Review Article

Theme: Targeted Proteomics Quantification for Membrane Proteins

Guest Editors: Marilyn E. Morris and Yurong Lai

Optimized Approaches for Quantification of Drug Transporters in Tissues and Cells by MRM Proteomics

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Abstract. Drug transporter expression in tissues (*in vivo*) usually differs from that in cell lines used to measure transporter activity (*in vitro*). Therefore, quantification of transporter expression in tissues and cell lines is important to develop scaling factor for *in vitro* to *in vivo* extrapolation (IVIVE) of transporter-mediated drug disposition. Since traditional immunoquantification methods are semiquantitative, targeted proteomics is now emerging as a superior method to quantify proteins, including membrane transporters. This superiority is derived from the selectivity, precision, accuracy, and speed of analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode. Moreover, LC-MS/MS proteomics has broader applicability because it does not require selective antibodies for individual proteins. There are a number of recent research and review papers that discuss the use of LC-MS/MS for transporter quantification. Here, we have compiled from the literature various elements of MRM proteomics to provide a comprehensive systematic strategy to quantify drug transporters. This review emphasizes practical aspects and challenges in surrogate peptide selection, peptide qualification, peptide synthesis and characterization, membrane protein isolation, protein digestion, sample preparation, LC-MS/MS parameter optimization, method validation, and sample analysis. In particular, bioinformatic tools used in method development and sample analysis are discussed in detail. Various pre-analytical and analytical sources of variability that should be considered during transporter quantification are highlighted. All these steps are illustrated using P-glycoprotein (P-gp) as a case example. Greater use of quantitative transporter proteomics will lead to a better understanding of the role of drug transporters in drug disposition.

KEY WORDS: drug transporters; LC-MS/MS; membrane proteins; MRM proteomics; quantitative proteomics.

INTRODUCTION

Drug transporters play critical role in drug absorption, distribution, metabolism, and excretion (ADME). By affecting target tissue concentration of a drug, transporters often determine drug efficacy and toxicity [1–3]. Therefore, it is important that contribution of transporters in drug disposition (*i.e.*, including tissue distribution) is characterized. Drug transporter function can be studied in humans by imaging (*e.g.*, positron emission tomography (PET)) using specific transporter substrates [4–6]. While this technique has the advantage of measuring drug concentrations directly in tissues, it has some limitations, *i.e.*, (i) lack of availability of transporter-specific probes, (ii) possible interference if metabolite(s) of the probe substrate are also detected, and (iii) costs of radiochemical synthesis and instrumentation. Human

hepatocytes are the only well-established primary cells that are available to predict transporter-mediated drug disposition. But this approach cannot be extended to other organs important in drug disposition, *e.g.*, the intestine or the kidney, as the primary cells from these organs are not validated for transporter function. Moreover, primary cells cannot be used to extrapolate data from one compound to another as the net transport in these cells is driven by multiple transporters. Therefore, alternative approaches are required to quantify drug transporter function to assess their role in drug disposition in various tissues. One such potential approach is physiologically based pharmacokinetic (PBPK) modelling that incorporates information on the expression of drug transporters [7]. That is, to estimate *in vivo* maximum transport velocity ($V_{\max}$) of a drug by (i) first measuring the *in vitro* $V_{\max}$ in a cell line (or in vesicles) expressing a single transporter and then (ii) scaling the *in vitro* $V_{\max}$ value by incorporating *in vitro* to *in vivo* (tissue) transporter expression data. Using such adjusted $V_{\max}$, one can determine transporter-mediated *in vivo* clearance ($V_{\max}/K_m$) assuming that the K_m (substrate affinity) remains constant (*in vitro* vs. *in vivo*). This approach is routinely used for reliable prediction of

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cytochrome P450-mediated hepatic drug metabolism, *i.e.*, inter-system extrapolation factor (ISEF) approach [8].

Although gene expression data can indicate the presence of drug transporters in tissues, these data cannot be used in PBPK modelling because of the poor correlation between mRNA and protein levels. Therefore, protein expression of transporters is the closest surrogate of transporter activity. While transporter protein quantification by Western blotting is semiquantitative, we [9–13] and others [14–24] have recently shown that liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can be successfully used to determine protein expression in tissues with greater selectivity, speed, sensitivity, and reproducibility. MS-based protein quantification, commonly referred as multiple or selective reaction monitoring (MRM or SRM) proteomics, relies on selective quantification of surrogate peptide(s) in a digested protein sample (Fig. 1) [25, 26]. The selectivity is achieved by the MRM characteristic of triple quadrupole MS instrument. In MRM analysis, two stages of mass selection, *i.e.*, selection of a precursor ion and monitoring of specific product ion(s) derived from the precursor, provide highest analytical specificity applicable to complex proteome analysis. Such a two-step selection allows greater signal-to-noise ratio necessary for the quantification of low-expressing transporter proteins.

One can conclude from the above discussion and Fig. 1 that MRM detection of surrogate peptides is similar to that of small molecules (*e.g.*, drug or metabolite in biological matrix). This is indeed true and any laboratory equipped with triple quadrupole MS instrument can adopt MRM proteomics for transporter quantification once a surrogate peptide is identified. However, MRM proteomics is still a new technique for transporter quantification, and there are many variables that should be taken into account for its successful implementation.

A number of research and review papers covering selective elements of transporter quantification by MRM have been published [16, 27–30]. Here, we have synthesized these elements to provide a step-by-step guide to develop reliable transporter quantification methods in tissues or cell lines with emphasis on following steps: (i) surrogate peptide selection, (ii) peptide qualification, (iii) peptide synthesis and characterization, (iv) membrane protein isolation, (v) protein digestion, (vi) sample preparation, (vii) LC-MS/MS parameter optimization, (viii) method validation, and (ix) sample analysis (Fig. 2). The variables that can affect these processes are discussed in detail. The available bioinformatic resources and their merits and applications are also discussed. All these

aspects will be illustrated using P-glycoprotein (P-gp) as a case example.

