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SHORT COMMUNICATION

Targeted Precise Quantification of 12 Human Recombinant UGT1As and -2Bs using NanoUPLC-MS/MS with Selected Reaction Monitoring

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Running Title

Targeted Precise Quantification of Human Recombinant UGTs

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Number of Words in Discussion: 407

Nonstandard Abbreviations: ADME, absorption, distribution, metabolism and excretion; CYP450, cytochrome P450; MRM/SRM, multiple/selected reaction monitoring; MS, mass spectrometry; recUGT, recombinant UGT; SIL, stable isotope labeled; UGT, uridine-diphosphate glucuronosyl transferase; UPLC, ultra-high performance liquid chromatography

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ABSTRACT

Background: Quantification methods employing stable isotope labeled (SIL) peptide standards and LC-tandem mass spectrometry are increasingly being used to measure enzyme amounts in biological samples. Isoform concentrations, combined with catalytic information, can be used in ADME studies to improve accuracy of *in vitro*/*in vivo* predictions. **Methods:** UGT1As and -2Bs were quantified in 12 commercially available recombinant UGTs (recUGTs) (n=49 samples) using nanoUPLC-MS/MS (multiple/selected reaction monitoring [MRM/SRM]). Samples were trypsin digested and analyzed using our previously published method. Two MRMs were collected per peptide and averaged. Where available, at least two peptides were measured per UGT isoform. **Results:** The assay could detect UGTs in all recombinant preparations; recUGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17, with LOD below 1.0 pmol/mg protein for all isoforms. The assay had excellent linearity in the range observed (2 – 15.5 pmol/mg, after dilution). Examples of concentrations determined were 1465, 537, 538, 944, 865, 698, 604, 791, 382, 1149, 307 and 740 pmol/mg protein for the respective isoforms. There was a 6.9-fold difference between the maximum and minimum recUGT concentrations. **Conclusions:** The range of concentrations determined indicates that catalytic rates per mg total protein *in vitro* will not accurately reflect isoform inherent specific activity for a particular drug candidate. This is the first report of a targeted precise quantification of commercially available recUGTs. The assay has potential for use in comparing UGT amount with catalytic activity determined using probe substrates, thus allowing representation of catalysis as per pmol of UGT isoform.

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Introduction

Uridine-diphosphate glucuronosyl transferase (UGT) enzymes catalyze formation of the glucuronide conjugates of Phase II metabolism and are important for the elimination of drugs, xenobiotics and endogenous molecules (Tukey and Strassburg, 2000; Rowland et al., 2013). In drug development studies potential drug candidates are tested with a range of metabolic enzymes, including UGTs, to determine possible routes of disposition. Catalytic activity of enzymes in the studies is normally presented as amount of substrate converted per unit of time (e.g. µmol min^{-1}) or, for specific activity, the amount converted per unit of time per amount of total protein in the enzyme preparation (e.g. µmol min^{-1} mg^{-1}) (Court, 2005; Wen et al., 2007). These units fail to account for differences in the actual amount of enzyme in a preparation which is generally only estimated or unknown. It is suggested, for example, that in recombinant UGT (recUGT) preparations the UGT content is approximately 5-15 % of the total protein content (personal communication, BD Biosciences). Targeted isotope dilution techniques with tandem mass spectrometry have recently been used to quantify a wide range of bioactive proteins including UGTs, cytochrome P450s (CYP450s) and transporters (Li et al., 2009; Harbourt et al., 2012; Ohtsuki et al., 2012; Picotti et al., 2013). The specificity and broad dynamic range of the methods are advantageous when compared to often semi-quantitative, non-specific and expensive traditional immunometric methods (Seppen et al., 1994; Ritter et al., 1999; Paine and Fisher, 2000; Fallon et al., 2008). In this study we present application of a previously described capillary LC-tandem mass spectrometry isotope dilution method (Fallon et al., 2013) for the targeted quantification of up to fourteen UGT isoforms to a series of commercially produced recUGT samples (BD Supersomes™ [baculovirus infected insect cell microsomes]) (12 isoforms; n=49 samples). We discuss the variation in concentrations determined between

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isoforms and between isoform batches and the implications of these variations for absorption, distribution, metabolism and excretion (ADME) studies. We believe that by measuring the amount of actual isoform in recombinant preparations the catalytic activity could be more appropriately described in units of activity per amount of isoform.

