ANNUAL REVIEWS

Annual Review of Analytical Chemistry

Mass Spectrometry of Human Transporters

Brahim Achour,[1,*] Zubida M. Al-Majdoub,[1,*] Amin Rostami-Hodjegan,[1,2] and Jill Barber[1]

[1]Centre for Applied Pharmacokinetic Research, School of Health Sciences, University of Manchester, Manchester M13 9PT, United Kingdom; email: jill.barber@manchester.ac.uk

[2]Certara, Princeton, New Jersey 08540, USA

Annu. Rev. Anal. Chem. 2020. 13:223–47

First published as a Review in Advance on February 21, 2020

The *Annual Review of Analytical Chemistry* is online at anchem.annualreviews.org

https://doi.org/10.1146/annurev-anchem-091719-024553

*These authors contributed equally to this work.

Keywords

transporters, mass spectrometry, proteomics, human tissue, drug disposition, PBPK modeling

Abstract

Transporters are key to understanding how an individual will respond to a particular dose of a drug. Two patients with similar systemic concentrations may have quite different local concentrations of a drug at the required site. The transporter profile of any individual depends upon a variety of genetic and environmental factors, including genotype, age, and diet status. Robust models (virtual patients) are therefore required and these models are data hungry. Necessary data include quantitative transporter profiles at the relevant organ. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is currently the most powerful method available for obtaining this information. Challenges include sourcing the tissue, isolating the hydrophobic membrane-embedded transporter proteins, preparing the samples for MS (including proteolytic digestion), choosing appropriate quantification methodology, and optimizing the LC-MS/MS conditions. Great progress has been made with all of these, especially within the last few years, and is discussed here.

INTRODUCTION: WHY MASS SPECTROMETRY OF TRANSPORTERS IS IMPORTANT

The Importance of Transporters in Drug Development

Over the last 10 years, the drug development community has witnessed hot debates considering a dominant role for transporters in permeation of drugs and other xenobiotics across various biological barriers versus passive diffusion (1, 2). While the two sides in the argument have produced evidence and counterevidence for and against passive permeability as a dominant driving force for transmembrane passage of chemical moieties circulating around the body and in and out of tissues (3, 4), many scientists in drug development have tried to position themselves somewhere between the two poles of the argument. For sure, everyone in the drug development space agrees that our knowledge of transporter-mediated permeability has increased several orders of magnitude compared to what we knew just over two decades ago (5). There are many parallels to the field of drug metabolism, where half a century ago only a handful of enzymes were assumed to be responsible for drug metabolism, whereas we are now familiar with many different families of enzymes playing specific roles in metabolism of various drugs and their relative contributions. Similar attempts to identify the relative role of transporters in the disposition of drugs have just started (6).

Drug–drug interactions due to competitive or noncompetitive engagement with metabolizing enzymes as well as genetic polymorphisms in the enzymes played a big role in bringing drug metabolism studies into the limelight within the pharmaceutical industry during the last 30 years. In vitro–in vivo extrapolation (IVIVE) of such observations through modeling has become routine to a degree that regulatory guidelines are issued on such practices [see US Food and Drug Administration (FDA), **https://www.fda.gov/media/108130/download**]. However, the concerns regarding transporter-mediated interactions or the impact of polymorphisms in relation to transporters have been much more limited in comparison (7, 8).

Nevertheless, studying transporters has been encouraged during drug development because of the fact that transporters can be involved in determining local concentrations and hence pharmacodynamics effects. Thus, investigating transporters during drug development links the activities associated with understanding drug disposition with attempts to establish the concentration-effect relationship. It is now recognized that similar kinetics of drugs in the systemic circulation in various individuals might not reflect the kinetics of drugs locally in a given organ. Interindividual variations (resulting from genetic or environmental factors) exist in transporter abundance, leading to disconnects between the drug concentrations in the systemic circulation and at the site of drug activity. Examples of the anomalies that are caused and their interpretations can be found in the seminal work by Rose et al. (9). Hence, as debated previously (10), PBPK (physiologically based pharmacokinetic) models that include transporter information are addressing not just the pharmacokinetic sources of variability but also the pharmacodynamics variations caused by differences in local concentrations at the site of effect and despite establishing similar drug concentrations in the systemic circulation. Interindividual variations of transporters in different patient groups, therefore, can have pharmacodynamic consequences even without major changes in the kinetics of the drug, as seen, for example, in the systemic circulation of drugs acting on the central nervous system in pediatrics when the transporters are not fully developed (11).

All evidence and trend analysis suggest that transporter-related studies during drug development, both through in vitro studies and clinical studies involving biomarker activities and genotyping, will continue to be a dominant feature of drug development practice regardless of the position that we may take on their prominence in relation to overall permeability and in spite of passive diffusion.

Table 1 Important transporters of xenobiotics in human cells. Data from Giacomini et al. (12)

Transporter(s)	Description
ABC transporters responsible for the efflux of xenobiotics from cells	
P-glycoprotein (P-gp, ABCB1, MDR1)	Substrates include lipids, steroids, and peptides Inhibitors include macrolide antibiotics erythromycin and clarithromycin
Breast cancer resistance protein (BCRP, ABCG2)	Substrates include anticancer drugs such as methotrexate and noncancer drugs such as nitrofurantoin Inhibitors include anti-HIV protease inhibitors such as nelfinavir
Multidrug resistance protein 1 (MRP1, ABCC1)	Substrates include many anticancer drugs, including methotrexate Synthesis of inhibitors is an active area of drug discovery
Multidrug resistance protein 2 (MRP2, ABCC2)	Transports a broad range of organic anions, including methotrexate Inhibitors include a number of antiretroviral drugs
Multidrug resistance protein 3 (MRP3, ABCC3)	Endogenous substrates include monovalent bile salts
Bile salt export pump (BSEP, ABCB11)	Eliminates bile salts from hepatocytes
SLC transporters responsible for both efflux and influx of xenobiotics from cells	
Organic anion-transporting polypeptide 1B1 (OATP1B1, SLCO1B1)	Its wide range of substrates includes statins Inhibited by macrolide antibiotics, including erythromycin, and several other drugs
Organic anion-transporting polypeptide 2B1 (OATP2B1, SLCO2B1)	Substrates include statins Cyclosporin is an inhibitor
Organic anion-transporting polypeptide 4A1 (OATP4A1, SLCO4A1)	Substrates include estrogens and benzylpenicillin Synthesis of inhibitors is an active area of drug discovery
Peptide transporter 1 (PEPT1, SLC15A1)	Substrates include di- and tripeptides and peptide-mimicking drugs
Urate transporter 1 (URAT1, SLC22A12)	Regulates the level of urate in the blood and is primarily found in the kidneys
Organic anion transporters 1 and 3 (OAT1, SLC22A6 and OAT3, SLC22A8)	Substrates include penicillins and nonsteroidal anti-inflammatory drugs Inhibitors include rifampicin
Organic anion-transporting polypeptide 3A1 (OATP3A1, SLCO3A1)	Transports bile acids
Organic cation transporter 3 (OCT3, SLC22A3)	Substrates include neurotransmitters, steroids, and hormones

Abbreviations: ABC, ATP-binding cassette; SLC, solute carrier.

