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Simultaneous absolute quantitation of ATP-binding cassette transporters in normal dog tissues by signature peptide analysis using a LC/MS/MS method

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Abstract

Membrane transport proteins are fundamental components of blood-tissue barriers and affect the absorption, distribution and elimination, and interactions of many of the drugs commonly used in veterinary medicine. A quantitative, simultaneous measurement of these proteins across dog tissues is not currently available, nor is it possible with current immune-based assays such as western blot. In the present study, we aimed to develop a sensitive and specific liquid chromatography tandem-mass spectrometry (LC/MS/MS) based quantitation method that can simultaneously quantitate 14 ATP-binding cassette transporters. We applied this method to a panel of normal canine tissues and compared the LC/MS/MS results with relative messenger RNA (mRNA) abundance using quantitative real-time polymerase chain reaction (qRT-PCR). Our LC/MS/MS method is sensitive, with lower limits of quantitation ranging from 5 to 10 fmol/μg of protein. We were able to detect and/or quantitate each of the 14 transporters in at least one normal dog tissue. Relative protein and mRNA abundance within tissues did not demonstrate a significant correlation in all cases. The results presented here will provide for more accurate predictions of drug movement in dogs through incorporation into physiologically based pharmacokinetic (PBPK) models; the method described here has wide applicability to the quantitation of virtually any proteins of interest in biologic samples where validated canine antibodies do not exist.

Keywords

ABC transporters; LC/MS/MS; Targeted proteomics; Signature peptide analysis

1. Introduction

Membrane transport proteins play critical roles in the absorption, distribution and elimination of drugs, impact drug-drug interactions and provide a key component of blood-tissue barriers such as the blood-brain barrier (International Transporter et al., 2010; Ohtsuki and Terasaki, 2007; Shitara et al., 2006). These proteins function to pump substrates in a single direction, generally from the cytoplasm out of the cell. For some hydrophobic

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substrates, these proteins act to remove drugs from within the membrane to the outside of the cell. This action can result in one of the most documented mechanisms of multi-drug resistance in cancer cells through the upregulation of drug efflux pumps, including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 1 (MRP1) (Chen et al., 2016; Gottesman et al., 2002). Aside from their functions in disposition of drugs, these proteins also play critical roles in normal mammalian membrane physiology such as translocation of sugars, amino acids, fatty acids, metal ions, peptides and proteins; mutations in these proteins leading to altered function are recognized as contributing causes of some genetic disorders in humans (Dean et al., 2001). Many of the drug efflux proteins present in cell membranes belong to the ATP-binding cassette (ABC) superfamily of transporters, the largest family of protein transporters, containing at least 48 characterized transporters in humans (Dean et al., 2001). Distribution across tissues in mice has been reported (Kamiie et al., 2008), but this information is currently missing in dogs which are one of the commonly used preclinical species for prediction of pharmacokinetics in human drug development. Species differences in substrate specificity, tissue distribution and relative abundance of drug transporters make cross-species extrapolations very complicated and quantitative information about distribution and abundance at the protein level has been suggested as imperative for the ability to develop physiologically based pharmacokinetic (PBPK) models in order to optimize cross-species extrapolations (Chu et al., 2013). The use of PBPK/PD modeling is increasing in support of drug development and is suggested to play a vital role in precision medicine by predicting inter-individual variability and the impact of covariates such as age or organ dysfunction on drug PK. However, a barrier to full utilization of these models is that current PBPK modeling platforms do not typically incorporate individual variability in drug metabolizing and transport protein quantities which are a major component of variability in PK and PD (Prasad et al., 2017). There is some evidence that confidence in human clinical predictions is improved when PBPK predictions for a drug have been verified in animal models, and better characterization of ADME proteins in pre-clinical species, such as the dog, will support an improved understanding of crucial species differences (Heikkinen et al., 2015). This will have the benefit of improving translation of PK and safety data from dogs to humans and thus, the quantitation of membrane transport proteins in dogs can be valuable to the drug development process.

