# Chapter 5
# Applications of Targeted Proteomics in ADME for IVIVE

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**Abstract**  Membrane transporters act as physiological "gatekeepers" that regulate the distribution of endogenous and exogenous compounds. It is therefore imperative that drug discovery/development research considers the function and expression of drug transporters, which can dictate drug concentration to pharmacological targets or may be the drug target themselves. Variation in transporter expression across species and in vitro models is recognized as a major complicating factor encountered during in vitro–in vivo extrapolations that can limit a model's predictive power. This is particularly problematic in scenarios such as biliary secretion that are dependent upon in vitro and preclinical data due to lack of clinical bile samples. Consequently, quantification of drug transport proteins becomes a fundamental element in establishing important correlations for pharmacokinetic predictions that are of significant interest during drug discovery. In this chapter we provide an overview of methodologies relevant to protein quantification and their important limitations, followed by a review of recent studies in which mass spectrometry-based targeted quantifications of drug transporters are applied in predictions of transporter-mediated drug clearance.

## Abbreviations

| | |
|---|---|
| ABC | ATP-binding cassette |
| ADME | Absorption, distribution, metabolism, elimination |
| AQUA | Absolute quantification |
| BCRP | Breast cancer resistance protein (human) |
| Bcrp | Breast cancer resistance protein (other species than human) |

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| BLAST | Basic local alignment search tool |
| BSEP | Bile salt export pump (human) |
| Bsep | Bile salt export pump (other species than human) |
| CHAPS | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| CYP | Cytochrome P450 |
| ELISA | Enzyme-linked immunosorbent assays |
| ESI-Q-TOF | Electrospray ionization quadrupole time of flight |
| IS | Internal standard |
| IVIVE | In vitro–in vivo extrapolation |
| LC | Liquid chromatography |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| MDCK | Madin–Darby canine kidney |
| MDR1 | Multidrug resistance protein (P-gp) |
| MRM | Multiple reaction monitoring |
| MRP2 | Multidrug resistance-associated protein 2 (human) |
| Mrp2 | Multidrug resistance-associated protein 2 (other species than human) |
| MS | Mass spectrometry |
| MSD | Membrane-spanning domain |
| NBD | Nucleotide-binding domain |
| OATP | Organic anion-transporting polypeptide |
| P-gp | Multidrug resistance protein (MDR1) |
| PK | Pharmacokinetics |
| PSAQ | Protein standard absolute quantification |
| PTM | Posttranslational modifications |
| RAF | Relative activity factor |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SCH | Sandwich-cultured hepatocyte |
| SDS | Sodium dodecyl sulfate |
| SIL | Stable isotope-labeled |
| SILAC | Stable isotope labeling by amino acids in cell culture |
| SLC | Solute carrier |
| SNP | Single nucleotide polymorphisms |
| TOF | Time of flight |
| WT | Wild type |

## 5.1 Introduction

Absorption, distribution, metabolism, elimination (ADME)-related proteins, such as cytochrome P450 (CYP) enzymes and drug transporters, represent versatile metabolism and transport systems that play a pivotal role in the disposition of xeno-biotics as well as endogenous substrates such as vitamins, peptides, and hormones. The expression of these proteins can be influenced by a number of factors such as disease, genetics, and exposure to inducers. The subsequent impact can include a

change in the total expression of a given family of proteins and can also involve perturbations in the tissue expression of specific isoforms. Since ADME-related proteins regulate the disposition of drugs, it becomes imperative to determine protein expression in various organs under various pathophysiological conditions in order to enable the prediction of disposition and adverse interactions with co-administered drugs. In this regard, the prediction of human pharmacokinetics (PK) remains an active and challenging area in drug discovery and development and consequently in vitro and in vivo preclinical ADME models have been investigated for their capability to predict human PK parameters. In such models, the Michaelis–Menten equation is one of the best-known to describe enzyme kinetics and it has been applied for in vitro–in vivo extrapolations (IVIVE) related to enzyme catalyzed clearance (Iwatsubo et al. 1997; Shimada et al. 1994), wherein $V_{max}$ and $K_m$ are the two determinants of clearance. While $K_m$ is a unique parameter of a designated substrate for a given protein, $V_{max}$ is derived from the catalytic rate constant and protein expression level in a given system. Accordingly, the application of a known amount of CYP enzyme, in an in vitro incubation study, is a key component for successful IVIVE when CYP-mediated metabolism is to be predicted (Obach 2001). While IVIVE is fairly well-established for the prediction of CYP clearance, similar approaches applied to predict drug transporter-mediated clearance are not straightforward. One of the main factors complicating prediction of transporter-mediated clearance is the large difference encountered in the clearance rates/routes across species and in vitro models (Lai 2009). Thus, a more comprehensive understanding of the mechanisms underlying the interspecies differences encountered in these models could increase confidence in human PK predictions. For instance, multiple in vitro systems, including transporter-over-expressed membrane vesicles and immortalized cell lines, have been widely used to determine transporter-involved drug disposition, but obtaining protein expression levels in these models is a recognized obstacle for translating the activity to in vivo. IVIVE is also not well-established from preclinical species to human, particularly in elimination routes such as biliary secretion that involve transporter-mediated clearance mechanisms yet to be fully characterized. This is further complicated by biliary secretion models that are highly dependent upon in vitro and preclinical data due to lack of clinical bile samples (Ghibellini et al. 2004). When coupled with kinetic parameter determinations, the quantification of drug transporters can facilitate scaling for extrapolation from preclinical models to human to further promote drug discovery and development. However, the hydrophobic nature of integral membrane protein domains, low expression levels, disconnects between protein and surrogate measures, and lack of reliable protein standards collectively underlie challenges with respect to protein quantification methods.