MRM PROTEOMICS VS. WESTERN BLOTTING FOR PROTEIN QUANTIFICATION

MRM proteomics has now become the gold standard for protein quantification [27, 31, 32]. MRM proteomics is superior over traditional Western blotting in several ways including selectivity, speed, ease of use, quality of data, and ability to confirm results (Table I). For example, MRM proteomics can quantify multiple proteins in a short period of time, *i.e.*, approximately ten proteins in 20 min. Because a synthetic peptide is used as a calibrator, MRM proteomics can be used for absolute protein quantification provided that the complete protein digestion is achieved. The sensitivity of MRM method depends on the MS sensitivity of the surrogate peptide; however, at its best, the sensitivity is comparable or better than Western blotting. The sensitivity of MRM method can be substantially increased by using stable isotope standard capture with anti-peptide antibodies (SISCAPA) [33, 34]. MRM offers better precision because the sample preparation steps in MRM proteomics are simpler and can be automated. In addition, the quality of data with MRM proteomics is better because the results can be confirmed by multiple signals generated from more than one MRM transitions and if possible from multiple peptides. However, finding multiple surrogate peptides can be difficult especially for homologous proteins (*e.g.*, Mdr1a and Mdr1b in rodents).

MRM METHOD DEVELOPMENT AND VALIDATION

Surrogate Peptide Selection

Selection of surrogate peptides for a protein is the first and most critical step in MRM proteomics. Until few years back, the surrogate peptide designing was based on experimental approach involving tedious protein fractionation by SDS-PAGE gel electrophoresis followed by capillary LC nano-electrospray ionization quadrupole time-of-flight (nano-ESI-Q-TOF) or Orbitrap MS instruments [23, 24, 35]. While this approach is accurate, it is a laborious, cost-intensive procedure, and requires a protein standard with reasonable purity and amount. To overcome these shortcomings, *in silico* approach, first adopted by Kamiie *et al.* [27] for drug transporters, is preferred these days. The uniqueness, LC retention, MS response, peptide stability, quantitative reliability, and signal quality are the important aspects that are considered for surrogate peptide selection. While integrated

Fig. 1. Outline of MRM analysis of a surrogate peptide after protein digestion. The most abundant parent ion is selected and then fragmented in the collision cell. The abundant product ions are selected and quantified

Fig. 2. Critical steps in method development and validation of a transporter quantification assay. Variability in protein quantification can be introduced in any one of these steps

software tools, *e.g.*, SRMatlas, MRMAid, or GPMDB (see below), are available for surrogate peptide design, these tools do not cover all the proteins across species. Therefore, we present below a basic generalized approach starting from finding a protein sequence to selecting a quantitatively reliable surrogate peptide for any transporter protein of interest.

1. *Protein sequence*: For targeted quantification of a transporter, first a full protein sequence is obtained from the databases such as NCBI, Ensembl, UniprotKB, or Protein knowledgebase (Table II). For example, the sequence for human P-gp (MDR1) was obtained from UniprotKB (Fig. 3) by putting protein name and species in the search box. The

protein accession number for the transporter (*e.g.*, P08183 (Uniprot) or ENSP00000265724 (Ensembl) for P-gp) can be obtained in this step for subsequent use.

2. *In silico peptide digestion*: For any protein, a list of potential peptides can be generated using *in silico* digestion tools listed in Table II. The parameters like protein accession number, corresponding database (*e.g.*, Uniprot), protease type (*e.g.*, trypsin), mass range (800–3,000), and minimum peptide length [7] are entered, and a list of potential peptides is obtained with information on the end and start amino acids and peptide molecular weights. We used Protein Prospector and Skyline (see below) to perform this task for P-gp. While these tools allow protein digestion by

Table I. Comparison of Western Blotting and MRM Proteomics for Protein Quantification [31, 32]

Category	Western blotting	MRM proteomics
Principle	Protein separation based on protein 3-D structure or the length of the polypeptide using gel electrophoresis. Then, proteins are transferred to a membrane (<i>e.g.</i> , nitrocellulose) and stained with specific antibodies	Selective quantification of surrogate peptide(s) in a digested protein sample by LC-MS/MS
Selectivity	Cannot distinguish between homologous proteins	Can differentiate proteins even when they differ by a single amino acid residue
Sensitivity	If good antibodies are available, this method is sensitive and can detect proteins up to picomole (per mg of total protein) level	If the best peptide is used, MRM method is comparable or better in sensitivity than Western blotting
Number of end points	Single end-point. Poor quality control	Multiple endpoints, <i>e.g.</i> , multiple MRM transitions of more than one surrogate peptides (if available)
Reproducibility	Poorer reproducibility than MRM because of the cumbersome procedure	Highly reproducible. Most of the steps are automated
Reagent quality	Selective antibodies are difficult to synthesize	Well-characterized synthetic peptides are readily available
Reagent availability	Antibodies are not always available	Peptides can be commercially procured
Costs	Quantification of a single protein is more economical than the MRM method	Instrument cost is high. However, it can quantify multiple proteins at a time, reducing the overall cost
Key advantages	Technique and expertise are routinely available in many laboratories	Selective, multiplex quantification, robust, large dynamic range
Major limitations	Nonselective, low dynamic range	Cost of instrument and sophisticated method development

LC-MS/MS liquid chromatography tandem mass spectrometry, MRM multiple reaction monitoring

various proteases (*e.g.*, trypsin, chymotrypsin, Lys-C, *etc.*), trypsin is commonly used in proteomics because of the following advantages. Trypsin specifically hydrolyzes peptide chains primarily at the carboxyl side of the amino acids lysine (K) or arginine (R), except when either is followed by proline (P). Trypsin forms peptides that are favorable for detection in triple quadrupole LC-MS/MS instrument (*e.g.*, peptides with mass range of 800–3,000 and charge (typically +2)). Further, most of the publically available MS proteomic data, *i.e.*, spectral libraries, are derived from trypsin-digested peptides.

3. *Exclusion of regions of uncertainty*: When peptides are generated by *in silico* prediction, it is important that only those candidates that are suitable for quantitative work are selected. Only peptides with 7–22 amino acids should be considered as this mass range is detectable in triple quadrupole MS. Also, potential (or known) transmembrane regions are excluded as they can affect protein digestion of transporter proteins. Where experimental information on the location of the transmembrane regions is not available, non-experimental methods can be used to predict these regions. For this, Uniprot marks transmembrane regions as (i) “potential regions” indicating some conclusive *in silico* evidence, (ii) “probable regions” with at least some experimental data, and (iii) “regions by similarity” indicating that some experimental information is available on a similar protein (or part of it). Uniprot predicted 12 transmembrane regions for MDR1, *i.e.*, positions 45–67, 117–137, 187–208, 216–236, 293–316, 331–352, 712–732, 757–777, 833–853, 855–874, 935–957, and 974–995 (Fig. 3).