Historically, evaluation of protein expression has been performed via immunohistochemistry or western blot however, these methodologies are limited in their ability to multiplex and they do not provide more than a relative quantitation. Moreover, identification and validation of antibodies for use in veterinary species such as dogs is a limiting factor in comprehensive analysis of protein families. Signature peptide analysis via high performance liquid chromatography-tandem mass spectrometry is a method that can overcome the flaws that are inherent in immunoassays with regard to specificity and precise quantitation (Hoofnagle and Wener, 2009). Multiple-reaction monitoring (MRM) mass spectrometry allows precise quantitative analysis of proteins by targeting specific peptide sequences that are unique to a particular protein and has the capacity for multiplexing hundreds of proteins in a single sample (Liebler and Zimmerman, 2013). This technique has become more common and has been utilized to study the expression profile of drug transporters in normal mouse tissues

(Kamiie et al., 2008) as well as drug transporters and metabolizing enzymes in human liver samples (Ohtsuki et al., 2012) and metabolizing enzymes in canine liver and intestinal sections (Heikkinen et al., 2012). This method is based on in silico selection of unique peptide sequences of a protein following trypsin digestion and subsequent development of a liquid chromatography tandem mass spectrometry based method for each unique peptide. Utilization of commercially prepared peptide standards and corresponding stable isotope labeled (SIL) standards then allows for precise and accurate quantitation of the peptides in a biologic sample.

Transporter and drug metabolizing enzyme expression has been performed at the level of mRNA extensively in human tissues, and mRNA expression of seven drug transporters was previously evaluated in canine lymphoma (Zandvliet et al., 2015). However, it has been demonstrated that mRNA expression levels do not necessarily reflect protein expression or functional activity of the corresponding proteins (Rodriguez-Antona et al., 2001). In fact, discordance between protein and mRNA levels was identified in 21 of 22 drug transport proteins in human liver (Ohtsuki et al., 2012). More specifically, the membrane transporter P-glycoprotein has been found to be regulated post-transcriptionally in leukemic cells with mRNA levels not indicative of active membrane protein levels (Yague et al., 2003). Without the use of a sensitive and specific method for quantitating protein levels simultaneously, the correlation between mRNA and protein cannot be readily determined.

The purpose of this study was to quantitate protein levels of 14 drug transporter proteins in normal dog tissues and compare those results to the tissue mRNA expression of these same transporters in order to 1) provide a quantitative atlas of protein expression that can be incorporated into PBPK models and 2) determine the suitability of using mRNA expression as a surrogate for analyzing differences in drug transporter protein expression.

2. Materials and methods

2.1. Materials

All organic solvents were purchased from Sigma-Aldrich (St. Louis, MO). Protease inhibitor tablets were purchased from Roche (Manheim, Germany) and the Mem-PER™ Plus kit and MS Grade Trypsin Protease were purchased from Thermo Scientific (Rockford, IL). Ammonium bicarbonate, dithiothreitol (DTT) and formic acid were purchased from Sigma-Aldrich. The protein quantification BCA kit and trypsin were purchased from Pierce Biotechnology (Rockford, IL). RNA^{later}® was purchased from Ambion (Rockford, IL), the RNeasy Mini Kit and QuantiTect® reverse transcription kit were purchased from Qiagen (Hilden, Germany). The iQ™ SYBR® Supermix was purchased from Bio-Rad (Hercules, CA). Peptide standards were purchased from Celtek Bioscience (Nashville, TN) and stable isotope labeled peptides AQUA peptides were purchased from Sigma-Aldrich. Primers for quantitative reverse transcriptase PCR were purchased from Integrated DNA Technologies (Skokie, IL). Pierce® C18 Tips (100 µL) were purchased from Thermo Scientific.

2.2. Selection of signature peptides

Full-length FASTA sequences for each of the ATP binding cassette transport proteins were used for in silico trypsin digestion with the freely available online software program MS-Digest in ProteinProspector (<http://prospector.ucsf.edu>). The resulting list of peptide candidates was then narrowed with the application of specific selection criteria that included length between 6 and 12 amino acids, lack of glycosylation sites and other post-translational modifications, no continuous sequences of lysine or arginine, and lack of methionine, cysteine and histidine residues for optimal peptide stability. The resulting lists were then subjected to an online program designed to predict high-responding peptides in electrospray ionization-based mass spectrometry assays (<http://genepattern.broadinstitute.org>). The signature peptides were then checked for unique identity by protein BLAST (blastp; <http://blast.ncbi.nlm.nih.gov>) to ensure no exact matches of amino acid sequence with any other transporter family members, or other proteins. The final set of signature peptides was then synthesized and used for high-performance liquid chromatography tandem mass spectrometry method development. Stable isotope labeled (SIL) peptides for Bcrp, Mrp1 and Bsep were synthesized and used as internal standards. The signature peptides selected for each of the proteins evaluated are listed in Table 1.