Human Transporter Nomenclature

Transporters have evolved to move endogenous materials in and out of cells and organelles, and most transporters are located in the plasma membrane. Many transporters can be hijacked by drugs and used for both influx to and efflux from the cell. In general, ATP-binding cassette (ABC) transporters efflux xenobiotics, including drugs, and may have a role in multidrug resistance. Solute carrier (SLC) family transporters can import or efflux drugs. The pharmacologically most important ABC transporters are P-glycoprotein (known as P-gp, ABCB1, or MDR1), breast cancer resistance protein (BCRP, ABCG2), and multidrug resistance proteins 1 (MRP1, ABCC1) and 2 (MRP2, ABCC2). Among SLC transporters, OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), OATP4A1 (SLCO4A1), and PEPT1 (SLC15A1) are of particular note (see **Table 1**).

Human Samples and In Vitro–In Vivo Extrapolation

In various translational pharmacology applications, extrapolation from in vitro measurements to in vivo outcomes requires reliable characterization of transporters in relevant tissue samples and in vitro systems, which is increasingly possible using mass spectrometry (MS) (13). It is important

to emphasize that the process of extrapolation in the case of drug transporters is different from that of drug-metabolizing enzymes largely because enzymes are concentrated primarily in liver and intestine, whereas transporters are expressed in all tissues where they control processes of drug absorption, distribution, specific targeting, secretion, and elimination, which impact both drug kinetics (i.e., exposure to the drug) and dynamics (i.e., drug effects) (5). In practice, successful prediction of drug transport kinetics from in vitro systems, e.g., the rate of drug transport in plated hepatocytes (14) or Caco-2 monolayers (15), is dependent on accurate IVIVE of activity, which presents several challenges when disposition is characterized by the combined outcome of passive diffusion, active transport, and metabolism (16). The integration of these data and other physiological parameters is routinely carried out using modeling and simulation, as demonstrated in various studies (17–19). The scaling of transporter-mediated disposition is achieved based on the abundance of the specific drug transporters in tissue relative to the in vitro system. For example, drug transport into the central nervous system is characterized in mammalian microvascular endothelial cells (20) and extrapolated to characterize transport across the human blood-brain barrier using relative expression data (21, 22).

Galetin (23) has previously reviewed in vitro tools relevant to liver drug disposition; however, there are currently no clear guidelines for optimal choice and application of in vitro cell systems in translational pharmacology (13, 24). This is important because there are differences in expression in vitro relative to tissue and between in vitro systems (14); for example, reduced expression of uptake transporters OATP1B3 (SLCO1B3) and OATP2B1 (SLCO2B1) and increased expression of OATP1B1 (SLCO1B1) have been reported in sandwich cultured hepatocytes compared to suspended systems (25). Differences in expression were also reported with different cell culture conditions (15, 26). In addition to differences in expression, Harwood et al. (27) pointed out that activity per unit of transporter can vary in vitro from in vivo and therefore this should also be incorporated into the extrapolation process. This was demonstrated recently by the effect of differences in membrane potential in vitro from in vivo, leading to different OCT3 (SLC22A3) activity levels in HEK293 and MDCKII cells relative to renal epithelial cells from human kidney cortex (28).

Transporters in Health and Disease

The importance of transporters in health and disease has been demonstrated. Transporters regulate the passage of both natural substrates and xenobiotics, including drugs and toxins, into and out of cells.

The human genome (**https://www.uniprot.org/**) contains nearly 400 putative SLC and 52 ABC transporter genes. Transporter abundance and activity can be affected by age, ethnicity, exposure to xenobiotics, and disease. Alone of the usual suspects, gender has not so far been found to correlate strongly with transporter function. A comprehensive analysis of the interplay of these factors on the abundance of even one transporter is daunting and, indeed, for all practical purposes, impossible.

A good example of the current state of play is given in **Figure 1**. Here, key transporter abundances in diseased tissue of Crohn's disease and ulcerative colitis patients have been compared with nondiseased tissue. In both cases, the important efflux transporters P-gp (ABCB1, MDR1) and BCRP (ABCG2) are downregulated in disease. Unfortunately, however, these data are not generated at the protein level but are derived from measurements of mRNA encoding the relevant transporters. mRNA concentrations are not ideal surrogates for protein concentrations because mRNA is inherently unstable, and its steady state concentration relates not necessarily to the absolute amount of its corresponding protein but rather to the amount of synthesis required. It has been shown that mRNA concentrations correlate poorly with protein concentrations for proteins involved in drug metabolism and transport (27).

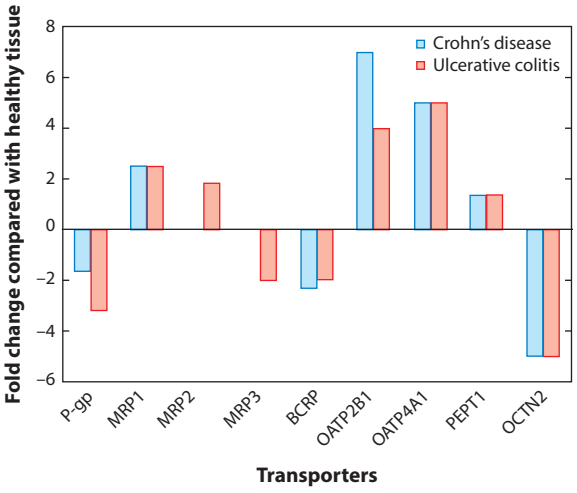

Figure 1

Abundance of mRNA-encoding transporters in inflammatory bowel disease expressed as fold change relative to healthy intestine. P-gp (ABCB1, MDR1), MRP3 (multidrug resistance protein 3, ABCC3), BCRP (breast cancer resistance protein, ABCG2), and OCTN2 (SLC22A5) are downregulated in disease. MRP1 and MRP2 (ABCC1 and ABCC2), OATP2B1 (SLCO2B1), OATP4A1 (SLCO4A1) and PEPT1 (SLC15A1) are upregulated.

There have been attempts to obtain protein concentrations more directly by various means, including, most notably, immunoblotting. MS-based proteomics offers some significant shortcuts and potential improvements because of its selectivity and sensitivity. It is possible to determine the abundance of many transporters simultaneously and therefore to uncover correlations between different transporters and between transporters and other proteins (29). Nevertheless, the number of proteomics-based reports of transporter abundance in disease is below 10 and all are recent.

Consider the difficulty in designing a suitable experiment, comparing, for example, liver transporters in healthy patients and cancer patients. Typically, the tumor and surrounding tissue will be removed surgically, and through appropriate tissue banking (which is administratively and ethically exacting but possible) the tissue may be made available to researchers. The acquisition of healthy control tissue is the first almost insurmountable hurdle. Nobody volunteers for a liver biopsy in the interest of science. Instead, we are reliant on postmortem donations from the victims of road traffic accidents and (in the United States) gunshot wounds. Even once the tissue has been obtained, transporters remain a protein chemist's nightmare. They are big: P-gp (ABCB1, MDR1) is a chain of 1,256 amino acids, and MRP1 (ABCC1) is 1,531 amino acids long. Even BCRP (ABCG2) is 655 amino acids long. Worse, they are, of necessity, membrane embedded, typically in the plasma membrane, from which they must be extracted with detergents whose most important properties include their ability to wreck chromatography columns used in liquid chromatography with tandem MS (LC-MS/MS). A few research groups have managed to overcome these problems in a small number of cases, and the methodology is described below.