Posttranslational modification (PTM) can change the mass of a peptide and hence should be avoided. When literature data on PTMs are not available, predictive tools, *e.g.*, NetNGlyc, NetOGlyc, and NetPhos, can be used to predict such PTMs (Table II). Using such tools, amino acid residues, 91, 94, 99, and 666 in MDR1 (Fig. 3), were excluded as they are expected to be either phosphorylated or glycosylated. It is also important to avoid any peptide with possible changes in amino acid sequence due to non-synonymous single nucleotide polymorphism (SNP) or other genetic variations. For example, a common variant rs2032582 in ABCB1 [36] causes changes in amino acid S→A (position 893) which restricts the use of ELEGSGK as a quantitative surrogate peptide. While this information can be obtained from literature, useful databases exist that compile such data (Table II). Consideration of genetic polymorphism is important, but this criterion can be relaxed if the allele frequency is low (*e.g.*, <1%) particularly in the absence of suitable alternative peptides. In general, all the peptides containing cysteine (C) and methionine (M) residues (underlined in Fig. 3) can be excluded because they are susceptible to rapid oxidation [37]. Accuracy of reported protein sequence is also important to be considered. For example, conflicting sequences can be submitted to the databases from different research groups, different types of experiments, or different biological samples, *e.g.*, positions 23 [38] and 336 [39] in P-gp. Conflicting information on protein sequence can be sometime obtained from databases (*e.g.*, Uniprot). Continuous sequence of arginine (R) or lysine (K) (*e.g.*, RR, KK,

Table II. Bioinformatic Tools Used in Various Steps of MRM Proteomics Method Development

Parameter	Bioinformatic tool	Website
Protein sequence	NCBI	http://www.ncbi.nlm.nih.gov/protein
	Uniprot	http://www.uniprot.org/
Digestion	Protein prospectus (MS-Digest)	http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest
	Expasy	http://web.expasy.org/cgi-bin/peptide_mass/peptide-mass.pl
	MRMPATH	http://tmpl.uab.edu/MRMPATH/submit_query_protID.jsp?num=P08183&enzyme=0
	Skyline	https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view (A)
Uniqueness	BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
	Protein prospectus (MS-homology)	http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology
Genetic polymorphisms	dbSNP	http://www.ncbi.nlm.nih.gov/SNP/
	Ensembl	http://uswest.ensembl.org/index.html
	Pharmacogenetics of Membrane Transporters Database	http://pharmacogenetics.ucsf.edu/
	e-PKGene	http://www.druginteractioninfo.org/applications/pharmacogenetics-database/
Retention time	SSRCalc	http://hs2.proteome.ca/SSRCalc/SSRCalcX.html
	Skyline	<i>See above</i>
Transmembrane topology	Phobius	http://phobius.sbc.su.se/
	TMHMM Server v. 2.0	http://www.cbs.dtu.dk/services/TMHMM-2.0/
	PeptideAtlas	http://www.peptideatlas.org/
Glycosylation	NetNGlyc	http://www.cbs.dtu.dk/services/NetNGlyc/
	NetOGlyc	http://www.cbs.dtu.dk/services/NetOGlyc/
Phosphorylation	NetPhos	http://www.cbs.dtu.dk/services/NetPhos/
Peptide MS response	ESPPredictor	http://www.broadinstitute.org/cancer/software/genepattern/modules/ESPPredictor.html
	PeptideSieve	http://tools.proteomecenter.org/wiki/index.php?title=Software:PeptideSieve
	CONSequence	http://www.ncbi.nlm.nih.gov/pubmed/21813416
Fragmentation or MRM	Skyline	<i>See above</i>
	GPMDb	http://gpmdb.thegpm.org/mrm/index.html
	MRMaid	http://elvis.ccc.cranfield.ac.uk/mrmaid/faces/ResultsPage.xhtml
	PepNovo	http://bix.ucsd.edu/Software/PepNovo.html
	MRMPilot	http://www.absciex.com/products/software/mrmpilot-software?country=United%20States
	SRMATlas	https://db.systemsbio.org.net/sbeams/cgi/PeptideAtlas/GetTransitions?_subtab=1
	MRMer	http://proteomics.fhcr.org/CPL/MRMer.html
	MaRiMba	http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP-MaRiMba
Spectral libraries	NIST	http://peptide.nist.gov/
	BiblioSpec	https://skyline.gs.washington.edu/labkey/project/home/software/BiblioSpec/begin.view
	PRIDE	http://www.ebi.ac.uk/pride/
Sample analysis	AuDIT	http://www.broadinstitute.org/cancer/software/genepattern/modules/AuDIT.html
	SRM Collider	http://www.srmcollider.org/srmcollider/srmcollider.py
	Skyline	<i>See above</i>

NCBI National Center for Biotechnology Information, BLAST Basic Local Alignment Search Tool, GPMDb Global Paleomagnetic Database, NIST National Institute of Standards and Technology

RK, or KR) (ragged ends) should be excluded as it may cause incomplete trypsin digestion. While trypsin does not cleave K and R that are followed by P, this phenomenon is not always true [40]. Nevertheless, peptides containing such sequences should be excluded if other peptides are available for quantification.

4. **Uniqueness:** While specificity can be tested right after step 2 above, it can be exercised on the limited number of potential proteotypic peptides after excluding the regions of uncertainty. Specificity can be verified by using online tools like BLAST search analysis or homology analysis in Protein Prospectus (Table II). These tools can also confirm whether the peptide is conserved across species as shown for MDR1 in Table III. Use of the conserved peptides is advantageous when transporter expression is compared across

species. The use of the same peptide reduces variability introduced by trypsin digestion.