2.3. Membrane protein sample preparation

Canine tissue samples were obtained from the necropsy laboratory at the Colorado State University Veterinary Teaching Hospital and were taken only from dogs without a known disease condition to avoid potential effects of disease status on protein expression. Samples included brain, liver, kidney, duodenum, jejunum and ileum. Fourty milligrams (40 mg) of each fresh tissue was weighed out and rinsed with rinsing buffer from the Mem-PER protein extraction kit. Samples were then transferred to a new 1.5 mL eppendorf tube and frozen at -80°C until membrane protein extraction was performed. For quantitative real-time PCR experiments, 30 mg of each fresh tissue was weighed out and placed in a 1.5 mL eppendorf tube containing RNA*later*® and stored at 4°C until RNA extraction was performed. Membrane protein isolation was performed by homogenizing 40 mg tissue in Permeabilization buffer containing protease inhibitor using a PowerGen 700 tissue homogenizer. Samples were then transferred to 2 mL eppendorf tubes and incubated on ice, with shaking, for 10 min. The permeabilized cells were pelleted at $16,000 \times g$ for 15 min at 4°C and the supernatant containing cytosolic proteins was removed. The pellet was resuspended in 650 μL of Solubilization buffer containing protease inhibitor and pipetted up and down to obtain a homogenous suspension. Samples were then incubated on a rocker plate at 4°C for 30 min followed by centrifugation at $16,000 \times g$ for 15 min at 4°C . The supernatant containing the solubilized membranes and membrane proteins was then transferred to new, ice-cold, 2 mL eppendorf tubes. Five-hundred microliters (500 μL) was used for subsequent de-lipidation/detergent removal steps using a chloroform:methanol procedure outlined previously (Mirza et al., 2007). Briefly, 400 μL methanol was added to each tube and vortex mixed briefly followed by centrifugation at $9000 \times g$ for 15 s. Next, 200 μL chloroform was added to each tube and briefly vortex mixed followed by centrifugation at $9000 \times g$ for 15 s. For phase separation, 300 μL of ddH₂O was added and samples were vortex mixed again followed by centrifugation at $9000 \times g$ for 2 min. The upper (methanol and water) phase was carefully removed via aspiration and discarded. Another 300 μL of

methanol was added to the rest of the chloroform layer and interphase with precipitated protein and samples were vortex mixed followed by centrifugation at $9000 \times g$ for 3 min to pellet protein. Supernatants were removed and the samples were allowed to dry on ice. Dried samples were resuspended in 250 mM ammonium bicarbonate (pH 7.9) to complete dissolution using brief sonication in a water bath and vortex mixing. Protein concentration was determined using the BCA assay with 10 μ L of sample. To each sample 10 mM DTT was added and the samples were put through five cycles of sonication in water bath (1 min) followed by incubation on ice (2 min) before being boiled at 95 °C for 3 min. Samples were then transferred immediately to an ice bucket. Alkylation was carried out by the addition of 10 mM iodoacetamide (IAA) and incubation at 37 °C for 30 min. Trypsin digestion was then carried out in 30% methanol at 60 °C for 2 h with shaking. Trypsin digestion was stopped with the addition of 1% formic acid and then 2.5 pmol of stable isotope labeled peptides was added to each tube. Samples were spun at $15000 \times g$ for 3 min, transferred to a new 2 mL eppendorf tube and evaporated to dryness. Samples were then resuspended in 80 μ L of 0.1% formic acid and samples were cleaned up using C18 pipette tips. Samples were eluted from C18 tips with 20 μ L 70% acetonitrile, another 180 μ L of 0.1% formic acid was added and samples were transferred to HPLC autosampler vials with glass inserts.

2.4. Isolation of brain microvessels

Brain capillary fractions were collected from frozen tissues using adaptation of methods previously published (Vernon et al., 2011; Yousif et al., 2007). Briefly, cortical brain samples were minced into small pieces and homogenized in phosphate-buffered saline containing 1% bovine serum albumin (PBSA) using a Dounce homogenizer and passing samples through 25 mL and then 10 mL serological pipettes. Samples were then centrifuged at $5000 \times g$ for 20 min at 4 °C. Samples were then underlaid with lymphocyte separation media (Corning, Manassas, VA) and centrifuged at $5000 \times g$ for 10 min. The resulting pellets were washed in PBSA and passed through a double-filter system consisting of an initial 85 μ m filter followed by a 40 μ m filter which was washed with PBSA to remove isolated microvessels. Aliquots of the resulting samples were cytospun onto slides for fluorescence immunocytochemistry to verify isolation of microvessels by staining for Factor VIII with a DAPI counterstain. Brain microvessel fractions were then split into two samples for membrane protein isolation as described above and for RNA isolation and quantitative real-time RT-PCR (described below).