Among the protein quantification methods, mass spectrometry (MS)-based targeted quantification can readily provide the relative amounts of transporters expressed in different systems that are necessary for scaling transporter-mediated clearance from in vitro to in vivo or from preclinical to clinical. Quantification of transporter proteins may increase the understanding of the variability of transporter-mediated clearance across species or specific populations, although differential

binding affinities must still be taken into account by other means. In this chapter we highlight some of the unique advantages of MS-based protein quantification and discuss important experimental methods, applications, and limitations with an emphasis on the recent targeted quantifications reported for transporters of particular relevance to drug disposition.

## 5.2  Protein Quantification Approaches

Attempts to quantify proteins of interest have incorporated a variety of approaches. Gene quantification methods including Northern blot, quantitative RT-PCR, and DNA array analyses have all been applied as measurements of protein expression (Vander Borght et al. 2006; Sun et al. 2002; Tanaka et al. 2005; Figge et al. 2004; Bleasby et al. 2006). The outcomes have indeed captured organ gene expression patterns and transcriptional regulation of genes via nuclear receptors (Teng and Piquette-Miller 2005; Nishimura and Naito 2005). Despite this success, these methods are ultimately a surrogate assessment of protein expression levels, which can be complicated by differences in stabilities and expression rates, as well as lack of information regarding posttranslation modifications (Haynes et al. 1998). Noteworthy examples of such oppositions between mRNA transcript and protein levels have been reported (Bleasby et al. 2006; Belinsky et al. 2005; Diao et al. 2010; Haimeur et al. 2004). Immunoblotting techniques, such as western blot analysis or enzyme-linked immunosorbent assays (ELISA), have also been popular approaches to directly characterize protein expression. While these assays can be sensitive and robust, the applications can be limited by lack of pure protein standards or the cross-reactivity and availability of suitable antibodies (Michaud et al. 2003).

Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS)-based quantitative proteomics has been increasingly employed for targeted protein quantification within the enzyme and transporter disciplines (Kamiie et al. 2008; Li et al. 2008, 2009a, b, c, 2010; Sakamoto et al. 2011; Shawahna et al. 2011; Uchida et al. 2011a, b; Zhang et al. 2011; Kawakami et al. 2011; Seibert et al. 2009). Generally, intact proteins are digested into peptides with subsequent separation and detection by LC-MS/MS to measure peptide ion intensities as a surrogate measurement of protein levels. In contrast to surrogate transcripts, surrogate peptides are derived directly from proteins and hence are decoupled from posttranslation disconnects. These advantages render MS-based quantification a useful tool to help elucidate remaining gaps not addressed by previous approaches. Furthermore, unlike immunochemical methods, which can be limited by restricted access to an appropriate antibody, MS-based quantifications use peptides unique to the protein of interest. Such unique peptides can be readily obtained from commercial sources and serve as surrogate standards for the protein of interest and consequently overcome the absence of protein standards. Despite these advantages, significant analytical challenges in MS-based quantifications remain as certain ADME proteins, in

particular drug transporters, are present at relatively low expression levels and encompass multiple hydrophobic domains. Improvements in sample preparation methods (including a combination of immunoaffinity protein enrichment as well as anti-peptide antibodies (Anderson et al. 2009)), MS instrumentation, and ultra performance LC have emerged as a means to overcome low expression levels. It is however important to note that structural differences among proteins can result in varying levels of proteolysis. Since proteolysis has a significant impact on accuracy at the protein level, it may thereby limit *absolute protein* applications, especially in the absence of protein standards as further discussed in Sect. 5.4.2.

## 5.3  Targeted Proteomics

### 5.3.1  Overview of Mass Spectrometry-Based Targeted Protein Quantification

Quantitative targeted proteomics represents a subset of proteomic analyses in which highly sensitive and reproducible multiple reaction monitoring (MRM) MS methodologies are commonly used to detect specific peptides in a complex mixture (Yocum and Chinnaiyan 2009; Elschenbroich and Kislinger 2011). These peptides are generated through the digestion of intact proteins with subsequent separation and detection by LC-MS/MS (Fig. 5.1). The detection of intact proteins by MS-based analyses, beyond the scope of this chapter, is also used to study membrane proteins (Whitelegge et al. 2006). However, the drug transporter quantifications reviewed here all utilize a *bottom-up* approach in which peptides produced from the digestion of proteins are used as a surrogate measure of the original protein. While the low abundance of a given transporter protein may still underlie detection sensitivity challenges, with respect to LC column capacity, the targeted nature of these analysis
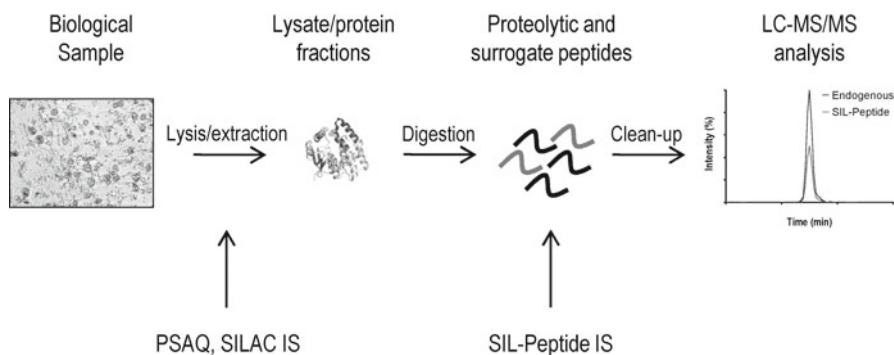


**Fig. 5.1** Overview of experimental workflow and different methods for internal standard introduction

provides the means to detect specific peptides present in a complex mixture using routine triple quadrupole instrumentation (Chalkley 2010).