Materials and Methods

Materials and Instrumentation. Materials, instrumentation and methods were as previously described (Fallon et al., 2013) with slight modifications. Briefly, synthetic stable isotope labeled (SIL) proteotypic peptides of known concentrations were purchased from Thermo Biopolymers (Ulm, Germany) to serve as internal standards for calibration. Where possible a minimum of two peptides were obtained per isoform. MRM Pilot (AB SCIEX) and Skyline (MacCoss Lab, University of Washington) software were used to select and optimize MRM acquisition parameters on a QTRAP 5500 (AB SCIEX). Digestion was with trypsin (Promega, Madison, WI) and chromatographic separation was by nanoUPLC (Waters nanoAcquity, Milford, MA). All recombinant samples were obtained from a commercial source (BD Supersomes™ [see description above], BD Biosciences, San Jose, CA). Rat liver microsomes were from XenoTech, LLC. (Lenexa, KA). Human liver, kidney and intestinal microsomes were obtained from BD Gentest™ (liver), Celsis IVT (kidney and intestine) and XenoTech, LLC (kidney and intestine). Total protein concentrations were measured in all samples using the Pierce BCA Protein Assay kit. Results were adjusted according to variation from the nominal concentrations (Fallon et al., 2013).

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Sample Preparation and Analysis. Recombinant samples (5 mg/mL nominal protein concentration) were diluted 100-fold with 50 mM ammonium bicarbonate. To 10 µL aliquots (0.5 µg protein) of each in duplicate was added ~19 µg of rat liver microsomes to bring the total amount of protein per sample to ~19.5 µg. Standard to assess tryptic cleavage (β-casein, 0.5 µg) was also added. Samples were reduced with dithiothreitol and denatured by heating at 65 ºC for 40 min. Samples were carbamidomethylated with iodoacetamide and cleaved with trypsin (1:20 w/w) by incubating at 37 ºC for 4 h. Reactions were stopped by the addition of acetonitrile (75 µL) and a pooled solution containing 1 pmol of each SIL proteotypic UGT peptide standard was added. Following centrifugation the supernatant was taken to dryness, reconstituted in 50 µl of modified mobile phase A (2 % acetonitrile in water w/ 0.1 % formic acid), centrifuged to remove particulates and transferred to vials for injection. Sample (2 µL, <1 mg total protein digest) was loaded onto a trap column at 15 µL/min for 1 min, then eluted through the analytical column at 2 µL/min, from 0 – 42% B (acetonitrile) over 24 min. The mass spectrometer was equipped with a NanoSpray III source and Analyst 1.5 software was used to acquire data via scheduled MRMs. Twenty four native UGT peptides and 24 SIL UGT peptides monitored in the method were used in the analyses (Fallon et al., 2013). Two MRMs were collected per peptide. For each isoform one peptide was used to report the isoform concentration as previously described (Fallon et al., 2013). For UGTs 1A3 and 1A8 only one peptide was available for use in the analysis. For all other isoforms at least two peptides were available (Fallon et al., 2013).

Treatment of Data, Quantification and Validation. MultiQuant 2.0.2 software (AB SCIEX) was used for data analysis employing smoothing (2.0 points; Gaussian Smooth Width) and peak splitting (2 points). Enzyme isoform concentrations were calculated by

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comparing the response for known amount of SIL peptide (1 pmol) with unknown amount of unlabeled (tryptic) peptide. Responses, following the dilution (100-fold), were within the linear range. Linearity, inter- and intra-day variation, and LOD were as previously described (Fallon et al., 2013).