2.5. Mass Spectrometric Analysis

Tryptic digests of membrane protein samples were analyzed with an HPLC system consisting of a NexeraX2 LC-30 AC liquid chromatography pump (Shimadzu, Kyoto, Japan) with a NexeraX2 SIL-30 AC autosampler (Shimadzu) coupled to an ESI-triple quadrupole mass spectrometer (AB Sciex QTRAP® 6500, Applied Biosystems, Foster City, CA). Chromatographic separation was performed with and Aeris Peptide 1.7 μ XB-C18 (150 $\times$ 2.1 mm) column (Phenomenex, Torrance, CA) using a SecurityGuard™ ULTRA guard cartridge (Phenomenex) in a column oven maintained at 35 °C. Gradient elution was utilized to separate peptides with mobile phase A consisting of 0.1% formic acid in Milli-Q H₂O and mobile phase B consisting of acetonitrile with 0.1% formic acid. Initial mobile phase B concentration was 2% for 2 min, then a linear increase to 33% B at 30 min and held at 33%

until 32 min, linear increase to 75% B at 36 min and held steady at 75% until 38 min and a linear decrease to 2% B at 38.5 min followed by re-equilibration at 2% B until 45 min. Chromatogram peaks for peptides were identified using Analyst® v1.6.2 software (AB Sciex) and quantitation of peptides was performed based on linear regression analysis and extrapolation from peptide standards (0.25 to 200 fmol on column) in a bovine serum albumin tryptic digestate with SIL peptides as internal standards using MultiQuant™ v3.0 software (AB Sciex). To verify tryptic digestion of peptides, cleavage control peptides consisting of the signature peptide with the two to three amino acids on the C- and N-terminal ends (Table 2) were incubated in solutions of bovine serum albumin in place of signature peptides and subjected to the trypsin digestion procedure. Mass spectrometric analysis was used to confirm that all cleavage control peptide was converted to signature peptide by the method. The chromatography gradient was developed to maximize the separation of the 14 peptides and allow for determination of retention times in a solution containing all 14 peptides together. For the selection of transitions to follow, each peptide was infused directly into the mass spectrometer and the most abundant ion was selected as the parent; either the (M), (M + H)⁺, (M + 2H)²⁺, (M + 3H)³⁺ or (M + 4H)⁴⁺ ion. These ions were then fragmented to identify the most abundant daughter ions detected by the spectrometer and the three most abundant Y-ions for each peptide were incorporated into a scheduled multiple reaction monitoring (sMRM) method along with the retention times identified from the peptide mixture.

2.6. Quantitative real-time RT-PCR

RNA was extracted from canine tissue samples stored in RNA^{later}® by homogenization of samples in buffer from the RNeasy Kit (Qiagen) and RNA isolation was performed with the RNeasy kit following manufacturer's recommendations. Reverse transcription of RNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen) with a no-RT control for each sample. Each reaction contained iQ™ SYBR® Green Supermix (Bio-Rad), 100 nM forward primer, 300 nM reverse primer and 10 ng template cDNA. HPRT was used as the housekeeping gene. Primers for canine gene sequences corresponding to each membrane transporter are listed in Table 1. PCR reactions were carried out in duplicate on an Mx3000P™ thermal cycler (Stratagene, La Jolla, CA) programmed to run an initial stage at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 1 min with fluorescence monitoring (42 cycles), and 72 °C for 30 s. Following the final cycle, temperature ramped back up to 95 °C for 1 min, then 55 °C for 30 s and back up to 95 °C with continuous fluorescence monitoring for dissociation curves. Average threshold values (Ct) were then used to compare expression of each individual gene to expression of Bcrp/Abcg2 in each tissue via the 2^{-Ct} method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

Accuracy of the calibration curves, coefficient of variation between calculated values for each of the transitions used for individual peptides, and precision of calibration curves was calculated. Table 4 shows the performance of the sMRM assays and the lower limit of quantitation for each signature peptide. Standard curves using 1:2 dilutions of cDNA generated from reverse transcriptase reactions using each primer pair showed accuracies for each of > 90%. Concordance between protein quantity and gene expression levels relative to

Bcrp/Abcg2 in each tissue was evaluated by Spearman correlation with significance set at $p = .05$.