Terasaki and colleagues (30) at Tohuko University in Japan have established methods for transporter analysis in several human and animal tissues and have associated reduced levels of ABCA8 with the buildup of cholesterol and taurocholate levels within liver cells. Using targeted MS-based proteomics (see the section titled Methodology in Proteomics Research), they were able to demonstrate changes in protein expression of the ABCA group of transporters associated with a mouse model of obstructive cholestasis. The approach to the difficult problem of transporter

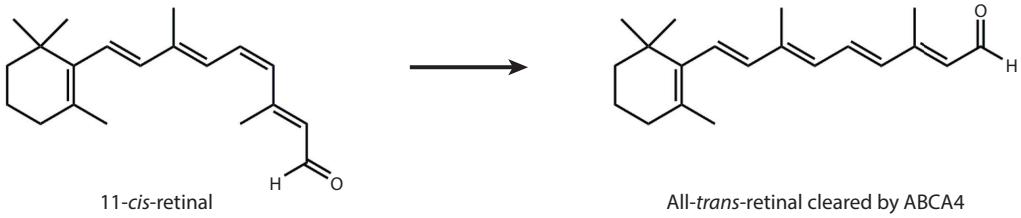

Figure 2

Conversion of 11-*cis*-retinal to all-*trans*-retinal in response to light in the eye.

proteomics of human disease is inventive: Human tissues and cell lines are aligned with rodent disease models and proteomics carried out on the animal model. There is, however, one obvious limitation: The biochemical variability seen in humans of different ages, ethnicity, and diet is not sampled.

MS-based proteomic measurements have been applied to cell lines in which mitochondrial DNA is absent. These cells represent a large group of so-called respiratory chain diseases, which are estimated to affect 1 in 5,000 live births. A heroic set of measurements by Aretz and coworkers (31) established that many proteins, including SLC transporters, were deubiquitinated relative to normal cells. Ubiquitination is a process that marks (often damaged) proteins for destruction, and its failure indicates that a cell is poorly regulated.

Two notable investigations of eye disease were dependent on animal tissue. ABCA4 is a photoreceptor-specific transporter, implicated in clearance of all-*trans*-retinal (**Figure 2**). Mutations in *ABCA4* have been linked with Stargardt disease, a rare inherited form of macular degeneration that presents early in life (usually before age 20). ABCA4 was isolated from bovine retinas and the sites of posttranslational modification were mapped using MS of a tryptic digest. Seven N-linked glycosylation sites and five phosphorylation sites were identified (32). Further experiments based on mutant human *ABCA4*, localized in vesicles, were used to suggest that phosphorylation regulates the function of ABCA4.

Another interesting study of eye disease involved a chick model of shortsightedness and also focused on the retina. Here, a full-scale proteomic analysis was carried out, and chicks with induced shortsightedness were found to have upregulated transporters in the retina (33). A huge data set has been generated, but its implications for human health are not yet clear. Pelkonen et al. (34) have also quantified transporters in the blood-retinal barrier, using a cultured cell line. The use of targeted proteomic measurements allows for the quantification of transporters specifically. Here, the focus was on drug delivery to the retina and understanding the barrier between the retina and the systemic circulation.

Neurodegenerative disease is in some ways more accessible to MS-based proteomics than other disease. This is because dementia is primarily a disease of old age, and brain banks are able to recruit donations from patients who die of dementia and those who die of other age-related causes. Transporters in the blood-brain barrier are of especial interest because changes, such as those resulting from neurodegeneration, have the potential to affect both the effectiveness and the toxicity of psychoactive drugs. Key to obtaining good data is good sample preparation, beginning with the isolation of microvessels (35, 36). Thus, following pioneering small-scale studies (see 35), two groups published the first medium-scale analysis of brain transporters by MS almost simultaneously; Billington et al. (37) focused on two different healthy brain regions, whereas our group (38) considered both healthy brains and brains from donors with dementia. "Healthy" is, of course, a relative term; brain tissue is always obtained postmortem. These donors, however, had

lived to at least middle age, and some died aged 90+. A key finding by Billington et al. was that mean transporter abundance was in the order GLUT1 (glucose transporter 1, SLC2A1) > BCRP (ABCG2) > P-gp (ABCB1, MDR1), although BCRP and P-gp showed overlapping abundances. We (using frontal lobe rather than occipital and parietal lobes) placed P-gp (ABCB1, MDR1) and BCRP (ABCG2) as having very similar abundances, with P-gp just ahead. We used both targeted and global proteomic measurements, and the targeted analysis revealed highly significant upregulation of OAT1 (SLC22A6) in Alzheimer's disease.

We expect many more reports of transporter analysis in health and disease to emerge in the next few years as problems with sample preparation are solved. Obtaining suitable normal controls, especially for diseases of young people, is likely to remain a problem and may require that organ donation schemes are extended to include tissue donation where organs are unsuitable for transplant.

A BRIEF CONSIDERATION OF THE HUMAN TRANSPORTERS LITERATURE

Transporters are key proteins that determine interindividual differences in exposure to drugs. Therefore, the expression levels of functionally important transporters have been a focus of interest for pharmacokinetic research and provide fundamental information in extrapolation of in vitro results to in vivo. Here, we summarize the most recent studies on transporter quantification. Assessment of transporter expression is performed either in vitro (cell lines) or in vivo (tissues) by the use of MS-based targeted proteomics or global analysis, both of which are proving to be very promising. Targeted approaches, in which isotopically labeled peptides are used as standards, have been used to measure several ABC and SLC transporters in different human organs. These include liver (39–42, 43), brain (35, 37, 38), kidney (42–46), intestine (39), and lung (47), and in animal tissue such as brain, kidney, and liver (36, 48–50). Interspecies comparisons have been made (51). Transporter abundance levels have also been measured in human cells, including hepatocytes and transfected cell lines (52). MRP2 (ABCC2), BSEP (ABCB11), and BCRP (ABCG2) abundances were measured in hepatocytes over various time points and compared with liver transporter expression. There was a reduction in animal MRP2 in cell lines but no change in human MRP2 in hepatocytes as compared to liver. Importantly, the expression level of BSEP decreased but BCRP increased in both human and rat hepatocytes, while MRP2 expression was reduced in rat and increased in human sandwich cultured hepatocytes (26, 53). These results helped to improve the prediction of hepatobiliary clearance in rats (54), illustrating the importance of IVIVE in drug kinetics research.

LC-MS/MS quantification of transporters in animal liver tissue and hepatocytes has allowed interspecies and intraspecies comparisons between beagle dogs, cynomolgus monkeys, Sprague-Dawley rats, and Wistar rats (51). Tumor cell lines have been used to chart absolute changes in MRP1 (ABCC1) transporter abundance (55), and changes in bile acid transporter OATP3A1 (SLCO3A1) in cholestatic liver tissues from patients and rodents (compared with healthy liver tissue) have been quantified (56). Downregulation of MRP1 (ABCC1) in 3D colon cancer cell cultures has been demonstrated (57). Other examples include evaluation of the presence of P-gp (ABCB1, MDR1) and BCRP (ABCG2) transporters in red blood cell membranes, their partitioning in drug metabolism and disposition, and potential drug–drug interactions (58).