5. **Additional criteria:** Amino acids that are prone to deamination (asparagine and glutamine [41, 42]) can be avoided. N-terminal glutamine and glutamic acid can form cyclic pyroglutamate under acidic conditions [43]. Aspartic acid, when paired with glycine, proline, or serine, can undergo hydrolysis and cause peptide cleavage in acidic environment [44]. Tryptophan and histidine residues are also prone to chemical degradation [45–47]. Despite these stringent selection criteria, it is sometime difficult to control modification of peptide. In that case, a combination of both unaltered and altered peptides can be selected for reliable quantification. Some peptides are difficult to synthesize. For example, it is challenging to remove

10	20	30	40	50	60
MDLEGDRNGG	AKKKNFCKLN	NKSEKDKKEK	KPTVSFVSFM	RYSNWLDKLY	MVVGTLAAII
70	80	90	100	110	120
HGAGLPLMML	VFGEMTDIFA	NAGNLEDLMS	NITNRSDIND	TGFFMNLEED	MTRYAYYYSG
130	140	150	160	170	180
IGAGVLVAAY	IQVSFWCLAA	GRQIHKIRKQ	FFHAIMRQEI	GWFDVHDVGE	LNTRLTDDVS
190	200	210	220	230	240
KINEGIGDKI	GMFFQSMATF	FTGFIVGFTR	GWKLTLVILA	ISPVGLLSAA	VWAKILSSFT
250	260	270	280	290	300
DKELLAYAKA	GAVAEVLAA	IRTVIAFGGQ	KKELERYKNK	LEEAKRIGIK	KAITANISIG
310	320	330	340	350	360
AAFLLIYASY	ALAFWYGTTL	VLSGEYSIGQ	VLTVFFSVLI	GAFSVGQASP	SIEAFANARG
370	380	390	400	410	420
AAAYEIKIID	NKPSIDSYSK	SGHKPDNIKQ	NLEFRNVHFS	YPSRKEVKIL	KGLNLKVQSG
430	440	450	460	470	480
QTVALVGNSS	CGKSTTVQLM	QRLYDPTGEM	VSVDGQDIRT	INVRFLREII	GVVSQEPVLF
490	500	510	520	530	540
ATTIAENIRY	GRENVTMDEI	EKAVKEANAY	DFIMKLPKHF	DTLVGERGAQ	LSGGQKQRIA
550	560	570	580	590	600
IARALVRNPK	ILLLDEATSA	LDTESEAVVQ	VALDKARKGR	TTIVIAHRLS	TVRNADVIAG
610	620	630	640	650	660
FDDGVIVEKG	NHDELMKEKG	IYFKLVTMQT	AGNEVELENA	ADESKSEIDA	LEMSSNDSRS
670	680	690	700	710	720
SLIRKRSTRR	SVRGSAQDR	KLSTKEALDE	SIPPVSWFRI	MKLNLTWPY	FVVGVFCAII
730	740	750	760	770	780
NGGLQPAFAI	IFSKIIGVFT	RIDDPETKRQ	NSNLFSLLFL	ALGIISFITF	FLQGFTFGKA
790	800	810	820	830	840
GEILTKRLRY	MVFRSMLRQD	VSWFDDPKNT	TGALTTRLAN	DAAQVKGAIG	SRLAVITONI
850	860	870	880	890	900
ANLGTGIIIS	FIYCWQLTLL	LLAIVPIIAI	AGVVMKMLS	GQALKDKKEL	EGSGKIA TEA
910	920	930	940	950	960
IENFRTVVS	TQEQKFEHMY	AQSLQVPYRN	SLRKAHIFGI	TFSFTQAMMY	FSYAGCFRFG
970	980	990	1000	1010	1020
AYLVAHKLMS	FEDVLLVFS	VVFGAMAVGQ	VSSFAPDYAK	AKISAHHIIM	IIKPTPLIDS
1030	1040	1050	1060	1070	1080
YSTEGMLPNT	LEGNVTFGEV	VFNYPTRPDI	PVLQGLSLEV	KKGQTLALVG	SSGCGKSTVV
1090	1100	1110	1120	1130	1140
QLLERFYDPL	AGKVLLDGKE	IKRLNVQWLR	AHLGIVSQEP	ILFDCSIAEN	IAYGDNRSRV
1150	1160	1170	1180	1190	1200
SQEEIVRAAK	EANIHAFFIES	LPNKYSTKVG	DKGTQLSGGQ	KORIAIARAL	VRQPHILLD
1210	1220	1230	1240	1250	1260
EATSALDTES	EKVVEALDK	AREGRTCIVI	AHRLSTIQNA	DLIVVFQNGR	VKEHGTHQQL
1270	1280				
LAQKGIYFSM	VSVQAGTKRQ				

Fig. 3. P-gp protein sequence (<http://www.uniprot.org/uniprot/P08183>). Residues highlighted in gray represent predicted transmembrane regions. Underlined residues represent either unstable residues, SNPs, ragged ends, or sequence conflicts

asparagine N-terminal-protecting agent during peptide synthesis [48]. A peptide containing multiple serine or proline residues is also difficult to synthesize. Optimum solubility of peptide is important for LC retention as well as for solubilization of peptides during various steps (synthesis to LC-MS/MS analysis) of quantitative proteomics. The incomplete solubility of peptide might be due to hydrophobicity of peptides or due to β sheet formation [49]. It is generally difficult to dissolve a peptide if more than 50% of the amino acid residues are hydrophobic (e.g., valine, isoleucine, leucine, phenylalanine, tryptophan, or cysteine). A series of glutamine, isoleucine, leucine, phenylalanine, threonine, tyrosine, or valine can form β sheets that result in incomplete solvation [37]. These criteria are important, but if applied stringently, potential MS-sensitive peptides might be excluded. Therefore, if peptides with such red flags are selected, it is important to test their stability and solubility before using them for protein quantification.