3. Results

3.1. Identification of peptide mass transitions and development of sMRM method

Direct infusion of each synthesized signature peptide into the ESI-MS/MS system identified the most abundant precursor ion for each peptide in a Q1 scan (Fig. 1A) and subsequently, the three most abundant product ions (y-ions) were identified in Q3 (Fig. 1B) which provided the best sensitivity. A method was built to include three transitions for each peptide along with the retention time identified by injection of 10 fmol of peptide onto the chromatography column (Fig. 2). The resulting transitions and retention times for the scheduled MRM method are listed in Table 3. Trypsin digestion of the cleavage control peptides resulted in only MS/MS identification of the signature peptides and a lack of signal corresponding to the cleavage control peptide transitions, indicating complete trypsin digestion during sample processing.

3.2. Isolation of microvessels from canine brain tissue

Immunofluorescent staining for Factor VII and DAPI nuclear stain demonstrated that brain capillary fractions were effectively isolated from fresh and frozen brain tissues (Fig. 3) and provided adequate protein concentrations for trypsin digestion and LC-MS/MS analysis. The presence of DAPI stained nuclei in cells that had negative staining for Factor VII may represent co-isolation of microglial cells in the microvessel isolation protocol.

3.3. Protein expression levels of ATP-binding cassette transporters in canine tissue samples

The expression levels of 14 ABC transport proteins were determined in canine tissue membrane preparations obtained from 10 dogs. The chromatographic peaks identified from the sMRM method were quantified against the calibration curves generated from each peptide transition (Fig. 4) to calculate the fmol of peptide per milligram of protein. As can be seen in Table 4, 12 of the 14 transporters were quantifiable in at least one tissue type. Among all transporters, the MRP family demonstrated the lowest overall expression; for example, Mrp1 was identified in 4 of 6 tissues but was below the limit of quantitation in each of those tissues. Mrp9 was identified below the limit of quantitation in brain microvessels but was not detected in any of the other tissues and Mrp5 was not identified in any of the tissues examined. Mrp 2, 3 and 4 were only identified in renal cortex samples and all three had relatively similar expression levels (0.44 ± 0.11 , 0.32 ± 0.12 and 0.74 ± 0.6 fmol/ μ g protein, respectively). Similarly, Mrp 6 and 7 were only found in a single tissue each (liver and duodenum, respectively). Bcrp and P-gp were the most abundant proteins across tissues with quantifiable levels in all tissue types except ileum. While overall expression levels were highest in the brain microvessel samples, the largest degree of variability was also found in this sample set, particularly for Bcrp and P-gp. This result is similar to a study where transporters and metabolizing enzymes were quantified in freshly isolated human brain microvessels and, among ATP-binding cassette transporters, Bcrp and P-gp had the highest expression. With regard to tissue types, ileum had the lowest levels of

protein identified with only Mdr2 being found in quantifiable levels. Mdr2 (Abcb4) was also identified in canine liver and brain samples and was absent from duodenum samples which is in agreement with a previous study evaluating Abcb4 in canine tissues by mRNA and western blot (Spencer et al., 2010).

3.4. RNA expression levels of ATP-binding cassette transporters in canine tissue samples

Quantitative RT-PCR was used to compare the mRNA expression levels of the 14 membrane transport genes in the same tissues utilized for signature peptide analysis. The accuracy of amplification reactions was > 90% and melt curve analysis revealed only a single product for each reaction. Relative expression levels were calculated by comparison of threshold cycle (CT) values for each gene in comparison to Bcrp in each tissue. Bcrp was chosen as the reference because the mRNA was present in all tissues and, for analysis of correlations between mRNA and protein, Bcrp protein was measurable in more tissues than any other transporter. In all tissues we looked at, mRNA was present for more of the genes than were signature peptides. The relative expression of each mRNA along with the corresponding relative protein expression calculated from the signature peptide quantification is shown in Fig. 5. There was no significant correlation between relative mRNA and protein for any of the tissues examined.