Recently, quantitative-targeted absolute proteomics was used to determine the transporter protein content of the blood-brain barrier (36–38). In one of these studies (38), a targeted quantitative method was combined with global analysis, and 19 transporters were quantified for the first time.

Vildhede et al. (59) also applied multiplexed global proteomics combined with the total protein approach, measuring abundance of several uptake transporters, including OAT2 (SLC22A7),

OAT7 (SLC22A9), and OATP1B1 (SLCO1B1), and showed agreement with targeted proteomics. The total protein approach has not found widespread acceptance yet, but it has the advantage of not requiring exogenous standards.

Targeted proteomics has proved very successful in measuring the expression of transporters in the kidney and in demonstrating that transporter expression is species dependent (42, 44). Limited kidney transporter abundance data in adults are available and in most cases are conflicting. The available human transporter expression data on ontogeny were, until recently, only from rat and mice models. A recent study of 37 kidney samples from children, adolescents, and adults indicated no major changes with respect to age in aquaporin 1 and aquaporin 2 (45). Cheung et al. (46) investigated different transporters over a wide age range (starting with newborns) using mRNA analysis as well as proteomic analysis. An increase in transporter abundance with age was shown for P-gp (ABCB1, MDR1), urate transporter 1, URAT1 (SLC22A12), organic anion transporter 1, OAT1 (SLC22A6), organic anion transporter 3, OAT3 (SLC22A8), and organic cation transporter 2, OCT2 (SLC22A2).

Differences in transporter expression in different regions of brain (37) and intestine (60) have been evaluated using targeted proteomics. The results showed 1.4-fold differences in GLUT1 (SLC2A1), BCRP (ABCG2), P-gp (ABCB1, MDR1), ENT1 (SLC29A1), and OATP2B1 (SLCO2B1) transporter expression in the blood-brain barrier between the two brain regions (occipital and parietal), and the data can be used for IVIVE to predict drug concentration in brain. For intestine, efflux transporters such as P-gp (ABCB1, MRD1) and BCRP (ABCG2) and the uptake transporter PEPT1 (SLC15A1) were more highly expressed in jejunum and ileum than in the colon. However, the abundance level of MRP2 (ABCC2), MRP3 (ABCC3), and OCT3 (SLC22A3) was found to be highest in colon (60).

Species differences in protein expression are a key element in scaling for variations in animal models used for human extrapolation. Following Hoshi et al. (61) to quantify rat and marmoset blood-brain barrier transporters with high-throughput quantification, Uchida et al. (62) developed a protocol for quantitative targeted proteomics applied to quantify expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in three species. They found that the similarities of protein abundance involved in transport are greater between human and marmoset than between human and rat.

On the other hand, Ohtsuki et al. (63) investigated hCMEC/D3 cells as a blood-brain barrier in vitro model and compared them with isolated human brain microvessels. They established that the expression levels of most transporters in hCMEC/D3 cells are similar to human brain microvessels. Although this cell line proved to be a good model, the expression levels of several transporters, including MRP2 (ABCC2), were very high in rat liver compared with human, while in mouse BCRP (ABCG2) was very abundant compared with human, casting doubt on the suitability of mouse as a preclinical model (53, 63).

Targeted proteomics can be used to quantify specific groups of proteins determined prior to the experiment (because of the necessity of preparing suitable standards). The advantage of global proteomics is that it can be used to quantify very large numbers of proteins without prior information. To illustrate this point, we recently quantified >2,000 proteins, including 66 transporters, in a panel of 24 human liver samples (29). In a study of microvessels from the human blood-brain barrier, we identified >3,000 proteins with 53 quantifiable transporters, 19 of which were quantified for the first time (38).

The 30 papers cited in this section include 6 each from 2019 and 2018 and only two prior to 2012. This illustrates that the use of MS to quantify mammalian transporters is a small but rapidly growing area of research.

METHODOLOGY IN PROTEOMICS RESEARCH

Sample Preparation

Sample preparation is the most critical step in proteomics research and hugely affects the outcome of any experiment. Transporters are especially challenging because of a wide dynamic range spanning up to six order of magnitude, low abundances of several important proteins (e.g., ABCC5/MRP5, which is responsible for transport of cyclic nucleotides and the carnitine carrier SCL22A4/OCTN1), and the fact that they are embedded in membranes. Treatment prior to MS therefore includes protein enrichment, and fractionation of proteins is required.

Sample Quantity and Quality

Sample quantity is a limiting factor in any workflow. Approximately 100 mg of tissue is usually required to generate sufficient protein for analysis. This can be especially limiting for rodent tissue, and pooled samples may be required to generate sufficient material (36).

The quality of sample is also critical. Fresh samples are the first choice in transporter quantification studies, but when samples are derived from biopsy, the first priority of the surgeon is the well-being of the patient, so pathological investigation takes precedence over research. Similarly, the harvesting of tissue for research cannot be expected to be uppermost in the minds of grieving relatives when a patient dies. Fresh frozen samples are the second choice, and can often be obtained, especially when a research nurse is employed to take consent from patients and to process samples out of normal working hours. An increasing number of surgeons recognize the value of storing excised tissue, and hospitals are developing small tissue banks. As a last resort, paraffin-embedded tissue (64) can be used for proteomic measurements, and procedures have been developed for this purpose.

In this review, we focus on transporters, which are membrane proteins and therefore poorly soluble in water. We discuss the process of converting large, amphipathic, membrane-embedded proteins into small water-soluble peptides suitable for analysis by MS.

Protein Isolation, Enrichment, and Digestion

Solubilization of transporters requires both physical and reagent-based approaches (65). Typically, and depending on the tissue, freeze/thaw cycles, cryopulverization, glass bead-beating, high-speed homogenization, sonication, and pressure-assisted (French press cell) approaches are used to disrupt membranes. Afterward, enrichment processes such as chloroform/methanol partitioning or acetone precipitation can be used to isolate membrane proteins (66, 67).

Membrane protein enrichment and solubilization for LC-MS/MS analysis can be thwarted by protein interactions, aggregation, precipitation, and degradation by endogenous proteases. Most solubilization approaches therefore use freshly made lysis buffers containing detergents (68), such as sodium dodecyl sulfate, deoxycholate, or acid-labile surfactants such as RapiGest (69). In fact, other detergents such as Triton X-100, NP-40, or CHAPS are more effective for membrane disintegration and protein solubilization (70), but they are difficult to remove from the sample prior to LC-MS/MS and can contaminate the spectra.

Protease inhibitors must be added to the lysis buffer to prevent protein degradation. Chaotropes such as urea, guanidine, and thiourea can assist protein extraction and denaturation and disrupt protein–protein interactions. At low concentration, they can facilitate the action of digestive enzymes, but digestive enzymes are, of course, proteins and susceptible to denaturation by chaotropes. Trypsin, the most widely used enzyme in proteomics, is denatured by high concentrations (>2 M) of chaotropes, which need to be diluted before protein digestion (71).