The first step of a targeted quantification consists of selecting candidate peptides that can serve as a surrogate measure for the protein of interest. This can be accomplished through the use of in silico predictive tools (Kamiie et al. 2008; Zhang et al. 2011), which can be complemented with experimental tools discussed below (Li et al. 2008). Although there are several proteases available (as well as chemical cleavage methods), trypsin can be an ideal initial choice as it often produces fragments amenable to detection by MS in terms of size and amino acid composition. As a general guide, the $m/z$ value of a doubly charged precursor will need to lie within the detectable range of the mass spectrometer being used, although triply charged precursors may be detected in some cases. The vast majority of the theoretical peptides produced by an in silico digestion (http://prospector.ucsf.edu/prospector/mshome.htm) can be excluded on the basis of size, stability, and sequence identity. Resources such as the PeptideAtlas can also assist in identifying proteotypic peptides (Deutsch et al. 2008). In terms of sequence, peptides containing cysteine/methionine residues or *N*-terminal glutamine residues, with the potential for chemical modifications and spontaneous cyclizations, respectively, should be excluded during selection. In the interest of optimizing digestion efficiency, the adjacent sequence can also be screened to avoid any continuous segments of arginine and lysine, which could potentially hinder trypsin digestion. Given the complexity of sample digests, a surrogate peptide with a sequence unique to the target protein is necessary. Among the unique peptides that are identified using protein BLAST/homology searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome; http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology) against a pertinent species database, those not known to contain post-translational modifications (PTM) or mutations derived from single nucleotide polymorphisms (SNP) make optimal candidates unless a particular PTM or SNP is purposely being targeted. It is also important to keep in mind that not all modifications have undoubtedly been reported. Although the detection of peptides derived from the transmembrane domain can be of interest (Eichacker et al. 2004), an ideal target peptide will be located in the more exposed and soluble domains of the protein. In cases where explicit information regarding soluble domains is not available, the consensus of common topology predictions algorithms (Punta et al. 2007; Nam et al. 2009) can assist in discriminating between soluble and transmembrane segments during candidate peptide selection.

Peptide stability is the secondary criteria for the peptides that are filtered through above criteria. The selection process can generally be complemented by target peptide verification in sample digests analyzed by a high-resolution instrument such as a quadrupole time of flight (TOF) MS or linear ion-trap (Chalkley 2010; Prakash et al. 2009). The high-resolution instrumentation can take advantage of high mass accuracy that is needed to confidently identify a peptide in the absence of standards and known fragmentation patterns. The peptide with the best apparent detection sensitivity can then be selected as the quantification probe and the corresponding synthetic peptides can be used for analytical optimization of the

precursor-to-product transitions. Typical quantifications are conducted on a triple quadrupole mass spectrometer by utilizing MRM analyses, which offer two stages of mass filtering for each targeted transition (Elschenbroich and Kislinger 2011; Lange et al. 2008). Although these are directed toward unique peptides, due to the resolution of typical instrumentation, adequate resolution at the LC level is also important to separate interfering factors. Since matrix complexity represents such a significant obstacle for quantification of endogenous material in which a true blank is not available, in addition to LC separation, multichannel MRM analyses should be conducted to verify results with at least three transitions per peptide. The stable isotope-labeled (SIL) peptides that will serve as the co-eluting internal standard (IS) are also monitored in this manner. These isotopes can be incorporated into MS-based quantifications at various stages of the workflow as further outlined in Sect. 5.3.2.

Once validated, the resulting surrogate peptide and MRM method can be used for the digestion and targeted quantification of samples derived from whole cell lysates, membrane extractions, or other relevant preparations that are generally reduced and alkylated to help prevent the reformation of higher-order structure and thereby facilitate protease access. Due in part to the variable composition of integral membrane proteins that reside in a given location, different membrane compartments have their own characteristic properties. The concentration of active transporters present at the cell surface plasma membrane is presumably the most relevant with respect to predictions; however, it should be noted that total membrane protein fractions may include protein beyond the functional transporter on the cell surface. The plasma membrane can be difficult to completely isolate but procedures such as cell surface biotinylation can be incorporated into the process to enrich this fraction (Elschenbroich et al. 2010; Qiu and Wang 2008).