4. Discussion

We have developed a method for the quantitative, multiplexed and focused proteomic analysis of membrane transporter proteins and applied this method to the analysis of ABC transporter proteins in normal dog liver, renal cortex, brain capillary endothelial cells, duodenum, jejunum and ileum. While this method assumes that there is a linear relationship between the amount of signature peptide identified and the amount of protein from which the peptide originated in vivo, it has been used successfully in the past to develop an atlas of membrane transporters in mouse tissues and to identify and quantitate liver cytochrome P450 enzymes and integral membrane proteins in human tissues including the brain (Kamiie et al., 2008; Kawakami et al., 2011). This method has also been applied to the analysis of CYP enzymes in beagle dogs with the goal of establishing metabolic scaling factors across species (Heikkinen et al., 2012). To overcome some of the problems that are inherent to mass spectrometric analysis of membrane-bound proteins, namely dissolution and ionization inhibition by lipids, we employed a de-lipidation step by chloroform extraction which has previously been shown to produce higher quality mass spectral data (Mirza et al., 2007). The full-length amino acid sequence of the 14 proteins was entered into the in silico trypsin digestion program to generate a list of potential signature peptides for further selection. Our peptide selection criteria were adapted from those previously reported; specifically, to allow the doubly charged precursor ions to fall within the detection window of the mass spectrometer (up to 1250 m/z), lack posttranslational modification such as N-glycosylation, and avoid the possibility of chemical modification of the peptide during operation by avoiding methionine and cysteine residues (Kamiie et al., 2008). A secondary set of selection criteria was then applied which eliminated peptides with stretches of arginine or lysine, included those peptides that were amenable to stable isotope-labeling (i.e. including

either leucine, isoleucine, valine or proline) and finally, avoiding peptides with histidine residues that can have a low sensitivity to electrospray ionization-mass spectrometric analysis. The peptides selected in our study provided strong signal intensities, and the use of multi-channel MRM analysis with more than one transition per peptide allowed for the differentiation of peptides from background or secondary peaks present in biological samples. In this study we identified that the highest levels of Bcrp and P-gp were found in the brain microvessels and that liver and renal cortex had the largest number of transporters expressed compared to other tissues. These results differ slightly from those seen in mouse tissues where Bcrp was identified in the highest concentration in renal cortex, while P-gp was found to have the highest level of expression in brain capillaries (Kamiie et al., 2008). We also identified the Abcb4/Mdr2 protein and mRNA present in brain and liver of dogs but not in duodenum or jejunum. This is consistent with an earlier report of canine Abcb4 which found the cDNA and protein by western blot to be present in liver and brain but not duodenum (Spencer et al., 2010). Interestingly, the previous report of canine Abcb4 identified ten sequence variations among four mixed-breed dogs and ten additional purebred dogs. Five of the identified variants were non-synonymous with altered amino acid sequences. It is important to note that the methods we applied here have specificity such that a variant amino acid within a signature sequence would result in no protein detected in a sample. Because canine reference sequences available in NCBI for protein blast are all derived from a single dog (the boxer), it is possible that the lack of expression of some transporters in some tissues will be due to signature peptide sequences containing variants unique to the boxer. With increasing amounts of information available on single nucleotide polymorphisms in dogs, the methods we describe here can be applied to the identification and quantitation of variant proteins in canine tissues without the need to develop and validate specific antibodies. With regard to metabolizing enzymes, this would lead to an ability to correlate levels of wild-type or variant proteins with enzymatic capability.

We have also attempted to identify the degree of correlation between the level of transcript and protein for these 14 transporters. In no cases were we able to detect protein without also identifying the presence of mRNA, which suggests that there is a low level of false positive protein identification. In some cases there was measurable mRNA in a tissue but protein levels were either not detectable or were below the lower limit of quantitation, preventing the ability to correlate their changes together relative to Bcrp. For those tissue and transporter combinations where both the mRNA and protein were detectable and could be compared relative to Abcg2/Bcrp, we found the direction of relative change to correlate only 61% of the time (11 of 18). This may be explained by different levels of control for gene products as has been described for Mdr1/Pg-p in leukemic cells and for multiple genes in yeast leading to discordance in the amount of mRNA and protein (Gygi et al., 1999; Yague et al., 2003). Furthermore, our peptide selection criteria did not include identifying those that were conserved across all known isoforms of the proteins and, as such, it is possible that in some cases only one protein isoform was measured and then compared to the relative mRNA expression, which could have led to identification of discordance. However, our results do suggest that relative mRNA levels may not be universally applied as a surrogate for measurement of protein levels in tissues, and direct quantitation of protein will be a more desirable method.

5. Conclusion

We have developed a sensitive and highly specific, simultaneous quantification method for membrane proteins and have applied this to a panel of 14 ATP-binding cassette transport proteins in normal dog tissues. These results can be incorporated into the development of physiologically based pharmacokinetic models in dogs to improve the cross-species predictions from these models. In addition, the methods here can be applied to the study of virtually any protein and could allow for the simultaneous, discrete quantitation of different protein isoforms within the same biologic sample, an application that is not currently possible using antibody-based assays.