6. Prediction of chromatographic (LC) parameters: Only those peptides that are retainable in the LC column

should be selected for the next phase. Generally, a reverse phase column is used for peptide quantification. Software tools, e.g., SSRCalc, could be used for predicting peptide LC retention. In general, peptides with hydrophobicity index below (HI) 7 are not retained on the column, and those with HI above 50 strongly bind with reverse phase material. Such peptides should be excluded. Skyline can also predict retention time (RT) by regression equation generated from experimental data. Typical LC mobile phase conditions involve initial elution of polar interferences by high aqueous mobile phase (e.g., 98% aqueous) followed by gradually increasing organic phase (e.g., up to 60% acetonitrile). For P-gp, we predicted RT for its surrogate peptides based on peptide sequences and Skyline. Only 11 peptides that have optimum RT in conventional reversed-phase columns were considered for the next stage (Table III). Alternatively, Thermo Scientific Pierce peptide RT calibration mixture contains 15 standard peptides that can be used for RT prediction based on peptide hydrophobicity.

Table III. Selected Surrogate P-gp Peptides and Their Predicted and Observed LC-MS/MS Parameters and Specificity in Human and Rodents

Peptide	Residue position	Parent ion	Product ions				RT (min)	Fragmentor (V)	Collision energy (eV)	Specificity	SSRT
			1	2	3	4					
IIGVFTR (P1)	735–741	403.2	579.4	423.3	692.5	276.2	12.5	120	12	hMDR1	26.3
IIGVFTR (SIL-P1)		409.3	589.4	433.3			12.5				
ELLAYAK (P2)	243–249	404.2	565.3	452.3	381.2	338.2	10.7	120	12	hMDR1	18.7
ELLAYAK (SIL-P2)		408.3		460.3	389.2		10.7				
GAAYEIFK (P3)	360–367	449.7	699.5	536.4	770.5	407.4	12.5	125	13	hMDR1	24.3
GAAYEIFK (SIL-P3)		453.8	707.5	544.4			12.5				
ILKGLNLK (P4)	409–416	449.8	672.4	785.5	544.3	752.5	10.3	125	13	hMDR1, rMdr1b, rMdr3, mMdr3	24.2
ILKGLNLK (SIL-P4)		453.8	680.4	793.5			10.3				
FYDPLAGK (P5)	1,086–1,093	455.7	485.3	600.3	426.3	763.4	13.3	125	13	hMDR1	21.7
FYDPLAGK (SIL-P5)		459.7	493.3	608.3			13.3				
ILSSFTDK (P6)	235–242	455.8	684.4	597.4	510.4	797.5	10.6	125	13	hMDR1, mMdr1a	20.6
ILSSFTDK (SIL-P6)		459.8	692.4	719.4			10.6				
NTTGALTTR (P7)	809–817	467.8	719.4	618.4	490.3	561.3	6.9	125	13	hMDR1,	10.4
NTTGALTTR (SIL-P7)		472.5	729.4	628.4			6.9			mMdr1a, rMdr1	
IAIARALVR (P8)	539–547	491.8	685.4	614.4	458.3	798.5	14.6	130	14	hMDR1, rMdr1,	24.1
IAIARALVR (SIL-P8)		496.8	695.4	624.4			14.6			mMdr1a/b	
TINVRFLR (P9)	460–467	509.8	804.6	690.5	591.5	435.4	14.0	130	14	hMDR1	28.5
TINVRFLR (SIL-P9)		514.8	814.6	700.5			14.0				
STVVQLLER (P10)	1077–1085	522.8	757.5	658.4	530.3	417.2	13.9	130	14	hMDR1, rMdr1,	27.1
STVVQLLER (SIL-P10)		527.8	767.5	668.4			13.9			mMdr1a/b	
IATEAIENFR (P11)	896–905	582.3	749.5	678.5	565.4	436.3	12.2	140	16	hMDR1, rMdr1,	25.7
IATEAIENFR (SIL-P11)		587.3	759.5	688.5			12.2			mMdr1a/b	

Key: Residues shown in bold letters (*i.e.*, K and R) are labeled with $^{13}\text{C}_6^{14}\text{N}_2$ -lysine and $^{13}\text{C}_6^{14}\text{N}_4$ -arginine, respectively Other LC-MS/MS parameters are discussed before [11]. One missed cleavage site is present in P4, P8, and P9
RT retention time, SSRT Sequence specific retention time

7. *Prediction of relative MS response:* The MRM signal intensity depends on the MS response of parent as well as product ions. Not all peptides are MS responsive and not all MS/MS fragments are equally abundant. Therefore, real spectral libraries or experimental MRM data from databases (Table II) can be used to select the best peptide and fragment ions. However, as these data are not always available for drug transporters, bioinformatic tools such as ESPPredictor, PeptideSieve and CONSeQuence can be used for this purpose. These tools typically work based on the machine learning algorithms that are taught to identify highly abundant peptides from experimental data. This is an active area of research and it is anticipated that improved bioinformatic tools, developed based on larger datasets, will be available in the future. Fragmentation pattern with relative intensities of product ions can be obtained or predicted by tools, such as PeptideART, MRMPilot, PinPoint, Skyline, and SRMATlas (Table II). The potential high abundant fragment ions can also be generated for heavy labeled peptide at this stage using these tools. We used SRMATlas and PeptideART to predict the most intense fragments of P-gp peptides (Table III). We found that >90% of the top five fragments predicted by both the software were similar. The collision energy and fragmentor (cone) voltage for each peptide were predicted using SRMATlas. Typical collision energy for MRM peptides is from 10 to 30 eV, and fragmentor (cone) voltage is from 100 to 160 V in Agilent triple quadrupole instrument. These parameters generally increase with peptide length. Dwell time can be optimized to allow at least 15 data points for each peak. If multiple peptides or transitions are used, dynamic or scheduled

MRM can be adopted which allows targeted data acquisition for a given transition only around the predicted RT.

While the above discussion is about theoretical criteria-based manual method of surrogate peptide selection, more advanced bioinformatic tools are emerging for this purpose. Integrated bioinformatic tools Skyline, GPMDB, PeptideAtlas, SRMATlas, and MRMAid for surrogate peptide selection are discussed below:

Skyline

Skyline is a Windows-based MRM transition design tool developed by MacCoss Lab, University of Washington. It is installed in a local computer before use. To start, one has to first create a blank document. The protein or peptide sequence is then pasted into the document in the FASTA format. Skyline automatically transforms this information into *in silico*-predicted peptide ions and MRM transitions for each peptide. Thus far, this all is based on theoretical calculations without considering spectral data. However, Skyline is flexible and the users can select settings such as desired peptide size and exclusion of specific amino acid(s). An important and unique advantage of Skyline is it allows the use of spectral data from databases or MS instrument from different vendors to verify the predictions. As mentioned above, Skyline also predicts RT for peptides more accurately than SSRCalc alone based on instrument-specific data. Skyline data can be exported to create method files for direct upload into the instruments from major LC-MS/MS vendors. Quantitative data analysis can also be facilitated using Skyline. Panorama, an open-source repository server developed by the Skyline group can be used for Skyline data management and sharing.

