmol/mg protein to 9.4 pmol/mg protein) in the intestine. Isoforms that were determined to be below detection limits within the pooled microsomal studies, excluding UGT1A4, were also not detected in any of the individual library specimens.

To further validate the nanoLC LIT-TOF based assay for UGT expression, UGT1A1 and UGT1A6 enzyme rank orders and enzymatic levels were compared to the corresponding results obtained using our previously developed assay (on the LC coupled to an ABI 3000)[25]. Tests for both isoforms indicated a strong Pearson correlation (r=0.926 for UGT1A1, Figure S-2; r=0.854, Figure S-3 for UGT1A6, n=9 for each isoform) between platforms. Furthermore, the rank orders for each isoform within the library specimens were similar and most of the enzyme levels obtained using the current method varied <20 % from the values determined using the previous assay[25].

In recent years, the use of stable isotope internal standards with tandem MS methods to quantify biologically active proteins has become more common[25, 34,35]. The goal of this report was to develop a method that could successfully quantify all of the human UGT1A enzymes within a single chromatographic run using nanoLC-MS/MS, the preferred platform for many researchers in proteomics[5, 18, 36]. While triple quadrupole mass spectrometry has been applied for quantification, other hybrid instruments, including the LIT-TOF used in this study, have also been used and have displayed the selectivity and sensitivity desired to quantify biological enzymes in complex matrices. Thus a range of MS platforms may be successfully employed for targeted quantitative proteomics, though sensitivity may vary between platforms.

The UGT1A protein sequence is ~530 amino acids in length and consists of five exons, of which amino acids residing in the C terminus (exons 2-5) are shared between each 1A isoform[1]. The N terminal region is subject to individual enzyme splicing, resulting in the 9 unique active isoforms and 4 pseudogenes in humans[1, 2]. Because of this, amino acid sequences unique to each isoform are often a small list, making proteoytpic peptide selection limited. It is desired in targeted quantitative proteomics to select two unique peptides per protein and at least two MRMs per peptide over 500 *m/z* for optimal sensitivity and selectivity[37]. In our study the limited selection window for UGT1A8 and UGT1A9 resulted in the selection of only one unique peptide for each of these isoforms. A second peptide of sequence TYSTSYT**L*** EDLDR, was found to be shared between the two isoforms. Furthermore, peptides containing reactive/unstable amino acids such as methionine and

cysteine, which are normally excluded based on selection criteria, were selected and employed due to a lack of unique peptides for some UGT1A isoforms (Table 1).

Peptide 15 (FFTLTAYA**V***PWTQK), representative of UGT1A4, that contains a tryptophan residue, would normally be excluded based on selection criteria, yet the peptide produced consistently linear standard curves (r^2>0.97, data not shown) and acceptable C.V. values on interday and intraday variability testing (Table S-1, S-2). Reproducible product ions could not be obtained for peptide 16 (VTLGYTQG**F***FETEHLLK) and peptide 17 (GHQVVV**L***TLEVNMYIK) because the parent ions from these peptides were from the +3 to +5 charge states, making both MRM analysis and chromatography cumbersome and often unresolvable. In addition, peptide 5, representing UGT1A3 (HVLGHTQ**L***YFETEHFLK), could not be used. This peptide was quadruply charged as parent ion as a result of multiple histidine residues within the sequence. Along with peptides 5, 16 and 17, peptide 10 (TYSTSYT**L***EDLDR) was excluded from UGT analysis due to lack of specificity for a particular isoform since its peptide sequence was shared by both UGT1A8 and UGT1A9. Often the best peptides of those employed for UGT analysis contained single or multiple proline residues that exhibit the well documented “proline effect” that is responsible for favorable cleavage near proline sites under collision induced dissociation (CID) MS conditions[38]. Based on our experience, optimal peptide selection is of the utmost importance for accurate quantification of proteins digested to peptides. While some guidelines recommend that peptides as large as 16 mer may be selected, it is often smaller peptides between 8-10 mer in length containing proline and no reactive residues (C, M, W) that perform better for high sensitivity MRM based peptide quantification[7, 28, 34].