Although membrane protein analyses have also included techniques involving gel-based protein resolution (Rabilloud 2009; Wu and Yates 2003), many recent drug transporter quantifications focused upon herein exploit the targeted detection of hydrophilic peptides produced upon the in-solution digestion of complex samples prior to LC-MS/MS. But as reported with gel-based techniques, hydrophobic protein segments resist exposure to aqueous environments, which can lead to aggregation and sample loss. Therefore, solubilization and denaturation limitations still remain with respect to facilitating protease access and digestion efficiency, the implications of which are discussed in Sect. 5.4.2. When reliable standards are not readily available, which is often the case for the integral membrane drug transporters of interest, it is important to appreciate the effect of each portion of the experimental design as primary and secondary structural differences among proteins can amount to different levels of proteolysis. Standard protein solubilization and denaturation tactics involve the use of chaotropes (urea and guanidine), detergents (triton, SDS, CHAPS, and more recently, MS-compatible detergents such as RapiGest™ SF surfactant (Waters, Milford, MA)), bile acids (deoxycholate), organic solvents, and organic acids (Speers and Wu 2007; Helenius et al. 1979; Lin et al. 2008; Proc et al. 2010). Since these reagents can interfere with proteolysis, their initial concentrations are reduced to a compatible level during the digestion. Evaluations including an assessment of the time course for a digestion are valuable to ensure the

digestion is complete with respect to what is achievable under a given set of conditions. After the digestion is quenched (this is often the point of IS introduction), various peptide enrichment and separation strategies can be used, which at the very least include LC separation prior to MS detection. As alluded to above, several IS options exist. Regardless of which option is incorporated, the IS will be distinguishable by mass spectrometry and can be used to normalize the results (the extent of normalization depends on what type of IS is used), while unlabeled synthetic peptides can be used to construct a standard curve to determine quantification values for each sample.

## 5.3.2    Internal Standard Strategies

Different *bottom-up* techniques related to the isotope dilution concept have been described for MS-based protein quantification (Gerber et al. 2003; Barr et al. 1996; Zhang et al. 2010; Brun et al. 2009), wherein the identity and the timing of IS introduction offer different advantages (Fig. 5.1). Among the most commonly employed are SIL methods where a SIL synthetic peptide with an identical sequence to the proteotypic peptide is used as the IS during analyte peak area normalization. Other IS approaches have been described, some of which include chemical derivatization, metabolic incorporation of heavy-labeled amino acids, and protein standard absolute quantification (PSAQ) (Elliott et al. 2009). Because peptides can only serve as a surrogate of protein levels, quantitative studies with more precise and accurate methodologies represent advancements with respect to errors derived from variability in native membrane protein extraction, denaturation, and digestion. The stable isotope labeling by amino acids in cell culture (SILAC) approach is one such method which offers a metabolic-labeling strategy for label incorporation during culture (Ong et al. 2002). In this situation, both heavy and light proteins can be combined at the beginning of the experiment and digested together, after which the heavy isotope-labeled peptide serves as the co-eluting IS. Thus, SILAC is recognized for the ability to normalize for losses/enrichments derived from any portion of the workflow and increase the precision across different measurements (Ong et al. 2002; Geiger et al. 2011; Hanke et al. 2008; Harsha et al. 2008; Ong and Mann 2006, 2007). PSAQ methods are similar in concept to SILAC in that the IS is derived from a labeled protein; however, in the case of PSAQ (or "absolute SILAC"), the concentration of the IS is known, usually by quantitative amino acid analysis of purified material, thereby allowing for better accuracy at the protein level (Hanke et al. 2008; Brun et al. 2007; Ishihama et al. 2005; Lebert et al. 2011). In contrast, due to different biochemical properties, SIL peptides must be added either during or post-digestion to serve as the IS for the remainder of the experiment. Consequently, this is the least accurate approach at the *protein* level, as variability prior to digestion cannot be accounted for. Nonetheless, SIL methods such as the absolute quantification (AQUA) method (Gerber et al. 2003) are usually the most feasible and rapid methods that may be perfectly suited to extract scaling factors from relative

quantification studies. Indeed, SIL peptides are among the most common IS techniques used to evaluate the expression of drug transporters thus far (Kamiie et al. 2008; Li et al. 2008, 2009a, b, c, 2010; Sakamoto et al. 2011; Shawahna et al. 2011; Uchida et al. 2011a, b; Zhang et al. 2011).

## 5.4 Applications

Given that the expression of ADME-related proteins is known to vary with species as well as developmental and pathological characteristics (age, sex, disease state) (Meier et al. 2006; Renton 2001, 2004), it is necessary to monitor fluctuations in protein expression in order to understand the in vivo disposition of many drugs and improve the predictability of in vitro models (Li et al. 2010). In 1997, Crespi and Penman developed the relative activity factor (RAF) method to characterize specific CYP isoform contributions (Crespi and Penman 1997), which has also been implemented for the characterization of transporter-mediated drug disposition (Kitamura et al. 2008; Maeda et al. 2010). While the RAF approach provides a useful tool to characterize the contribution of enzyme/transporter isoforms, routinely observed overlaps in substrate specificities still underlie major hurdles with respect to identifying "clean" reference substrates. In turn, the quantification of ADME proteins becomes a key factor in establishing the correlations between in vitro models, preclinical species, and human, which could ultimately accelerate the early stages of drug discovery. The potential applications of targeted proteomics can span several stages of drug discovery and development (recently reviewed by Ohtsuki et al. 2011) and encompass other areas of research such as protein drugs and protein drug targets, biomarker validation, and PTM investigations. In addition to the obvious applications, other intriguing avenues within the scope of MS-based proteomics have emerged in protein topology and interaction research (Wu and Yates 2003; Wu et al. 2003). This additional research may provide underlying mechanistic information that can increase our understanding of transporters, which can ultimately be applied to advance predictions. However, to date, the vast majority of quantification studies within the drug transporter discipline have emphasized the targeted determination of transporter levels in species, tissues, and in vitro models in the interest of understanding expression differences which are among the confounding factors encountered in IVIVE.