Results

Total and extracted ion chromatograms for a representative sample (rUGT1A4-21161) are shown in Fig. 1 with addition of standard to assess tryptic digestion (β-casein). UGTs could be quantified in all samples (Table 1), with all concentrations of intended UGT isoform being in the 2.0 – 15.5 pmol/mg total protein range after (100-fold) dilution. The recombinant UGTs quantified were UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17. There was a 7-fold difference between maximum and minimum average (n=2) concentrations determined between all isoforms (maximum and minimum concentration samples are circled in Table 1). The standard deviations (± SD) and percentage co-efficients of variation (% C.V.) for UGT content within batches (duplicates averaged) for the isoforms listed are shown in Table 1. For UGT1A4 the highest concentration batch was 77 % higher than the lowest concentration batch (Table 1). Between and within isoform concentration variations are demonstrated in Fig. 2, A) and B). Mean isoform concentrations determined in liver and kidney microsomes, using the method, are shown in Fig. 2, C) and D) (each liver sample was prepared at least in duplicate and each kidney sample was prepared five times). UGT concentrations determined in intestinal microsomes (n=3; each sample was prepared at least in duplicate) were 7.5, 3.4, 2.5 and 10.0 pmol/mg protein, respectively for UGTs 1A1, 1A10, 2B7 and 2B17.

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Discussion

The UGT content of a range of commercially available recUGTs (BD Supersomes™, 12 isoforms, n=49 samples) has been successfully quantified (Table 1) using a previously described isotope dilution targeted quantitative proteomic method (Fallon et al., 2013). This is the first report of such an analysis and provides additional information for the use of recUGT and other enzyme preparations in ADME and drug development studies. The range of concentrations determined suggests that catalytic activity rates measured *in vitro* per mg of total protein may not accurately reflect isoform specific activity for a particular drug candidate due to wide variability between isoforms and between lots (batches) of isoforms. The concentrations were found to vary ~7-fold between isoforms (1465 vs 211 pmol/mg) (Table 1). An appropriate use of the data could be to express catalysis as per pmol of UGT isoform, thus improving knowledge of the substrate activity of new chemical entities or drugs. The observation of variation between isoform batches (the highest recUGT1A4 concentration was ~77 % higher than the lowest concentration, Table 1, Fig 2, B; this was the highest variation observed) further indicates the possible limitation of the assumption of uniform isoform content, including when considered in relation to isoform kinetics.

A multiplexed (Fig. 1) targeted precise method for quantification such as this has broad applicability in ADME and in the support of drug development. The method allows the quantification of proteins involved in drug disposition, including recUGTs, that have not been previously measurable using traditional immunologically based methods (Fallon et al., 2008; Li et al., 2009; Harbourt et al., 2012). The equivalence of recUGT and organ specific UGT (hepatic, intestinal, renal) catalytic rates on a per pmol basis (Fig. 2, C) and D)), employing the data presented here, is currently being determined. However, variation is expected due to, for

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example, post translational modifications or protein interactions within the membrane. The prospect of employing enzyme catalytic activity measurements denoted as per amount of enzyme isoform in ADME studies is accordingly feasible. The availability of concentration/abundance data for UGTs in recombinant systems and tissue fractions should prove useful for scientists attempting to determine inter-system extrapolation factors, or more specifically to determine fractional metabolism (fm) by UGTs, or attempt *in vitro-in vivo* extrapolation. In addition, the development of physiologically based pharmacokinetic models where interindividual variation of UGT abundance in hepatic or extrahepatic drug metabolizing organs is incorporated depend on knowledge of enzyme abundance in the relevant drug clearing tissues.

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Authorship Contributions

Participated in research design: Fallon, Smith, Neubert

Contributed new reagents: Fallon, Smith

Conducted experiments: Fallon

Performed data analysis: Fallon, Smith, Goosen

Wrote or contributed to the writing of the manuscript: Fallon, Smith, Neubert, Goosen

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Footnotes

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Figure Legends

Fig. 1. TIC and extracted ion chromatogram for digested BD Supersomes™ recombinant sample UGT1A4-21161, 1st replicate, including all the stable isotope labeled peptide standards and β-casein control peptides. The 100-fold diluted hUGT1A4 concentration represented in the lower panel by the peptide YLSIPAVFFWR was 3.08 pmol/mg protein.

Fig. 2. UGT protein concentrations in A) BD Supersomes™ UGT1A1 samples analyzed in duplicate (n=4), B) BD Supersomes™ UGT1A4 samples in duplicate (n=4), C) a library of BD Gentest™ human liver microsome samples (mean, n=60) and D) human kidney microsome samples (mean, n=2). Each liver and kidney sample was analyzed at least in duplicate.

