Lost in Centrifugation! Accounting for Transporter Protein Losses in Quantitative Targeted Absolute Proteomics (QTAP)

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Introduction:
Incorporating the abundances of enzyme and transporter proteins into Physiologically-Based Pharmacokinetic Models (PBPK) facilitates a fully mechanistic In Vitro–In Vivo Extrapolation approach for the prediction of whole body drug disposition. Transporter protein abundances obtained from Quantitative Targeted Absolute Proteomics (QTAP) strategies are generally reported in membrane preparations obtained by differential centrifugation. However, it is inevitable that target protein losses occur during the fractionation procedure. Thus, accounting for losses of target proteins by application of recovery factors (RFs) is necessary for meaningful incorporation of protein abundance in biological applications, i.e. in PBPK models, that aim to accurately reflect levels in the biological system. A strategy to account for protein losses is proposed to facilitate the determination of absolute protein abundances in cells and organs for their subsequent incorporation into PBPK models.

Methods:
• Caco-2 cells (ATCC, HTB-37, passage 25-35) were grown on 44-cm² Transwell filters (0.4 μm pore size) for 10, 16 and, 29 days on filters.
• Cells were scrape harvested, lysed overnight and the starting whole cell lysate (total protein (TP), fraction 1) was sampled. Differential centrifugation of the lysate isolated a total membrane (TM, fraction 2) and an end-point plasma membrane (PM, fraction 3) fraction. A BCA assay was run in TP, TM & PM fractions to determine protein content.
• The protein samples were digested using an adapted deoxycholate, Lys-C and trypsin digest protocol and standard isotope labelled (SIL) proteotypic peptides for the basal membrane marker protein, Na⁺/K⁺-ATPase (ATPA1), and the apical membrane marker protein, Villin (VILI), were spiked into the digested Caco-2 fractions.
• Digests were analysed using an LC-MS/MS system and selected reaction monitoring in a positive ionisation mode to determine peptide abundances.

Results:
A. The Expected Enrichment
Estimates of the protein content of cell or tissue fractions are prerequisites of individual transporter quantification (Fig. 1). The expected protein enrichment of the sub-cellular fraction from differential centrifugation is ascertained by measuring the protein content in the initial TP and subsequent TM and PM fractions (Eq. 1). The Caco-2 PM fraction constitutes 1% of the TP fraction, thus, VILI and ATPA1 abundances are expected to be 100-fold greater in the PM fraction compared to the TP fraction.

B. The Actual Enrichment in Abundance
To determine the actual enrichment in abundance a QTAP LC-MS/MS strategy was applied to the TP, TM and PM fractions. The abundances of ATPA1 and VILI are provided in Fig. 2. There is a step-wise increase in ATPA1 and VILI abundances from TP (fraction 1) to PM (fraction 3) as expected. However, ATPA1 and VILI abundances (Eq. 2) do not reach the expected 100-fold enrichment in the PM fraction (Fig. 1), indicating that losses of target protein occur during the fractionation procedure.

Discussion and Conclusion:
• An approach to correct for targeted protein losses after centrifugation is postulated by combining data generated from protein assays and QTAP abundance methods.
• We show that for the membrane markers ATPA1 and VILI there is only 4.9-fold and 15.5-fold increase, respectively, in abundance when applying RFs.
• Membrane-associated proteins losses are likely to arise from contamination of;
  1) discarded soluble fractions with ‘non-soluble’ membrane components
  2) discarded nuclear/mitochondrial laden pellets with ‘non-soluble’ membrane components prior to obtaining the TM fraction
  3) gradient centrifugation layers contaminated with PM fractions
→ The corrected abundance values are required for incorporation into PBPK models.

References:
1. Harwood et al., 2013, BDD, 34, 2-8
2. Balogh et al., 2012, Proteomics & Bioinformatics, 54, 1-5