## 5.4.1 *LC-MS/MS-Based Quantification of Drug Transporters*

The application of targeted LC-MS/MS-based proteomics has facilitated the detection and quantification of numerous proteins with relevance to drug disposition. Our focus here is devoted to transporter proteins; however, other notable ADME-related proteins such as the CYP enzymes are an active area of targeted quantification

analyses (Kawakami et al. 2011; Seibert et al. 2009; Langenfeld et al. 2008). Although the challenges derived from low abundance and a proteolytically resistant nature presumably contributed to an initial lag in integral membrane protein quantifications as compared with soluble protein studies, SIL peptide-based methods have been increasingly incorporated to evaluate drug transporters. Two popular approaches have emerged, the first of which involves the characterization of individual transporter proteins that is often coupled with method optimization. The second approach utilizes a higher-throughput strategy that can rapidly quantify numerous proteins, although individual secondary characterizations may eventually be required for certain applications, as this approach does not include time-consuming sample preparation optimizations that may be necessary with respect to detection sensitivity for some proteins.

### 5.4.1.1   Individual Characterizations

Over the course of a series of studies by (Li et al. 2008, 2009a, b, c, 2010), SIL peptides were implemented to examine individual ATP-binding cassette (ABC) transporters in order to characterize the different cell models that are routinely used to assess transporter involvement with potential drug candidates. These analyses also aimed to determine the relative transporter expression across species and tissues. In the earliest of these studies (Li et al. 2008), multidrug resistance-associated protein 2 (MRP2) immunoprecipitation-enriched samples were initially digested and detected with high resolution using nanospray ESI-Q-TOF analyses in order to select the proteotypic peptide with the best apparent detection sensitivity. The resulting peptide was used to quantify MRP2-transfected Madin–Darby canine kidney (MDCK) cells as well as the endogenous canine Mrp2, which was notably undetected by immunoblotting. Since the proteotypic peptide identified was able to selectively recognize the MRP2/Mrp2 protein in multiple species (Fig. 5.2), they subsequently utilized this method to examine the hepatobiliary transporter in liver tissues and hepatocytes from human, dog, rat, and monkey (Li et al. 2009c). In total, the amount of MRP2/Mrp2 in livers was found to rank rat≫monkey>dog≈human, wherein Mrp2 was approximately tenfold higher in rat than MRP2 in human. Interestingly, a 40 % loss of Mrp2 was also observed in cryopreserved hepatocytes, which provides insight regarding the potential differences between in vivo circumstances and important models of transport even within a single species. Li et al. 2009b also extended their quantifications to include measurements of bile salt export pump (BSEP) and breast cancer resistance protein (BCRP) in livers and hepatocytes. The proteotypic peptides identified in this study determined the amount of BCRP/Bcrp and BSEP/Bsep ranked dog>rat>monkey≈human and rat≈monkey>dog≈human, respectively.

To the extent that interspecies differences can limit a model's predictive power, these results represent key findings with respect to translational gaps in IVIVE for substrates undergoing transporter-mediated elimination. The sandwich-cultured hepatocyte (SCH) model, which provides the proper orientation and localization of