The Global Proteome Machine Database (GPMDB)

This is a web-based proteomic tool that has a dedicated MRM database, which is supported by an experimental mass spectral library. To get the MRM data for peptides related to a given protein, Ensembl protein accession number (or protein name as keyword) and species name are entered into the search box. For example, using P-gp Ensembl accession number (ENSP00000265724), a list of MRM peptides can be predicted. While the tool gives a list of all predicted peptides, the recommended peptides are highlighted with asterisk based on peptide length, charge state, and amino acid composition. GPMDB also predicts RT. The experimentally observed MS/MS information (consensus spectrum) for each peptide can be obtained by clicking on the peptide, which then can be used to generate MRM method. GPMDB also provides information on potential interferences, *i.e.*, it generates a list of peptides in the given species that can give at least one similar product ion. The application of GPMDB is limited to those peptides where spectral data are already available. For example, MRM transitions are available for P-gp but not for OATP1B1 in GPMDB.

PeptideAtlas

This is a web-based proteomic repository developed by the Institute for Systems Biology (ISB), Seattle (WA). To get information to generate MRM assay, one needs to put the protein name as keyword or accession number and target species in the search box. Within “Distinct Observed Peptides” panel, it shows details of real experimental data for peptides, *e.g.*, number of times the peptide has been observed and number of samples in which the peptide is seen. On clicking “Annotated Transitions” panel, PeptideAtlas provides a list of observed transitions, *i.e.*, peptide sequence, charge state, parent and product ions, product ion intensity, collision energy, instrument used, and RT. Similar to GPMDB, one has to download these data onto a local computer for future use. Peptide ranking is provided to help users select the best potential peptides. It also provides the information on the predicted transmembrane regions.

SRMATlas

This is a sister software of PeptideAtlas developed by the same group. It is a database strictly designed to create MRM methods for protein quantification. The data are based on MRM experiments conducted mostly on triple quadrupole instrument, and therefore, it is a great resource for targeted proteomic method development. Though SRMATlas is limited to few species (human, mouse, yeast, and *Mycobacterium tuberculosis*) to date, it provides comprehensive information on MRM transition, product ion intensity, collision energy, and predicted retention time. To retrieve a list of transitions, one needs to put the protein accession number onto the search button and select parameters such as number of peptides and transitions required and instrument type (Agilent QQQ, AB Sciex QTRAP5500/4000, Thermo TSQ or QTOF). The software can also calculate MRM transitions for heavy peptides. Batch processing can be done to collect MRM transitions for multiple proteins or peptides at a same time.

MRMaid

Similar to SRMATlas, MRMaid is a web-based tool which was also specifically designed (by Cranfield University and Queen Mary University) for MRM method developed. The software relies on experimental data available in PRIDE database (Table II). To get the MRM information, protein name or accession number and species name are entered into the search box. A table of MRM transitions is obtained by clicking on “MRMaid Search.” Similar to SRMATlas, one can select instrument type, number of peptides, and product ions per peptide region. The output also shows the number of times a product ion is observed in PRIDE and relative intensities of product ions. While RT is calculated based on SSRCalc algorithm, it provides option of calculating RT for a particular LC type.

Qualification of Surrogate Peptide

Once a surrogate peptide is predicted by the *in silico* tools, it is important to test whether this peptide works in the real sample. One can procure the synthetic surrogate peptides at this stage and tune LC-MS/MS parameters using standards. However, peptide synthesis generally takes 4–6 weeks, and sometime the selected surrogate peptide turns out to be less sensitive in MS. Therefore, an alternative approach that is independent of peptide standard can be used to facilitate method development. The latter involves screening potential quantitative surrogate peptides in the real samples (membrane fractions from tissues or cells) using generic LC and MS conditions. For transporter protein, vesicles or cells expressing transporters are preferred for qualification as they generally have higher transporter expression. For example, 11 surrogate peptides of P-gp were detected in cell expression P-gp in a single dynamic MRM method (Fig. 4). The peptide signal can be qualified by (i) superimposability of multiple product ion chromatograms and (ii) elution of peptides at predicted RT (Fig. 5). Reproducibility of (i) and (ii) with another column generally adds confidence to the results. MS sensitivity can be optimized by playing with critical MS parameters such as collision energy, resolution, and dwell time for any given set of parent to product ion. An alternative approach is to purchase crude peptide libraries for tuning LC-MS/MS parameters. Such peptide libraries are currently available from Thermo Scientific Pierce Biotechnology (PEPotec SRM Peptide libraries) as aliquots of ~0.1 mg/peptide with purity between 75 and 125%. The cost of these peptides is much lower, \$15–25/peptide, than the analytical grade peptide standard (~\$600/peptide). Once two to three best peptides are qualified, *e.g.*, IATEAIENFR, and NTTGALTTR (Table III) for P-gp, these can be used for protein quantification.

Peptide Synthesis and Characterization

Peptide can be procured from custom peptide synthesis companies (*e.g.*, New England Peptides, Thermo Scientific Pierce Biotechnology, or Sigma Aldrich). The corresponding internal standard peptide that is labeled by incorporation of [¹³C₆¹⁵N₂]lysine or [¹³C₆¹⁵N₄]arginine can also be purchased. For absolute quantification of transporters, the purity and concentration of standard solutions are important. Amino acid analysis (AAA) is a critical test to ensure the absolute quantity of peptide standard used as calibrator.

Fig. 4. LC-MS/MS chromatograms showing superimposable peaks representing various transitions of P-gp surrogate peptides and corresponding internal standards. Peptides P4, P5, P7, P10, and P11 were detectable with high intensity

Chromatographic purity should also be established at this stage. Peptide aliquots should be stored at temperature < -20°C . New England Peptides, Thermo Scientific, and Sigma Aldrich also offer AAA for an additional cost of $\sim \$250/\text{peptide}$.