Although they are generally considered necessary, several drawbacks are associated with using even the most benign detergents, including enzyme inactivation, LC interference, and ion suppression (72). Dilution or removal of detergents is therefore important prior to LC-MS/MS. Detergents can often be removed by dialysis, protein precipitation, and gel electrophoresis, but filter-aided sample preparation (FASP) is now widely considered to be superior, especially for membrane proteins (73). Several studies have demonstrated the applicability of FASP in the analysis of membrane proteins (38, 74). We have also experimented with the alternative GASP (gel-aided sample preparation) method, which has some advantages but requires further optimization (see below) (75). Novel, easily removed surfactants that are cleavable using heat or low pH, such as ProteaseMax (Promega) and RapiGest (Waters), have been developed (69, 76), and these yield products that are compatible with LC-MS/MS analysis.

The use of acetonitrile and guanidine has been reported to improve the trypsin activity and digestion efficiency of membrane proteins (77). Formic acid was also shown to be very powerful in the solubilization of membrane proteins (78); it has the disadvantage of being incompatible with trypsin at 37°C, causing protein decomposition, and solubilization is therefore performed at room temperature (79).

The bottom-up proteomic workflow involves subjecting complex mixtures of proteins to proteolytic digestion and analyzing the resultant matrix with LC-MS/MS. This has been likened to solving a 5,000-piece jigsaw puzzle by first putting every piece through a shredder, and is, at first sight, madness. However, a digest has several advantages in terms of MS over a top-down analysis of a mixture of proteins. Most proteins, when treated with (for example) trypsin, will yield a suitable number of peptides of optimum length and detectability by MS, and these peptides can be used for quantification. The most widely used enzymes, trypsin and endopeptidase Lys-C, give rise to peptides with a basic residue at the C terminus, the condition most likely to give good MS and MS/MS spectra (80). In general, peptides 7–30 amino acids long can be detected and quantified readily, and the basic residues arginine and lysine are present at 5.7% and 5.6% (81) of the human proteome, giving rise, normally, to peptides of appropriate length.

Chymotrypsin (V8 protease), elastase, pepsin and proteinase K, and the more expensive endoproteinases Asp-N and Glu-C have strengths and weaknesses in terms of digestion of membrane proteins. Undoubtedly, however, trypsin is the most useful enzyme for the analysis of soluble proteins (82), but trypsin alone may not be adequate for releasing transmembrane peptides because K (lysine) and R (arginine) are rare in hydrophobic transmembrane regions and are subject to glycosylation in plasma membrane proteins (83). This problem may be limited and the number of identified peptides increased by deglycosylation before digestion. Endopeptidase Lys-C can tolerate high concentrations (up to 8 M) of chaotrope and is widely used to facilitate both solubilization and digestion of transmembrane proteins before urea removal and trypsin digestion. This sequential digestion strategy (Lys-C followed by chaotrope removal, then tryptic digestion) has become a gold standard in membrane proteomics (36, 84). Endopeptidase Glu-C is also resistant to denaturation by chaotropes, but because it cleaves at acidic residues, which are rare in transmembrane regions, a lower number of peptides is usually generated (85). Chymotrypsin cleaves to the C-terminal side of aromatic residues, which are widely found in transmembrane regions. Here the limitations are a lack of specificity, leading to mixtures of peptides that are difficult to quantify. Elastase, pepsin, and proteinase K are all inherently nonspecific proteases producing a mixture of overlapping peptides that results in increased sample complexity.

Advances in the Application of LC-MS Proteomics to Transporters

Large-scale proteomic measurements of low abundance proteins, such as transporters, in biological samples are required to establish valid population data (biological variability) across various

sample batches and under different disease conditions (86). However, it is often technically challenging to achieve the required level of assay sensitivity, throughput, and selectivity (in complex matrices) when large numbers of samples are analyzed to quantify transporter proteins often expressed near the limit of quantification achieved by state-of-the-art technology (13, 87). In addition to improvements in sample preparation and protein extraction/purification techniques, advances in separation and MS technology as well as proteomic methods over the past decade contributed to the wealth of quantitative data of transporter expression in many types of tissues and cell lines. Generally, mass spectrometers suffer from detection bias toward the most abundant proteins in a sample (87), and therefore the proteomic community has focused significant efforts on developing methods and technologies that assist in maximizing peptide separation, efficiency of ion generation, and confidence in protein identification and quantification (88).

Peptide separation and ionization. LC coupled with electrospray ionization (ESI) MS is by far the most widely used proteomic technique to separate and analyze protein samples. Improvements of ESI equipment from high-flow sources, which were associated with poor analytical sensitivity, to more robust flow systems have allowed better ionization efficiency. Ionization is dependent on the size of charged droplets, with smaller droplets more readily desolvated to release peptides into the gas phase (89). Newer sources, mainly nanoESI, coupled with compatible LC systems, led to the development of highly sensitive instruments that can operate with miniaturized platforms capable of supporting electrospray as low as picoliters per minute (90). Compatible LC systems are used with analytical columns typically prepared by silica media packing in narrow-bore capillaries (91, 92) or porous layer open tubular LC columns (93, 94).

In addition to improvements in LC, the recent incorporation of ion mobility with MS offers an extra dimension of separation for resolving and analyzing peptides, owing to the synergy between the two techniques in gas-phase analysis (95). The speed of ion mobility (typically milliseconds) compared to other separation techniques, such as LC (minutes to hours), makes it an attractive alternative capable of high throughput (96). Ion mobility spectrometry separates ions based on their size and shape, which is then followed by MS resolution based on m/z (97). The orthogonality of the two techniques allows separation of isobaric/isomeric analytes, leading to improved selectivity in global analyses. For example, Creese & Cooper (98) successfully resolved coeluting (identical retention time) and isobaric (identical m/z) peptides glycosylated on different amino acids, allowing further structural elucidation of the posttranslational modification. Comigrating ions in the ion mobility device can present a resolution challenge, but these can often be separated by altering the timing/amplitude of applied voltages, using a different drift gas or adding dopants (volatile additives that change the ion molecule reactions in the gas phase) (99, 100). Another advantage of the extra layer of separation, which is particularly relevant to discovery proteomics, is increased depth and scope of analysis by lowering the limit of detection (removal of interference) and resolving peptides in the same m/z range (101, 102). Shliaha and colleagues (102) demonstrated increased numbers of identified peptides and proteins by ion mobility in conjunction with LC-MS compared to the use of standard LC-MS alone without affecting analysis time. Analysis of the complex data generated by these three dimensions of peptide characterization has become more manageable owing to availability of open-source, cross-platform software, such as Skyline (103). With novel designs and innovations in hybrid MS platforms, ion mobility is expected to enable routine proteome-wide analyses while limiting sample consumption and maximizing throughput (104). We have previously demonstrated applicability of ion mobility in conjunction with global proteomics for proteome-wide profiling of human liver samples (105).