Fig. 5.2 Schematic representation of membrane topology of BCRP/Bcrp and BSEP/Bsep and protein alignment across species. The proteotypic peptide for BCRP/Bcrp was selected from the intracellular *N*-terminal (**a**) or intracellular nucleotide-binding domain for BSEP/Bsep. (**b**) The stable isotope-labeled (SIL) internal standard was indicated with a single residue substitution of $[^{13}C_6, ^{15}N_1]$ Leu. Genebank number: human BCRP (NP_004818); rat Bcrp (NP_852046); dog Bcrp (NP_001041486); monkey Bcrp (AAX56948); human BSEP (NP_003733); rat Bsep (NP_113948) (with permission from Li et al. 2009b)
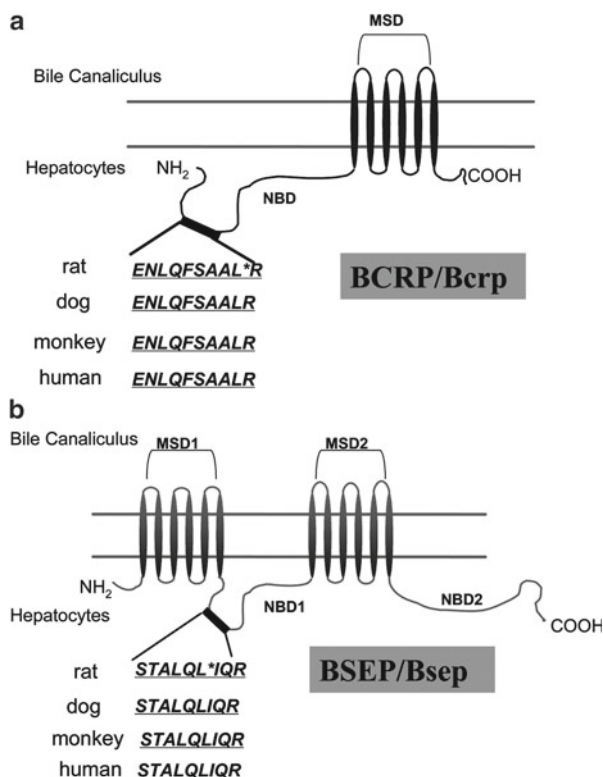
transporters along with the development of intact bile canaliculi, has become an important tool for investigating vectorial transport that cannot be assessed in hepatocyte models lacking cell polarity. However, the absence of quantitative information regarding transporter expression in SCH (which can also fluctuate during cell culture periods) complicates IVIVE. Collectively, the methods described for MRP2, BSEP, and BCRP were used to characterize SCH to compare levels across the cell culture periods and with in vivo findings (Li et al. 2009a). A number of alterations were observed and the results nicely illustrated that the amounts of MRP2/Mrp2 and BCRP/Bcrp could be correlated with the intrinsic clearance of test compounds in SCH (Fig. 5.3). Furthermore, by integrating a scaling factor to reflect the mass recovery of hepatobiliary transporters between in vitro SCH and in vivo and the respective contribution when multiple transporters are involved, the prediction of biliary secretion in rats was improved (Li et al. 2010). In total, the applications targeting the efflux transporters increase confidence for IVIVE of human biliary clearance and provide insight regarding the hepatocyte lot- and culture time-dependent expression of transporters that can underlie inter-experimental variation. The hepatic uptake transporters, which can perform the rate limiting step of clearance for specific compounds (Giacomini et al. 2010), also need to be addressed. As of this writing, to our knowledge efflux transporters have been the focus of
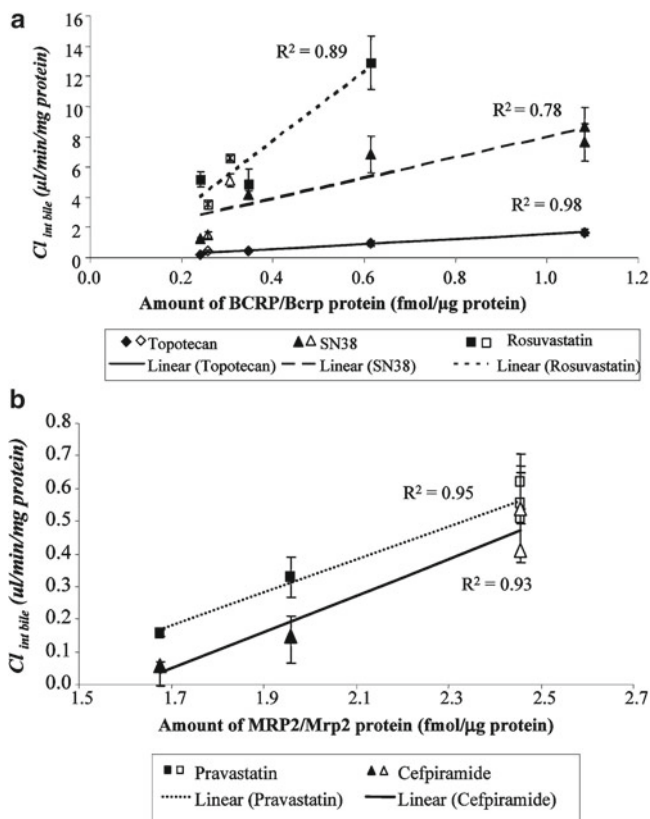
**Fig. 5.3** Correlation of protein amount and intrinsic biliary secretion in SC hepatocytes. (**a**) The intrinsic biliary secretion of SN38, topotecan, and rosuvastatin was plotted against the protein level of BCRP/Bcrp in difference lots of human or rat hepatocytes. *Solid symbols* represent freshly isolated human or rat hepatocytes, while the *open symbols* represent cryopreserved human hepatocytes. (**b**) The intrinsic biliary clearance of cefpriamide and pravastatin was plotted against the amount of MRP2/Mrp2 protein in the corresponding lot of SC hepatocytes. *Solid symbols* represent cryopreserved human hepatocytes, while *open symbols* represent fresh rat hepatocytes (with permission from Li et al. 2009a)

published studies correlating LC-MS/MS protein quantification with functional activity; however, organic anion-transporting polypeptides (OATPs) have also been examined by targeted protein quantification (Sakamoto et al. 2011; Uchida et al. 2011a; Niessen et al. 2009; Balogh et al. 2012) and will presumably be incorporated for IVIVE in future studies.

P-glycoprotein/multidrug resistance protein 1 (P-gp/MDR1) is also among the efflux transporters characterized by targeted protein quantification. Using a similar methodological approach as described above, Zhang et al. (2011) developed a LC-MS/MS method applied to quantify human P-gp in gene-transfected MDCK cells. The selected peptide was also able to provide a comparison with the

endogenous canine P-gp present in MDCK cell lines. In addition to the anticipated uses of this quantification method to enable more accurate predictions of in vivo drug disposition, the MS-based quantification of canine P-gp was recently used to assist in characterizing a new cell line, MDCKII-LE (low efflux), that was found to exhibit fivefold lower P-gp levels than MDCKII-WT (Di et al. 2011). This cell line is expected to offer an advantage over existing WT cells currently used for passive permeability measurements by diminishing the interference derived from endogenous canine P-gp activity. Most recently, P-gp was also examined by targeted quantification in a study aimed at assessing the brain distribution of P-gp substrates (Uchida et al. 2011b). P-gp-mediated efflux can ultimately affect brain distribution and hence pharmacological action. Accordingly, brain-to-plasma ratios can be used as an indicator in this regard during drug identification/selection processes. To this end, the level of P-gp quantified in both transfected cells and in mouse brain capillaries was integrated with in vitro transport activity (derived from efflux rates) and drug unbound fraction in order to reconstruct brain-to-plasma concentration ratios ($K_p$), which were found to provide a reasonable estimate (within threefold) of in vivo values for nine of the 11 compounds tested. As the level of transporter will vary from in vitro systems to brain endothelial cells, the results of this study demonstrate the utility of protein quantification information to further support IVIVE for prediction of drug penetration into the brain, which can then assist in identifying effective central nervous system drugs.