Sample Procurement and Membrane Protein Isolation

Endogenous factors such as genetic polymorphism, post-translational modification, disease condition, and medication can affect drug transporter expression. Transporter

Fig. 5. Chromatograms (two MRM transitions) of NTTGALTTR showing superimposability by comparing retention times at different peak heights at h (x), 0.75 h (y and y'), and 0.50 h (z and z')

quantification in tissues can also be affected by the quality of samples that depends on variables associated with sample procurement and storage (temperature, microbial contamination, *etc.*). Regarding transporter abundance in cell lines, factors such as passage number, confluency, or culture conditions can affect transporter expression. Cells grown in the presence of fetal bovine serum should be thoroughly washed if the total protein is used to normalize transporter expression.

Total transporter protein expression in the tissue homogenate or total membrane fraction (*i.e.*, plasma membrane plus membranes from intracellular organelles) may not all be functionally active because some of it might be in the trafficking process to the membrane. Therefore, isolation of pure plasma membrane is ideal for predicting transporter function. To do so, there are a few protocols and commercially available kits that purport to isolate the plasma membrane [50, 51]. However, in our experience, these kits/methods do not yield pure plasma membrane fraction reproducibly for quantitative applications. Therefore, we and others have used total membrane transporter expression for this purpose [9, 11, 12, 19, 22, 52–54]. Although it is possible that the transporter trafficking is altered by disease condition or drug treatment [55–57], we believe that obtaining pure plasma membrane is not necessary if the ratio of expression of transporter protein in the plasma membrane relative to that in the entire cell is assessed to be similar in tissues (*e.g.*, human livers) and in transporter-expressing cell lines. This assumption can be tested by crudely isolating various subcellular fractions from tissues and cells and comparing the expression of the transporters relative to the marker of plasma membrane (*e.g.*, Na⁺K⁺ATPase) in these fractions. Such studies are ongoing in our laboratory and have yielded promising results.

Protein Digestion

The surrogate peptide-based absolute quantification of protein completely relies on the efficiency of protein digestion. Typical trypsin digestion process involves (i) denaturation of transporter proteins by denaturant like urea or sodium dodecyl sulfate (SDS) and heating, (ii) reduction of cysteine residues by dithiothreitol (DTT), (iii) alkylation of thiols by iodoacetamide (IAA), and (iv) protein digestion by addition of sequencing grade trypsin. Optimization of protein/trypsin ratio, incubation time, temperature, pH, surfactant concentration, and stability of trypsin should be considered for the efficient trypsin digestion [58, 59]. Maximum digestion of transporter protein is achieved by incubating protein/trypsin in a ratio of 1:10 to 1:50 for 4–24 h at pH 7.5–8.5 and temperature 37°C. The use of surfactant is important to ensure solubilization of transporter protein. However, high concentration of surfactant can adversely affect trypsin digestion efficiency [58]. Trypsin digestion efficiency can be improved by using specialized equipment such as microwave [60], ultrasonic probe [61], and hydraulic pressure pulse activator [62]. Moreover, addition of serine protease (Lys-C) with trypsin improves digestion efficiency [63, 64]. The use of DOC is preferred over urea as a denaturant [58]. When urea is used, the temperature should not exceed 60°C as the latter results in carbamylation of proteins or peptides. Repigest is another denaturant that is becoming common in shotgun proteomics due to its compatibility with LC-MS/MS [65, 66].

Using the purified transporter protein as a calibration standard addresses the issue of less than 100% protein digestion (Table IV) [67]. For instance, based on pure P-gp protein as a quality control, we found that trypsin digestion of P-gp was close to 100% [11]. Digestion efficiency is not a problem in relative quantification if the variability in trypsin digestion is addressed.

Variability can be addressed theoretically by using labeled protein (SILAC protein) as an internal standard. However, we observed that if trypsin digestion is optimized and sample-to-sample variability in the digestion is low, labeled peptide behaves equally well or better than the labeled proteins as an internal standard [13]. Alternatively, QconCAT relies on concatamers of tryptic peptides in an artificial protein as standards and therefore addresses digestion efficiency with the assumption that the rate of trypsin digestion remains similar in the sample and the artificial protein (Table IV) [68–70].

To increase confidence in protein quantification, one can use multiple peptides (whenever available). Here, we should note that despite superior precision of the MRM technique, it might be misleading to use the term “absolute” quantification because the latter depends on the degree of protein digestion. Irrespective of the latter, if protein quantification is performed using the same peptide(s) and similar conditions to achieve maximum protein digestion, such data can be used to derive scaling factors for IVIVE from cell lines to human tissues. However, this is only possible if the ratio of intracellular to plasma membrane expression of transporter is similar in tissues and cells.

Sample Preparation for Analysis

Sample cleanup is important for reliable MS signal. Protein or peptides can be enriched by using ultrafiltration, solid phase extraction, or chloroform/methanol extraction described elsewhere [71–73]. If enrichment methods are used, recovery should be evaluated. To do so, an endogenous marker (Na⁺K⁺ATPase) or a spiked labeled protein can be used as an enrichment marker. Labeled peptide as an internal standard can be used to address variability in post-digestion steps (e.g., matrix effect). Nonspecific binding to sample

container and LC-MS/MS tubing should also be taken into consideration. It is generally preferred to use glass vials or LoBind plastic tubes to store samples and standards.

LC-MS/MS Parameter Optimization

LC separation is critical in MRM proteomics because of the complexity of tissue/cell protein sample. While nano-LC-MS/MS can be used for transporter quantification [14], our discussion is focused on conventional LC-MS/MS. A typical LC run is 15–60 min long in a reverse phase UHPLC column (e.g., 100×2.1 mm, particle size <2 μ). The initial mobile phase conditions are low organic solvents (e.g., 0–5% acetonitrile and 100–95% of water with 0.1% formic acid) for the first 3–5 min. This initial step washes polar interferences from the sample. During this step, the flow can be diverted to the waste to avoid MS source contamination and matrix effect. Mobile phase compatibility with the sample should be taken into consideration particularly if the analyte peptides are very polar. The organic phase can be gradually increased, and the slope of the gradient can be adjusted based on the hydrophobicity of the surrogate peptides. The predicted MRM parameters can be systematically tuned using the peptide standard similar to small molecules. Parent and product ions, collision energy, voltage, resolution, and dwell time are the key parameters that should be tuned using the standard.