Mass spectrometry analysis and protein quantification. The analysis of peptides is dependent on gas-phase fragmentation to acquire necessary sequence information, which is used with the

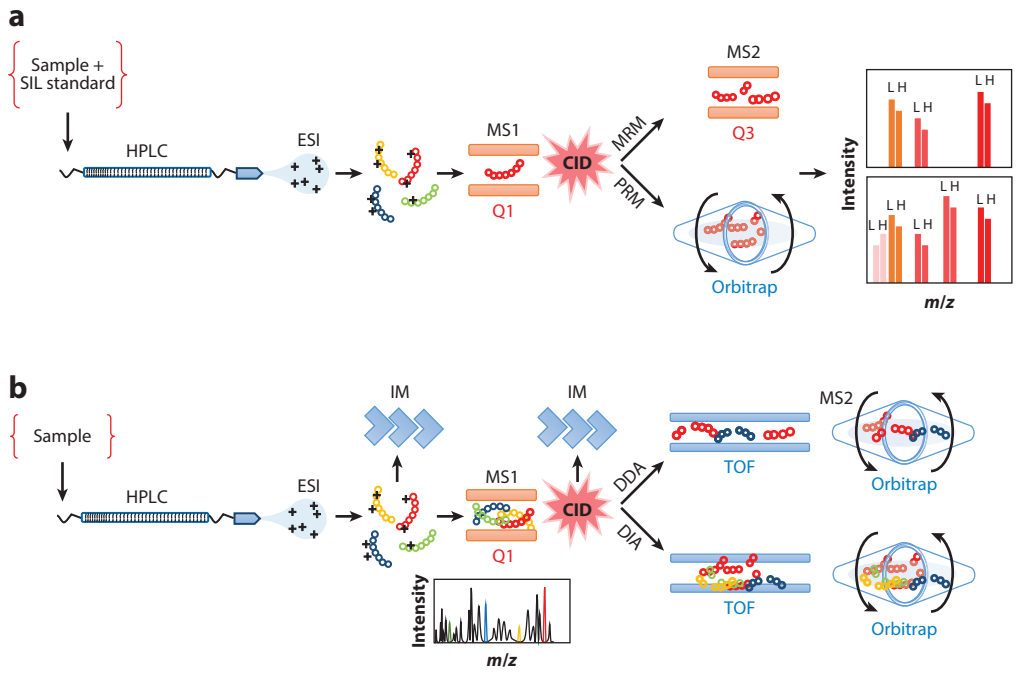

Figure 3

Workflows of state-of-the-art targeted (MRM/PRM) and global (DDA/DIA) proteomic methods used for the characterization of transporters in biological samples. (*a*) In targeted analysis, standards are routinely used with absolute quantification of a predefined set of proteins. (*b*) Global proteomics rely on an initial MS scan and subsequent fragmentation and quantification. Targeted proteomics is normally used for hypothesis-testing and quantitative applications; global proteomics is used for hypothesis-generating (discovery) and relative quantification experiments. Abbreviations: CID, collision-induced dissociation; DDA, data-dependent acquisition; DIA, data-independent acquisition; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IM, ion mobility (either peptide or fragment levels); MRM, multiple reaction monitoring; MS1, mass scan at the peptide level; MS2, mass scan at the fragment level; PRM, parallel reaction monitoring; Q, quadrupole; SIL, stable isotope–labeled; TOF, time-of-flight.

parent ion mass for accurate identification and quantification (106). The acquired data are compared to an organism-specific proteome database using a generic MS/MS search tool [e.g., MASCOT (107), SEQUEST (108), X!Tandem (109)] or to a custom-made data set generated for a preselected set of proteins using the same or a similar platform. The first approach is used in global proteomic analyses [data-dependent acquisition (DDA) and data-independent acquisition (DIA) methods] and the second is used in targeted [multiple/parallel reaction monitoring (MRM/PRM)] and sequential window acquisition of all theoretical mass spectra (SWATH) analyses. We have recently reviewed these methods and their use for the quantification of clinically relevant proteins (110).

Quantification of transporters by targeted proteomics. MRM-targeted methodology applied with stable isotope–labeled (SIL) internal standards (**Figure 3*a***) has remained the gold standard in the quantification of transporters over the past decade owing to its sensitivity, high throughput, and compatibility with multiplexed analysis (13, 111). This technique was instrumental for generating quantitative transporter data in different human tissues in early studies, carried out predominantly by Terasaki and coworkers (112–115). The introduction of scheduled MRM by Picotti et al. (116) further improved reproducibility and increased the number of peptides

analyzed in one experiment on standard triple quadrupole instruments, reducing both assay cost and time (117). Scheduled MRM has since been used by different laboratories worldwide to generate most of the reported transporter protein quantitative data in human tissue (e.g., liver, intestine, kidney and brain) as well as various useful cell lines (e.g., hepatocytes and Caco-2 cells) (15, 28, 37, 38, 43, 47, 48, 113, 118, 119). It is therefore not surprising that *Nature Methods* recognized this technique as Method of the Year in 2012 (120). Because of the extensive use of this method and the requirement for technical expertise in method development (**Figure 4*a***), the Clinical Proteomic Tumor Analysis Consortium (CPTAC) developed standard operating procedures and guidelines for preparation and application of MRM assays in clinical and pharmacology research (121, 122). Abbatiello et al. (123) demonstrated improved cross-laboratory reproducibility of quantitative plasma proteomic data in a large-scale study when standardized MRM assays are used. Concerted efforts toward standardization led to the creation of various MRM repositories for clinically relevant transporter proteins (112, 124–126), compiling MRM methods and quantitative data for >100 human (efflux and uptake) transporters. Rigorous assay validation, in line with guidelines from the US FDA (127) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (128) requires the definition of the assay's limit of quantification, technical variability, and range of linear response (**Figure 4*b***). This has increasingly been applied by various laboratories in recent years to establish robustness in targeted assays and confidence in subsequent measurements (38, 48, 118, 119).

Targeted transporter proteomic studies have generally used synthetic SIL peptides (AQUA) at known concentrations as internal standards to assess the levels of individual transporters (one standard per target protein). This limited the range of targets quantified by MRM studies due to the expense associated with synthesizing SIL peptides (129). The application of a concatenated standard (QconCAT, a recombinant protein comprising SIL peptides expressed on an artificial gene) by Russell at al. (130) to drug transporters in Caco-2 cells offered a solution to the sustainability limitation associated with synthetic SIL standards and opened the possibility for multiplexed quantification of a large number of transporters in the same experiment (up to 50 per standard). This approach was subsequently applied to drug transporters in liver (43), intestine (119), and brain tissue (36, 38). Access to higher-resolution platforms, such as time-of-flight (TOF) and Orbitrap mass analyzers, enabled the development of targeted assays capable of parallel fragment analysis by PRM methodology, also known as high-resolution MRM (MRM^{HR}), leading to higher specificity and target coverage than conventional MRM (131–133). In these assays, the initial MS1 scan is used to select the parent ions of interest, followed by simultaneous monitoring of all detectable fragments for both standard and analyte (**Figure 3*a***). The high resolution and mass accuracy achieved by PRM allow robust quantification of a wide range of low-abundance transporters, as demonstrated recently by Nakamura et al. (134).