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TABLE 1

recUGT protein concentrations determined in BD Supersomes™ recombinant samples analyzed in duplicate. One optimal peptide was used for each UGT. The maximum and minimum concentration samples are circled and demonstrate the wide range of concentrations obtained. Lot numbers are shown in the sample names.

recUGT	pmol/mg protein Conc.	Mean ± SD (% C.V.)	recUGT	pmol/mg protein Conc.	Mean ± SD (% C.V.)
rUGT1A1-1	1359.0	1209.8 ± 249.1 (20.6)	rUGT1A9-1	441.1	490.5 ± 77.7 (15.8)
rUGT1A1-10	1464.5		rUGT1A9-8	604.3	
rUGT1A1-11	914.6		rUGT1A9-9	476.4	
rUGT1A1-85244	1101.2		rUGT1A9-06831	440.2	
rUGT1A3-1	536.2	473.2 ± 75.9 (16.0)	rUGT1A10-80018	709.5	738.5 ± 45.8 (6.2)
rUGT1A3-9	366.7		rUGT1A10-19244	791.3	
rUGT1A3-10	421.3		rUGT1A10-59891	714.7	
rUGT1A3-11	504.9				
rUGT1A3-12	536.8		rUGT2B4-1	354.4	355.3 ± 25.6 (20.6)
			rUGT2B4-2	367.6	
rUGT1A4-9	537.5	417.3 ± 127.5 (30.6)	rUGT2B4-3	313.6	
rUGT1A4-06150	303.4		rUGT2B4-10714	381.9	
rUGT1A4-95375	517.3		rUGT2B4-33191	358.9	
rUGT1A4-21161	310.8				
			rUGT2B7-1	797.7	914.7 ± 125.8 (13.8)
rUGT1A6-1	911.8	839.5 ± 172.8 (20.6)	rUGT2B7-9	918.5	
rUGT1A6-7	943.6		rUGT2B7-10	1148.7	
rUGT1A6-8	921.6		rUGT2B7-11	926.8	
rUGT1A6-04294	581.1		rUGT2B7-12	819.4	
			rUGT2B7-95853	877.0	
rUGT1A7-1	865.0	706.9 ± 176.2 (24.9)			
rUGT1A7-13906	824.7		rUGT2B15-6	220.4	243.8 ± 43.1 (17.7)
rUGT1A7-10729	659.1		rUGT2B15-7	236.8	
rUGT1A7-68106	478.9		rUGT2B15-26316	306.5	
			rUGT2B15-36575	211.4	
rUGT1A8-7	697.8	598.8 ± 96.6 (16.1)			
rUGT1A8-05599	504.8		rUGT2B17-1	687.0	708.5 ± 28.0 (4.0)
rUGT1A8-21754	593.7		rUGT2B17-2	740.2	
			rUGT2B17-3	698.2	