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Fig. 1.

(A) Ion spectra for the parent signature peptide for Abcg8. (B) Fragmentation spectra with labeled Y-ions and B-ions determined from the table. This technique was used to determine the transitions providing the best signal to incorporate into the scheduled multiple reaction monitoring (sMRM) chromatography method for each of the 14 signature peptides.

Fig. 2. Chromatogram showing retention times of all peaks in a mixture containing each of the 14 signature peptides using 10 fmol of each peptide on column.

Fig. 3.

Factor VII staining following isolation of brain microvessels from frozen sections of brains obtained from normal dogs following necropsy. Microvessels were processed for protein isolation and trypsin digestion to measure signature peptides of membrane transport proteins.

Fig. 4. Example of scheduled MRM for P-glycoprotein signature peptide in a dog liver sample (top) and Multiquant® calibration curve regression for the three transitions identified for the P-glycoprotein signature peptide (bottom). Quantitation of all proteins was done by averaging the calculated concentrations from each of the transitions utilized in the regression analysis of the calibration curves.

Fig. 5.
Expression of protein and mRNA for membrane transporters in normal dog tissues relative to Bcrp/ABCG2. No significant correlation is found between the relative expression of mRNA and protein.

Unique signature peptides and forward and reverse qRT-PCR primers for each of the ATP-binding cassette transport proteins and genes evaluated in normal dog tissues.

Table 1

Gene/Protein	Signature Peptide	Forward Primer	Reverse Primer
Abcb1/Pgp	LANDAAQVK	CCCTCTGATTGACAGCTACAG	TCTGGTCGAGTGGGATAGTT
Abcb4/Mdr2	IATEAIENIR	AGCAGCTACCAAGACAAGAAA	GTGCCAGGGACATCAATAA
Abcb11/Bsep	STALQLIQR	GCAGCTTATTCAGCGTTTCTATG	CTCTTGCTCCACTATCCCAATC
Abcc1/Mrp1	NATFTWAR	CGGACAGAGATTGGTGAGAAAG	TCGAAGAGGTAGATGTCAGAGT
Abcc2/Mrp2	SSLTNGLFR	CGTCTATGGAGTTCTGGGATTAG	GTTGCCCTGTGAAAGGATGTTTG
Abcc3/Mrp3	ASGALIQEEK	GGTTTCTGTGTGTGTCTGT	GTAGAAAGGTGGTGAAGTGGAAAG
Abcc4/Mrp4	TATFIDVR	GGACCATCCGGGCATATAAAG	GGGACGTAGTCAGAAACAAGAA
Abcc5/Mrp5	SLSEASVAVDR	TCCAAGAGAGACCATCCGAGAA	GGCCAGCACCCATAATCCTATC
Abcc6/Mrp6	SSLAGGLR	CAACCTCCCTTCTTCCCTAAAG	CCAGAAACCCACCAGAACAT
Abcc10/Mrp7	GAVLNILYR	TCACCCATCACCTCTCTACAC	TCTCTGGTTCTCCTCCTCAAA
Abcc12/Mrp9	FTSVELLR	GGCTGCACGGTATTGACTAT	AGCAAGGACTTCAGGCTTTATC
Abcg2/Bcrp	SSLLDVLAAR	CGTGCTCGAGGAGAAAGAAA	TTGCTGTGCTGGAGTCTAAG
Abcg5	QTLNIER	CCAAGACGGATTGTACCAGAAAG	CAGTAGCACACACTGCTGAATA
Abcg8	ASLLDVITGR	GGCAACTTCACCATCCCTATT	CACCGATGCCAATGAGGATTA

Table 2

Cleavage control peptides used to verify complete trypsin digestion during the sample preparation protocol. Corresponding signature peptide sequence is underlined.