Quantification of transporters by global proteomics. DDA shotgun proteomics has been extensively used for protein discovery and quantification over the past two decades (106). In these methods, information from the initial scan (MS1) is used to select, based on intensity, a user-defined number of ions for sequential fragmentation, leading to generation of stochastic data due to MS/MS undersampling (87). For this reason, DDA typically suffers from inconsistent reproducibility, bias toward higher-abundance proteins, and considerable levels of missing data, especially as sample complexity increases (87, 135, 136). These limitations precluded early application of shotgun proteomics for the quantification of transporters. The development of proteome-wide metabolic and chemical labeling techniques, such as stable isotope labeling by amino acids in cell culture (SILAC) (137) and tandem mass tags (TMT) (138), enabled more reproducible relative quantification to be achieved by controlling for sample-to-sample technical variability.

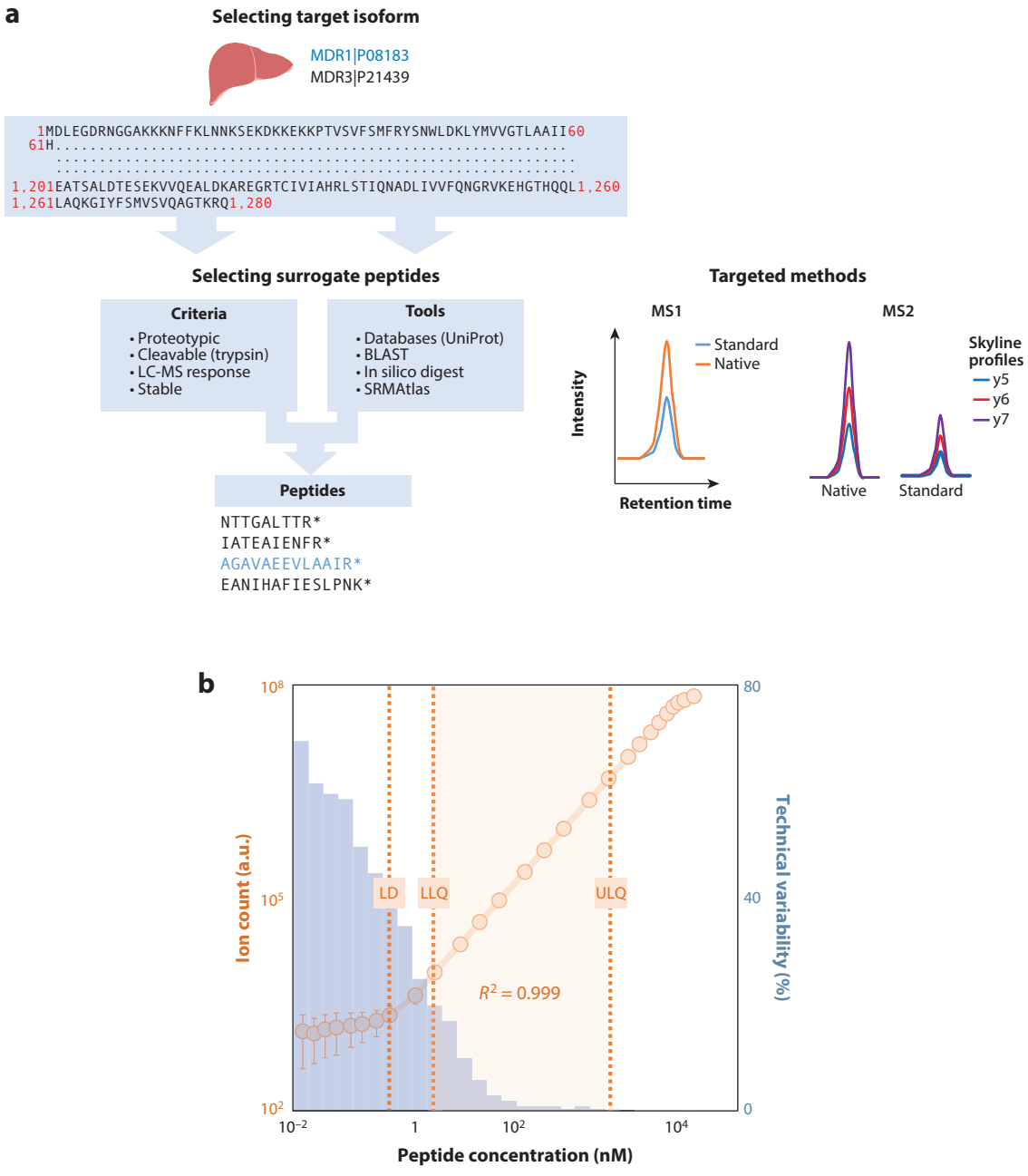

Figure 4

Assay development and validation in targeted proteomic quantification of transporters. (*a*) For a specific transporter isoform (p-gp, MDR1), surrogate (proteotypic) peptides are identified, and selective methods are designed (e.g., Skyline elution profiles). The sequence of MDR1 is accessed in UniProt (accession code P08183; 1,280 amino acids); peptide sequences are labeled on lysines (K) and arginines (R) as indicated by asterisks. Fragments monitored are y-ions because their sequences include the labeled amino acid. (*b*) Quality assessment of the developed methods include establishment of technical variability (CV, %), LD, LLQ, and linearity range (*shaded area*) for each surrogate peptide. Abbreviations: BLAST, basic local alignment search tool; LD, limit of detection; LLQ, limit of quantification; MS1, mass filter on the first quadrupole; MDR1 (P-gp), multidrug resistance protein 1 (gene name *ABCB1*); MS2, mass filter on the first quadrupole at the MS/MS level; ULQ, upper limit of quantification.

Prasad & Unadkat (139) demonstrated the use of SILAC standards for the quantification of liver P-gp (ABCB1, MDR1) and OATP transporters, with precision levels comparable to standard AQUA-MRM techniques. Using TMT labeling, Vildhede and colleagues (59) showed similar performance by DDA global proteomics to targeted methodology for the quantification of seven hepatic SLC transporters. To overcome the limitation of MS/MS undersampling, several pipelines that use only MS1 data (at high resolution) with sophisticated alignment and identification algorithms were proposed. For example, a scan method was reported by Cox & Mann (140) for data acquisition at improved mass accuracy (ppb levels) by iterative measurement and alignment using MaxQuant to enable reproducible quantification at the MS level. More recently, Shen et al. (87) developed a novel method, IonStar, for high-resolution proteome-wide MS1 analysis of large sample batches, which they applied to brain samples (hundreds) from a traumatic brain injury mouse model, reporting limited levels of missing data (<1%) and highly reproducible quantification (CV <20%). Extensive upstream fractionation of peptides is often required with these methods to increase proteome coverage and reduce sample complexity, leading to improved quantitative performance (140). Strict criteria are applied; consistent experimental conditions are maintained throughout the analysis, and the system is trained using trial runs of the same sample type to assign exact retention times and mass data, necessary for alignment in subsequent analysis. Using these caveats, we recently demonstrated applicability of QconCAT standards with high resolution MS1 scans to the quantification of xenobiotic transporters in human brain (38).