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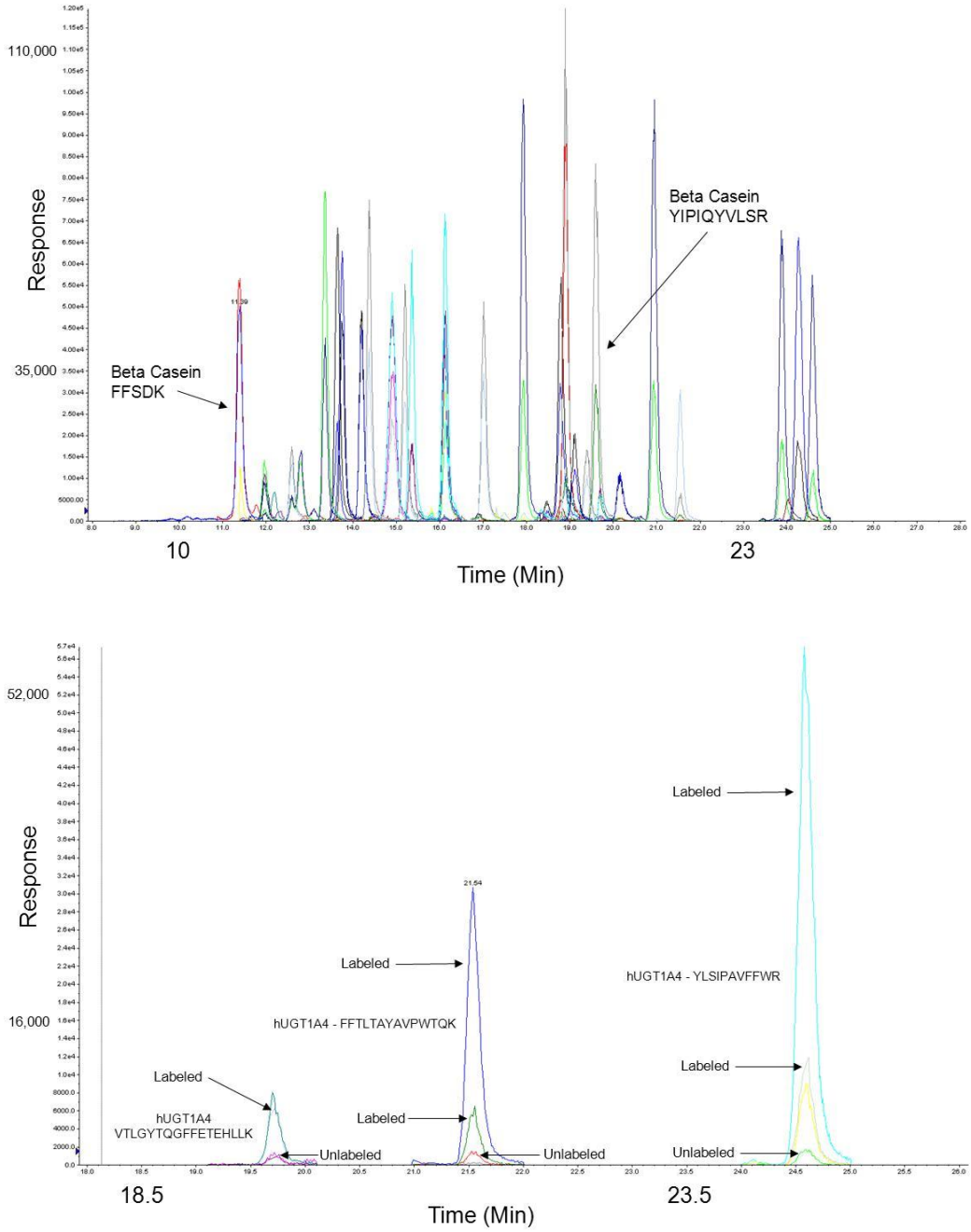

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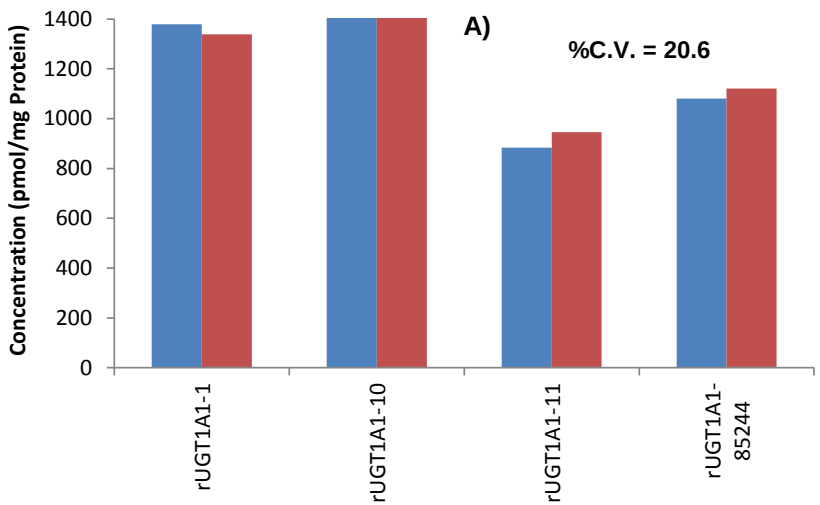