Protein	Cleavage control peptide
P-gp	TTRLANDAAQV <u>K</u> GAI
Mdr2	AGKIATENA <u>I</u> ENIRTVV
Bsep	SGK <u>S</u> TALQLIQRFYD
Mrp1	TVKNATFTWARS <u>D</u> P
Mrp2	AGKSSLTNGLFRI <u>L</u> E
Mrp3	AKASGALIQEE <u>K</u> AE
Mrp4	SKTATFTDVR <u>I</u> R
Mrp5	SVKSLSEASVAVDR <u>F</u> K
Mrp6	GKSSLAGGLLR <u>L</u> L
Mrp7	QARGAVLNILYRK <u>A</u> L
Mrp9	AKFTSVELLRE <u>Y</u>
Bcrp	SKSSLLDVLAARK <u>D</u>
Abcg5	YRQTLENIERT <u>K</u>
Abcg8	GRASLLDVITGR <u>D</u> H

Table 3

Unique amino acid sequences for each of the 14 ATP-binding cassette transporters, mass of peptide, three transitions chosen for the scheduled multiple reaction monitoring method and the chromatographic retention time of each. Stable isotope labeled peptides were utilized as internal standards.

Protein	Signature Peptide	Mass	Q1	Q3-1	Q3-2	Q3-3	Retention (min)
Mdr2	IATEAIENIR	1129.6	565.2	644.4	473.2	185.1	20.7
Bsep	STALQLIQR	1029.6	515.3	529.4	657.4	242.2	22.0
Mrp1	NATFTWAR	966.5	487.3	186.1	680.3	781.3	21.4
Mrp2	SSLTNGLFR	994.5	497.5	707.3	606.3	410.8	22.8
Mrp3	ASGALIQEEK	1046.2	523.2	646.3	533.2	759.3	16.2
Mrp4	TATFTDVR	910.5	455.5	637.3	274.2	173.0	17.4
Mrp5	SLSEASVAVDR	1133.6	567.3	646.4	201.2	558.2	20.7
Mrp6	SSLAGGLR	873.5	437.5	586.4	515.3	288.2	21.7
Mrp7	GAVLNILYR	1018.6	509.7	678.4	338.1	228.0	25.4
Mrp9	FTSVELLR	964.5	482.7	716.5	249.1	530.3	23.3
Bcrp	SSLLDVLAAR	1044.6	522.6	644.5	757.4	430.4	28.2
Abcg5	QTLNIENIR	1002.5	501.8	531.3	660.3	492.7	17.1
Abcg8	ASLLDVITGR	1044.6	522.8	660.4	545.3	446.4	30.1
P-gp	LANDAAQVK	929.1	464.9	745.2	631.2	516.3	13.8
Stable Isotope Labeled Internal Standards							
Bsep	STA[L ^{C13N15}]QLIQR	1036.5	518.8	657.4	NA	NA	22.0
Mrp1	NAT[F ^{C13N15}]TWAR	975.6	488.6	533.5	NA	NA	21.4
Bcrp	SSLLD[V ^{C13N15}]LAAR	1051.5	526.4	651.4	NA	NA	28.2

Signature peptide quantitation for 14 ATP-binding drug transport proteins in membrane fractions of normal dog tissue collected from 10 dogs.

Table 4

Protein	Tissue membrane protein concentration (fmol/ μ g protein)						
	Brain	Liver	Renal Cortex	Duodenum	Jejunum	Ileum	
Abcg5	NF	9.22 $\pm$ 5.0	NF	NF	NF	NF	
Abcg8	NF	11.12 $\pm$ 1.3	2.39 $\pm$ 1.4	NF	NF	NF	
Bcrp	145.54 $\pm$ 163.19	2.14 $\pm$ 0.50	1.26 $\pm$ 0.86	0.95 $\pm$ 0.20	1.17 $\pm$ 0.27	NF	
Bsep	NF	7.36 $\pm$ 2.1	< LLOQ	NF	NF	NF	
Mrp1	< LLOQ	< LLOQ	< LLOQ	NF	< LLOQ	NF	
Mrp2	NF	NF	0.44 $\pm$ 0.11	NF	NF	NF	
Mrp3	NF	NF	0.32 $\pm$ 0.12	NF	NF	NF	
Mrp4	NF	NF	0.74 $\pm$ 0.6	NF	NF	NF	
Mrp5	NF	NF	NF	NF	NF	NF	
Mrp6	NF	4.81 $\pm$ 1.3	NF	NF	NF	NF	
Mrp7	NF	NF	NF	1.17 $\pm$ 0.38	NF	NF	
Mrp9	< LLOQ	NF	NF	NF	NF	NF	
Mdr1	80.4 $\pm$ 48.5	2.2 $\pm$ 1.0	2.55 $\pm$ 0.63	0.17 $\pm$ 0.06	0.4 $\pm$ 0.16	NF	
Mdr2	28.7 $\pm$ 43.0	4.59 $\pm$ 1.2	NF	NF	NF	5.1 $\pm$ 2.5	