While MS1 proteome-wide quantification may present a promising development, generating MS/MS data for a large proportion of peptides detected in MS1 scans is essential for conclusive identification and low false discovery rates (88). The use of DIA methods has therefore been proposed as an alternative to DDA in recent years, offering advantages such as reduced bias and increased depth of analysis (135). Because all detected signals within a set *m/z* window are selected for fragmentation (**Figure 3*b***), complex data are generated, requiring specialized software for postacquisition data deconvolution (141, 142). A particularly promising DIA technique relies on sequential window acquisition of all theoretical fragment mass spectra by dividing the MS1 scan into *m/z* windows of predefined size, followed by sequential fragmentation of detected peptides and MS/MS data acquisition (143). Postacquisition data selection enables wide proteome coverage, typically achieved by shotgun methods, and highly reproducible quantification, comparable to targeted proteomic techniques (135, 144). The development of open access pipelines, such as OpenSWATH (145), has made the SWATH technique more widely applicable. Key applications of SWATH have recently been demonstrated for the analysis of posttranslational modifications, such as acetylation and glycosylation (146), and digital biobanking of tissue proteomic maps in health and disease (147). Recently, Jamwal et al. (148) applied SWATH to the characterization of liver enzymes, and Nakamura et al. (134) extended this application to the quantification of transporters in liver, intestine, and kidney at a level comparable to targeted analyses.

HELP PLEASE! OPPORTUNITIES AND LIMITATIONS IN THE APPLICATION OF MASS SPECTROMETRY TO TRANSPORTERS

The aim of this research is to get the right dose of the right medicine to the right patient. A patient's transporter profile affects the amount of any drug that reaches its target, and doses may need to be adjusted depending on the patient's transporters. Although it is impractical in the short-term to profile a patient individually, the stratification of patient groups based on age, gender, disease, habits, and genetic profile is a reasonable target.

This requires that we have access to statistically significant amounts of tissue from a wide range of donors. We have calculated that about 30 samples in each arm (e.g., diseased and healthy)

of a study gives statistical significance for most transporters and enzymes responsible for drug metabolism and disposition (B. Achour, unpublished). For a few poorly abundant transporters, around 70–80 samples are required. An extension of the organ donor scheme to permit tissue donation would be welcome and potentially has the social benefit of being inclusive; tissues from those with many serious diseases would be welcome.

Sample preparation is challenging but has seen many recent improvements. The development of improved detergents (69, 76, 77) is helpful, as is the FASP protocol (73), which has been adapted in several minor ways for these samples. Nevertheless, it remains the case that sample preparation is the territory of the highly skilled and dedicated. Several steps require 10-h days. The white paper "Toward a Consensus on Applying Quantitative Liquid Chromatography-Tandem Mass Spectrometry Proteomics in Translational Pharmacology Research" (13) arose from the International Society for the Study of Xenobiotics workshop on the same theme in September 2018. Among the recommendations is the provision of standard samples to ensure consistent standards in the field. It is worth drawing the attention of chemists to the GASP method (75). Currently, no mass spectrometer samples every peptide available, and different sample preparations therefore give different panels of peptides, but, if all goes well, the same protein quantification. Our preliminary data indicate that GASP is complementary to FASP (149), insofar as peptides detected in one technique may be invisible in the other, but GASP is subject to missed cleavage because the acrylamide in the gel incompletely modifies the lysine side-chain, and trypsin does not cleave at modified lysine (**Figure 5**). The obvious way to overcome this is to premodify (for example by acetylation) lysine residues completely. This modification has now been reported (150) and applied to prokaryotic cell lysates. It is notable that extensive optimization was required to yield near completion of reaction. It remains to apply the final protocol prior to GASP.

The same modification has the potential to improve the choice of enzyme. Protocols based on endopeptidase Lys-C and trypsin give rise to relatively short peptides because these residues appear on average every ninth residue in a protein. If cleavage at lysine is impossible, cleavage

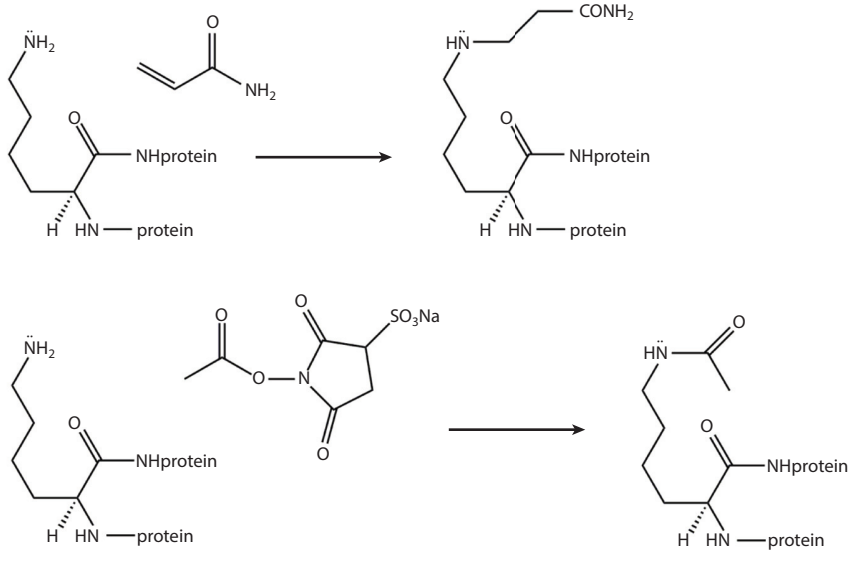

Figure 5

(*Top*) reaction (incomplete) of lysine side-chains with acrylamide (*bottom*) reaction of lysine side-chains with sulfo-NHS-acetate.

sites appear on average every seventeenth or eighteenth residue and must contain arginine, which is more basic than lysine. Longer, basic peptides have advantages in terms of improved detection sensitivity in the mass spectrometer and reduced complexity of analyte. Golghalyani et al. (150) report improved protein coverage using Arg-C-like digestion. Trypsin is, however, much more sensitive to chaotropes than Lys-C, and it is not clear whether omission of the Lys-C step will create more problems than it solves in the case of membrane-embedded proteins. Interestingly, Wiśniewski et al. (151) have recently reported improved quantification by the use of three enzymes, adding chymotrypsin to the usual Lys-C and trypsin.

The limitations of MS itself are more difficult to address. LC-MS/MS is improbably sensitive and selective. We can distinguish thousands of peptides and, with care, we can quantify the corresponding proteins. The remaining complaints, and there are many, essentially boil down to cost. A proteomic analysis can always be improved by running additional replicates, longer LC gradients, or more sample fractions, but with running costs of around £100 per hour, depending on the costing model used, each additional quality control is costly. Savings can be made by careful choice of experiment (133), but proteomics is not for the poorly funded. However, label-free global proteomics methods are now yielding more data than could possibly be analyzed adequately by one person or even one group. It is now quite possible to think in terms of spreading the cost of a well-designed experiment among groups with complementary interests. One aspect addressed by the white paper (13) is data sharing; we owe it to funders, and above all, to donors to use their data for maximum impact.

DISCLOSURE STATEMENT

A.R.-H. is employed part-time by Certara, a company that develops and supplies modeling and simulation software and services to the pharmaceutical industry. He is also the director of the Centre for Applied Pharmacokinetic Research (CAPKR) at the University of Manchester, whose research is funded by a consortium of pharmaceutical companies. The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors would like to thank Sarah Alrubia for sharing a figure and Eleanor Savill for her assistance in formatting the manuscript for submission.

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