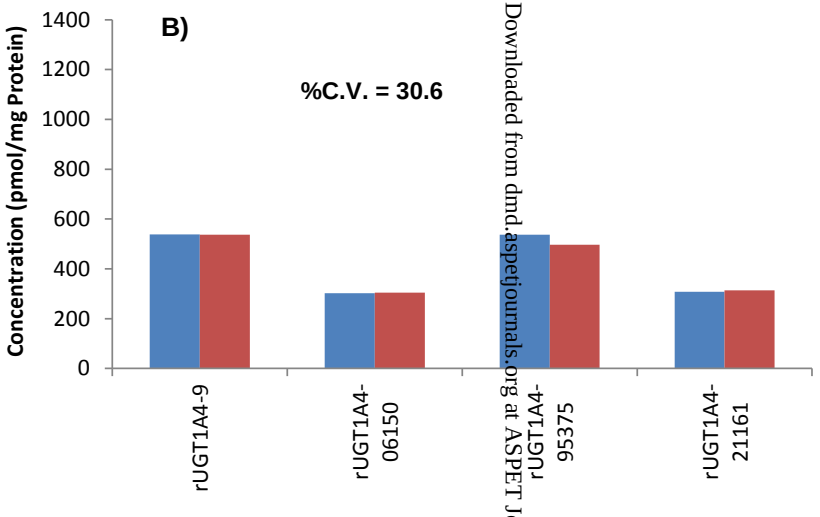

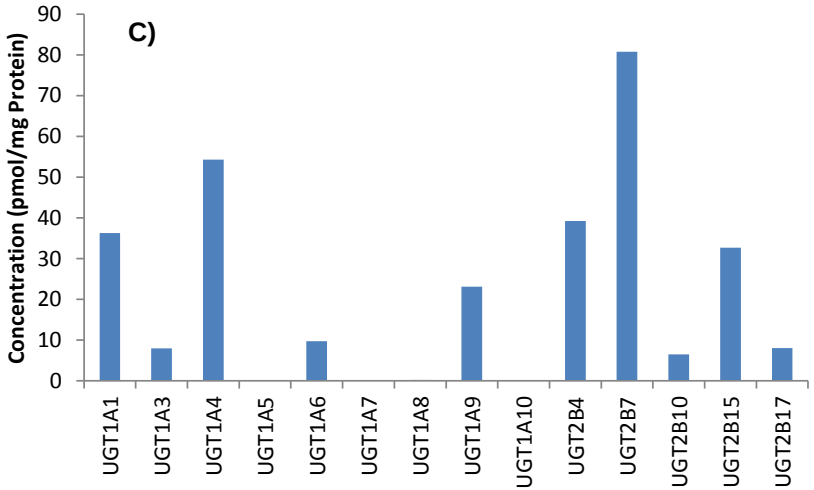

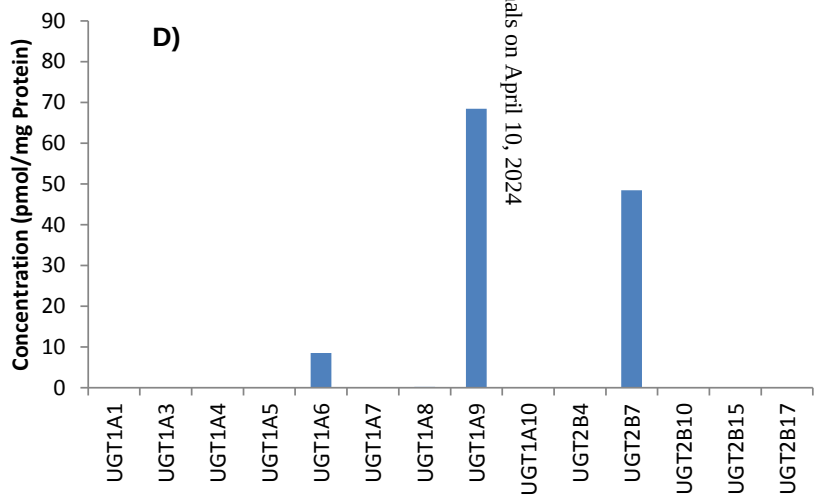

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