### 5.4.1.2 High-Throughput Characterizations

A series of publications reporting the quantification of an extensive number of membrane proteins have utilized a single set of sample preparation conditions combined with multiplexed selected reaction monitoring to conduct higher-throughput quantifications on brain, liver, kidney, and platelet samples (Kamiie et al. 2008; Sakamoto et al. 2011; Shawahna et al. 2011; Uchida et al. 2011a; Ito et al. 2011; Niessen et al. 2010), the precision and accuracy of which (at least at the peptide level) has been demonstrated to be reliable for various drug transporters and drug metabolizing enzymes (Sakamoto et al. 2011). Among the major studies, a paper by Kamiie et al. (2008) combined in silico peptide selections with the simultaneous analysis of 36 membrane proteins, including ABC and solute carrier (SLC) transport proteins, 26 of which were reported to be adequately detected/quantified in at least one of the tissue types analyzed (mouse brain capillaries, liver membranes, renal cortex membranes, and renal medulla membranes). As noted by the authors, since a major bottleneck encountered in drug discovery and development is the time elapsed between preclinical and clinical studies, this approach could serve as a useful tool for rapidly comparing a given protein's level across biological samples and species to help reduce the delay for advancement. Follow-up studies extended upon the surrogate peptide library to target an extensive number of membrane proteins in human and monkey brain microvessels (Shawahna et al. 2011; Uchida et al. 2011a; Ito et al. 2011). The detailed results of two of these studies were recently

highlighted in a review dedicated to targeted proteomics in ADME research (Ohtsuki et al. 2011). Collectively, these aforementioned studies provide vital information with respect to understanding species differences in transporter expression at the blood–brain barrier. However, it should also be emphasized that a large percentage of the targeted proteins in these studies could not be adequately detected/quantified. Among these presumably reside some transporters that are in fact present at significant levels, but are simply not detectable under the chosen sample preparation conditions. Due to the protein- and denaturant-dependent solubilization and digestion efficiency of these analyses, future method optimizations for individual proteins could result in a drastic increase of surrogate peptide production (Proc et al. 2010; Balogh et al. 2012) and reveal the relative levels of the undetected proteins reported in the species comparisons. The protein- and denaturant-dependent nature of these analyses also underscores a second limitation (expanded upon in Sect. 5.4.2) inherent to both individual and high-throughput characterizations with SIL peptides, which generally complicates reliable comparisons between the levels of *different* proteins.

### 5.4.2   Important Limitations of Targeted Quantifications in the Absences of Reliable Standards

In addition to the determination of scaling factors for individual proteins, there is significant interest in characterizing the contribution of co-localized transporters with overlapping substrate specificity in order to better understand the key determinants of drug disposition and predict drug–drug interactions. Due to the absence of reliable membrane protein standards of known concentration, the studies reviewed above ultimately use the AQUA of a surrogate *peptide* to analyze a *protein*, where the peptide levels, which can be determined with a high level of accuracy (Sakamoto et al. 2011), will reflect the relative amounts of the specific protein in different samples. However, despite the popular usage of phrases such as *absolute/accurate protein quantification*, the true accuracy at the protein level is generally not known and can vary to a significant extent from method to method and from protein to protein (Proc et al. 2010; Brun et al. 2007, 2009; Lebert et al. 2011; Balogh et al. 2012; Klammer and MacCoss 2006). Consequently, caution should be exercised during interpretation when a clear stoichiometrical relationship between the amount of proteolytic peptide detected and corresponding protein is not explicitly established. This issue derives from the fact that primary and secondary structural differences among proteins can amount to varying levels of proteolysis under the sample preparation conditions that are compatible with typical studies using trypsin digestion and LC-MS/MS-based peptide detection. Solubilization and denaturation limitations have been well-recognized within the general proteomic literature, particularly in studies and reviews tackling membrane proteomics that note the optimum conditions will vary across proteins (Rabilloud 2009; Wu and Yates 2003; Speers and Wu 2007; Helenius et al. 1979; Proc et al. 2010; Brun et al. 2007, 2009;

Lebert et al. 2011; Balogh et al. 2012; Klammer and MacCoss 2006; Agarwal et al. 2010; Arsene et al. 2008; Grant and Wu 2007; Zhang et al. 2009). However, the magnitude of impact on conclusions drawn from a subset of transporter-related quantifications has yet to be addressed in this respect. In particular, two of the studies highlighted above, which initially targeted >100 proteins, directly equate peptide quantification to protein levels in order to compare the abundance of different transporters in human brain microvessels (Shawahna et al. 2011; Uchida et al. 2011a). In this application, conclusions such as *BCRP was expressed 1.6-fold more than MDR1* (Shawahna et al. 2011), or *BCRP was the most abundant followed by MDR1* (Uchida et al. 2011a), rely on the assumption that the sample preparation conditions do not have a significant impact on the digestion efficiencies for each protein. Although differences in solubility are assumed to be negligible based on auxiliary studies with MDR1 and BCRP (Kamiie et al. 2008), tests with alternate preparations were not reported. Moreover, research surrounding the effect of multiple digestion schemes for several plasma proteins (Proc et al. 2010; Brun et al. 2007; Klammer and MacCoss 2006), as well as our in-house studies with OATP transporters (Balogh et al. 2012), indicates this assumption cannot be extrapolated to compare numerous proteins since surrogate-based results will generally not reflect the underlying endogenous ratios between different proteins. This is particularly concerning under the typical enzymatic digestions employed in these types of studies, which requires a balance between conditions that enhance proteolysis through denaturation of the target protein and conditions that will not significantly inhibit the proteolytic activity of the digestion enzyme.