Initial analysis was performed with an extended gradient of 2-60 % B over 60 min. Assay length (95 min run time) and lack of signal intensity resulted in several alterations, including a higher starting % B, but this was again inadequate due to peak splitting and band broadening with the stable isotope standards. Following further modification, a 5-55 % B gradient over 30 min provided both the highest signal intensity and shortest run time on the instrumentation. Peptides eluted between 17 and 32 min over five distinct time segments. To maximize MRM collections, eight to sixteen transitions were monitored in each segment, enabling quantification of all UGT1As within a single chromatographic run. Although the LIT-TOF used could only monitor a maximum of 100 MRM transitions within a single chromatographic run, this was sufficient to monitor the maximum 68 MRM transitions of interest in this study.

With the exception of peptides 5, 14, 16, 17 and 18, all peptides generated calibration curves that were linear for a range of recombinant microsomal protein. When the stable isotope calibrants were incorporated, this represented a range of 1.0 to 140 pmol/mg protein. Because determined concentration variability was <30 % between the peptide MRMs monitored, MRM values for each UGT1A isoform were averaged to generate expression datasets. For peptides with only one unique peptide, two MRM measurements were averaged from the single peptide. The highest detected UGT concentration was 96.4 pmol/mg protein of UGT1A9 within the kidney; however, even this concentration was well within the linear range of the assay. Wang *et al*.[35] demonstrated that some CYP isoforms are expressed in much higher concentration than UGT enzymes (>300 pmol/mg protein), indicating that the digested protein levels may need to be adjusted between assays to remain within the linear range of some instruments.

Enzyme expression data generated for the liver, intestine and kidney generally agree with previous reports for both mRNA expression and *in vitro* glucuronidation profiles[1, 3, 39, 40]. Within the liver, UGTs 1A1 and 1A9 were consistently expressed at a higher level than 1A3, 1A4 and 1A6 (Table 2, , Figure 3). While there have been conflicting reports of the

predominately extrahepatic UGTs 1A7, 1A8 and 1A10 being present in the liver, none of these enzymes were detected in either pooled or individual HLMs[33, 41, 42]. Within the liver, the highest variability in individual donor specimens was found with UGT1A1 and UGT1A9 isoforms (Table 2), both of which have well documented polymorphisms[1, 3, 29, 42].

Within the intestinal tract, expression of UGTs 1A1 and 1A6 was approximately 35 % of expression seen in livers, which concurs with our previous investigation into these isoforms[25]. Furthermore, previous studies by Wen *et al*[40] with etoposide, a UGT1A1 and 1A8 probe substrate, indicated that the V_{max} of etoposide glucuronidation rates in the intestine was approximately 30 % of liver levels. While UGTs 1A1, 1A6 and 1A9 were detected within the pooled intestinal digests, levels were appreciably lower than in the liver microsomes. UGT1A8 was expressed at a higher level in the intestine than in the kidney. Individual patient levels of UGT1A8 appeared highly variable within the intestine, yet there have been few UGT1A8 polymorphisms reported with appreciable effects on glucuronidation[43]**.** High variability was also seen between sample sets of the pooled human intestinal microsomes and the individual specimen donors. UGT1A4 was not detected within any of the pooled intestinal digests but was detected in each of the individual specimens (Table 2). Individual patient variability, drug interactions or genetic polymorphisms seen in UGT1A4 could be the source of the discrepancy[45].

Expression levels within the kidney microsomes were much higher than in the intestine and sometimes higher than seen in the liver. In particular, UGT1A9 was expressed at a level nearly four fold that of the liver (81 pmol/mg protein, Figure 3). While mRNA and protein correlation is often poor, recent reports characterizing UGT mRNA expression indicated UGT1A9 was highly expressed within the human kidney [32, 39]. However, it is important to note that when making comparisons between mRNA assays and our UGT assay, one must take into account the numerous polymorphisms within the UGT gene family that result in high expression variability in the tissue within the population. Altered protein expression affected by SNPs or other polymorphisms in human tissues could account for the low level discrepancies in 1A3expression within the intestine and kidney for both the UGT assay and mRNA values [32, 39] . Despite some differences in mRNA expression, UGT expression within the kidney coincides with mycophenolic acid (MPA) glucuronidation levels that are also three fold higher in the kidney compared to the liver and fifteen fold higher than those seen in the intestinal tract [44]. Not only were enzyme levels highest in the kidney for UGTs 1A4, 1A7, 1A9 and 1A10, but only UGTs 1A3 and 1A5 were not detected, making the kidney a potentially important organ for clearance via glucuronidation in the body.