Method Validation

US FDA bioanalytical method validation guidance can be adopted for LC-MS/MS protein quantification method (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf>). The validation should include demonstration of parameters such as selectivity, accuracy, precision,

Table IV. MRM-Based Protein Quantification Assays [67–70, 80–82]

Method	Principle	Advantages	Limitations
Peptide MRM	Protein is digested and surrogate peptide response is quantified in MRM mode. Synthetic peptide is used as calibration standard. Corresponding stable isotope labeled peptide is used as an internal standard	Simple, sensitive, high-throughput, reproducible, multiplexing capability	Incomplete digestion affects absolute quantification
SISCAPA	Combines the precision of peptide MRM with anti-peptide antibody enrichment to improve MRM sensitivity	Sensitivity, no matrix interference	Antibody production is expensive; incomplete digestion affects absolute quantification
QconCAT	Both light and heavy QconCAT concatamer construct are designed, expressed, and purified as standards. A known amount of the expressed, isotopically labeled QconCAT proteins are spiked into samples	Allows assessment of digestion efficiency and completeness by including an internal protein standard of a concatamer	Labor-intensive procedure; differences in the digestion of the standard and analyte proteins
Protein standard accurate quantification (PSAQ)	Purified intact protein standards (both light and heavy) are produced. A known amount of the purified proteins is spiked into samples	Allows assessment of digestion efficiency and completeness	Labor-intensive and expensive procedure; purification of transmembrane proteins is challenging
Conventional SILAC proteomics	Metabolic labelling using heavy amino acids is done in cell culture or <i>in vivo</i> for relative proteomic quantitation	Minimum bias due to handling errors in relative quantification	Generally suitable only for relative quantification

MRM multiple reaction monitoring, SISCAPA stable isotope standard capture with anti-peptide antibodies, SILAC stable isotope labeling with amino acids in cell culture

recovery, calibration curve, sensitivity, reproducibility, and stability. While MRM methods are generally selective, it is not surprising to observe multiple peaks due to similar parent-product pairs from tryptic digest. Therefore, it is important that a longer LC method and multiple transitions are used to confirm the target peptide signal. Nonavailability of a blank matrix is an important concern in the bioanalysis of endogenous proteins, including transporters. The potential interferences in a trypsin digest not only include other peptides but also include endogenous small molecules and xenobiotics. Therefore, fragments from each parent ion in the actual processed tissue samples (typically from multiple donors, *e.g.*, $n=6$) should be used to demonstrate the selectivity. Accuracy can be demonstrated by spiking standard peptide into the processed tissue samples. Within-run and between-run precision should be performed using processed tissue sample with and without adding synthetic standard. Considering the higher variability in protein quantification as compared to small molecules, acceptance criteria of $\pm 25\%$ for accuracy and precision can be used. Sensitivity can be demonstrated by measuring lowest analyte concentration (in sample buffer) that can be measured with acceptable accuracy and precision. It should be noted that the latter is not the actual lower limit of quantification (LLOQ) as the sample matrix does not represent the tissue sample. Therefore, if possible, a sample can be identified that lacks analyte protein (*e.g.*, due to genetic polymorphism or mutation), and LLOQ standard can be spiked into such blank sample to measure the real sensitivity of the method. Effect of freeze and thaw cycles, longer storage, and bench-top exposure on peptide stability should be established. The effect of the above conditions on protein quality can be demonstrated because the quality of protein can alter trypsin digestion. Processed sample stability in auto-sampler should be studied.

Sample Analysis

The ratio of analyte and internal standard response is calculated and plotted against concentration for a given peptide. The calibration equation is then used to calculate concentration in the actual sample. Assessment of peak quality is important as multiple interfering peaks can be observed in the biological sample. One way to assure quality of peaks is by calculating correlation between peptides (or fragments). Vendor-specific software are available for high-throughput post-acquisition data analysis. Tools such as Skyline, AuDIT, and SRM Collider are also helpful in the analysis of large sample size.

While the above discussion mainly focuses on the peptide MRM proteomics, the proposed approach can be extended to other MRM protein assays summarized in Table IV [67–70, 74–79].

P-gp Protein Quantification in Human Liver Tissue, Hepatocytes, and Cell Lines

We selected two surrogate peptides for P-gp quantification, NTTGALTTR and IATEAIENFR, based on the above-discussed approaches (Table III). P-gp protein expression was quantified in the School of Pharmacy, University of Washington human liver bank, hepatocytes, and LLC PKII cells (Fig. 6) using a validated method [11]. The cell line expressed ~120-fold higher P-gp protein than that expressed in the hepatocytes or human liver. NTTGALTTR was used for this quantification because it resulted in ~1.4-fold higher yield

Fig. 6. P-gp protein expression in human liver tissue ($n=64$), human hepatocytes [11], and human P-gp-expressing LLC PKII cells ($n=3$; passage number—approximately 45). Horizontal line, median; plus sign, mean; boxes, 25th–75th percentiles; whiskers, non-outlier range

than IATEAIENFR perhaps due to the better trypsin digestion efficiency of the former.

CONCLUSION

MRM proteomics has emerged as the gold standard for transporter protein quantification [14, 16, 27, 31]. For successful application of this technique, selection of the surrogate peptide(s) is a critical step in assay development. Selection of the peptide(s) can be facilitated using bioinformatic tools. A critical assumption in peptide MRM proteomics is that the protein digestion is considered complete. Although the term absolute quantification is commonly used in the field, it can be used only when complete digestion efficiency is assured. Such assurance can be obtained only when the pure protein standard is available. However, if the same peptide and similar digestion conditions are used for transporter quantification in cells and tissues, such relative quantification can still be used for IVIVE. Such an IVIVE approach is not limited to a particular organ (*e.g.*, liver), but it can be used to extrapolate drug disposition to any tissue. Taken together, the emergence of MRM transporter proteomic approach is an important milestone in systems pharmacology to better predict drug disposition and drug response/toxicity.

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