Since solubility remains one of the major hurdles in identification and quantification, optimizations tailored to a specific protein often encompass membrane solubilization strategies that typically examine organic solvents, detergents, and chaotropic agents, provided they are compatible with the route of digestion and subsequent MS analysis. The production of target peptide can also be monitored over the course of the digestion in order to ensure samples are taken at an optimal time point. This is an important evaluation to perform during method development; however, it is also important to understand that each method-protein combination can appear to reach completion at its own plateau, although the digestion may not be complete with respect to the maximum that is theoretically possible (Fig. 5.4) (Proc et al. 2010; Balogh et al. 2012). Incomplete digestion can at least be partially addressed by utilizing a combination of proteases and additional cleavage methods (Wu and Yates 2003). For example, the combination of Lys-C and trypsin can be advantageous to increase digestion efficiency. Due to the importance of membrane protein characterization, new tools are continually being explored. For example, lipid-based protein immobilization provides immobilization and digestion of bilayer-embedded native membrane proteins to rapidly probe the solvent-exposed domains in a flow cell format (Sui et al. 2011). But despite extensive research that will presumably be advantageous in the future, the efficient digestion of proteins is still limited under the routine conditions employed to obtain the majority of drug transporter levels reported thus far. Targeted method optimization can of course improve detection and accuracy by increasing the detection signal and closing the gap between the
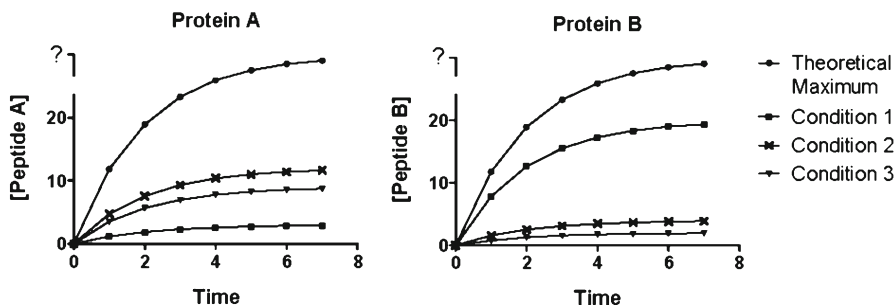
**Fig. 5.4** Digestion profile evaluation. The progress curves derived from a sample that is processed by different solubilization/denaturation methods may appear to reach completion, although the digestion may not be complete with respect to the maximum peptide production expected for the initial protein amount (which is not known in the absence of quality standards). The effect of sample preparation conditions with respect to digestion efficiency can also vary between proteins (Protein A vs. Protein B). The optimization of native protein processing for a targeted protein can significantly improve digestion efficiency and thereby improve detection and accuracy by closing the gap between the theoretical maximum and the observed peptide production under a given set of conditions

observed peptide production and the theoretical maximum. This approach can be valuable for relative quantification studies when sensitivity is a limiting factor, but it is emphasized that an unknown gap in accuracy still remains (Fig. 5.4).

In addition to screening numerous detergents and solvents, alternative attempts have examined digestion efficiency with extended surrogate peptides that contain a few amino acids from the surrounding sequence on either side. However, the proteolytic accessibility of a short peptide is unlikely to reflect that of a large integral membrane protein under protease-compatible conditions and hence can significantly overestimate digestion efficiency. Pursuit of purified protein, which structurally mimics endogenous material throughout the entire experiment, would offer the ideal IS to control for levels of protein loss/enrichment, extraction, denaturation, and digestion, as illustrated by PSAQ approaches (Hanke et al. 2008; Brun et al. 2007; Ishihama et al. 2005; Lebert et al. 2011). In this situation the accuracy would only be limited by the initial assessment of the standard material rather than the preparation-dependent nature of relative analyses. However, obtaining reliable membrane protein standards is not a trivial task. Therefore, to the extent that ratios determined under different sets of sample preparation conditions can underlie completely different conclusions, surrogate peptide-based quantifications are generally limited to relative determinations until further advancements are achieved.

## 5.5 Summary and Perspectives

Changes in ADME protein expression and associated function are realized as important components to understand in vivo drug disposition and improve the predictability of in vitro models. Many protein expression characterization options

exist, each with their own advantages and disadvantages such that there is no recognized "one-size-fits-all" approach that can accomplish every quantification task. For example, in situ immunohistochemical staining offers a unique opportunity to investigate the differential distribution of proteins in different parts of biological tissues. But in contrast to the aforementioned challenges and disconnects highlighted for immunochemical as well as mRNA transcript approaches, targeted protein quantification offers the opportunity to assess protein expression in various biological matrices by way of a sensitive and selective method amenable to high-throughput formats. The information obtained has the potential to fill gaps in understanding for transporter-mediated clearance across species, IVIVE, and populational distributions underlying variations in drug disposition. Reliable membrane protein standards are not yet readily available and caution is still warranted in relative peptide quantifications that may not directly reflect the relative abundance of different proteins. However, significant technological advances and individual method optimizations can indeed improve detection and accuracy by addressing the sample handling, digestion efficiency, and separation challenges that affect quantification in *bottom-up* proteomic workflows. Once established, these methods will undoubtedly continue to make LC-MS/MS-based quantifications more reliable and accessible in the ADME community.

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