Conclusion

By combining the advantages of nanoLC coupled with the new LIT-TOF instrumentation, we have developed and applied a method to detect eight of the nine active human UGT1A isoforms within a single chromatographic assay. Our assay demonstrates acceptable variability (<25 %) with comparable sensitivity relative to our earlier method performed using older triple quadrupole (API 3000) instrumentation and regular bore LC[25]. The use of nanoLC allowed for a dramatic reduction of protein needed for the assay without a loss of sensitivity. Furthermore, we are now able to compare absolute expression levels of UGTs within different tissues in humans that are comparable to previous reports of relative mRNA expression measured in RT-PCR studies. The significant levels of UGT expression within the kidney demonstrate that this organ could have a potentially important role in glucuronide disposition within the body. Liver expression was generally higher than levels seen within the intestine, but more UGT1A isoforms were detected in kidney compared with the liver.

While the liver is seen as the primary organ involved in metabolite disposition, the importance of other organ systems within the body should not be underestimated. The emergence of novel targeted quantitative proteomic methods based upon LC-MS/MS will allow the proliferation of protein expression studies, especially for the many proteins for which specific antibodies have not been successfully raised. The ability to quantify specific proteins within and between species without a reliance on antibodies opens many new avenues of research into xenobiotic metabolism including such areas as protein-protein interactions, enzyme regulation and induction/inhibition studies. These newly developed analytical methods will significantly improve the ability to evaluate and interpret in vitro and in vivo studies in drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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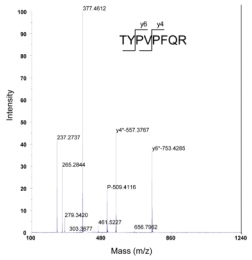

Figure 1.
LIT-TOF spectrum analysis of peptide 1 (Table 1-UGT1A1) following injection of 10 pmol of peptide on nanoLC-MS/MS showing optimum product ions 557.3 (y4) and 753.4 (y6) for use in MRM transitions.

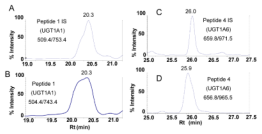

Figure 2.

Representative extracted ion chromatograms showing peptide 1 (T_{78}YPVP**F***QR_{85}), labeled and unlabeled (A and B, respectively), from UGT1A1, eluting at 20.3 min, estimated as 22.7 pmol/mg protein, and peptide 4 (S_{103}FLTA**P***QTEYR_{113}) (C and D, labeled and unlabeled, respectively), from UGT1A6, estimated as 8.5 pmol/mg protein.

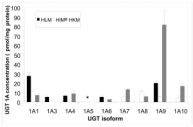

Figure 3.

Comparison of human UGT enzyme expression from five replicate measurements of digests of pooled human liver, intestinal and kidney microsomes (HLM, HIM and HKM respectively). Data are presented as mean values of each enzyme obtained from averages of two MRMs from one or two peptides as detailed in Methods. Standard deviation bars are shown. * UGT1A5 was not measured.

Table 1

Peptide standards employed showing their sequences and optimized MRM transitions used for UGT analysis by nanoLC LIT-TOF. MRM transition values were obtained following infusion and analysis of the stable isotope internal standards. Bold amino acid indicates presence of C13, N15 heavy label to generate a mass difference of between four and ten daltons.

UGT Isoform	Sequence	MRM No.	IS (labeled)	Endogenous (Unlabeled)	Rt (min)
1A1 (peptide1)	T_{78}YPVP**F***QR$_{85}$	1	509.4/557.3	504.4/547.3	20
		2	509.4/753.4	504.4/743.4	
1A1 (peptide 2)	D_{70}GA**F***YTLK$_{77}$	1	462.7/526.3	457.7/526.3	18.50
		2	462.7/681.4	457.7/671.4	
1A3 (peptide 5)	H_{92}VLGHTQ**L***YFETEHFLK$_{108}$		*a*	*a*	*a*
1A3 (peptide 6)	Y_{164}LSI**P***TVFFLR$_{179}$	1	681.3/885.5	678.3/879.5	30.1
		2	681.3/1085.6	678.3/1079.6	
1A4 (peptide 15)	F_{74}FTLTAYA**V***PWTQK$_{87}$	1	839.9/764.4	836.9/758.4	27.5
		2	839.6/1020.5	836.9/1014.5	
1A4 (peptide 16)	V_{92}TLGYTQG**F***FETEHLLK$_{108}$		*a*	*a*	*a*
1A5 (peptide 17)	G_{55}HQVVV**L***TLEVNMYIK$_{70}$		*a*	*a*	*a*
1A5 (peptide 18)[b]	Y_{164}LSIPA**V***FFLR$_{179}$	1	666.3/855.4	663.3/849.4	29.8
		2	666.3/1055.6	663.3/1049.6	
1A6 (peptide 3)	D_{44}IVE**V***LSDR$_{52}$	1	526.5/724.6	523.5/718.6	21
		2	526.5/823.6	523.5/817.6	
1A6 (peptide 4)	S_{103}FLTA**P***QTEYR$_{113}$	1	659.8/799.3	656.8/793.3	26.0
		2	659.8/971.5	656.8/965.5	
1A6 (peptide 7)	I_{77}YPV**P***YDQEELK$_{88}$	1	750.5/1027.5	747.5/1021.5	30.5
		2	750.5/1223.8	747.5/1217.8	

UGT Isoform	Sequence	MRM No.	IS (labeled)	Endogenous (Unlabeled)	Rt (min)
1A7 (peptide 8)	T_{76}YSTSYT**L***EDQDR$_{88}$	1	793.8/1046.4	790.3/1039.4	18.5
		2	793.8/883.4	790.3/876.4	
1A8/1A9 (peptide 10)	T_{76}YSTSYT**L***EDLDR$_{88}$	1	786.3/518.3	786.3/518.3	18.8
		2	786.3/647.3	786.3/647.3	
1A8 (peptide 11)	E_{89}FMD**F***ADAQWK$_{99}$	1	699.3/990.4	694.3/980.4	24.1
		2	699.3/875.4	694.3/865.4	
1A9 (peptide 12)	A_{92}FAH**A***QWK$_{99}$	1	481.7/536.2	479.7/532.2	18.1
		2	481.7/744.3	479.7/740.3	
1A10 (peptide 13)	T_{76}YSTSYT**L***EDQNR$_{88}$	1	792.8/661.2	789.3/661.2	20.2
		2	792.8/882.4	789.3/875.4	
1A10 (peptide 14) [a]	E_{89}FMV**F***AHAQWK$_{99}$	1	702.8/740.3	699.3/740.3	24.3
		2	702.8/897.4	699.3/897.4	

[a] Indicates that MRM analysis aborted due to insufficient product ion sensitivity following infusion or analysis by nanoLC.

[b] Data obtained using this peptide was limited due to the non availability of recombinant UGT1A5 for method development and validation.

Table 2

Mean UGT concentrations obtained from three individual intestinal microsomal donors (HIM) and nine individual donors of liver microsomal protein (HLM) analyzed by nanoLC LIT-TOF in duplicate[a].

	UGT Isoform (pmole/mg protein)							
	1A1	**1A3**	**1A4**	**1A6**	**1A7**	**1A8**	**1A9**	**1A10**
HIM								
AVG±SD	7.2±3.7	*	5.3±0.7	2.3±1.2	8.4±2.4	6.1±3.8	6.6±1.4	4.7±2.8
%C.V.	51.3	*	13.4	49.8	28.0	62.8	21.7	59.3
HLM								
AVG±SD	18.3±7.8	9.9±7.4	4.6±2.0	5.2±2.3	*	*	26.7±25.6	*
%C.V.	43.0	75.2	44.9	43.4	*	*	95.7	*

*Indicates enzyme concentrations were below detection limits (0.5 pmol/mg protein).

[a]Variability between enzyme measurements within a samples was <25%.