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[1]. 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 (RF's) 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-cm^2 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[2] 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 ionsiation 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.

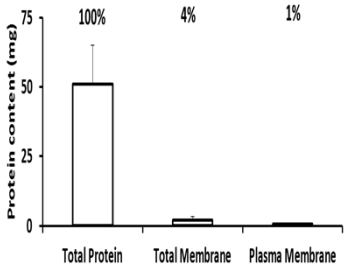

Fig 1. The protein content (mg) and yield as a percentage of the original TP fraction (values above bars) is shown for each Caco-2 fraction (n=4). **From this data it is expected that there is 100-fold enrichment from the original total protein fraction to the plasma membrane fraction.** Therefore, the abundances of the plasma membrane proteins, ATPA1 and VILI, should also show 100-fold enrichment when measured in TP compared to PM fractions.

Equation 1. $$\text{Expected Enrichment} = \frac{\text{Total Protein Content}}{\text{Plasma Membrane Protein Content}}$$

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.

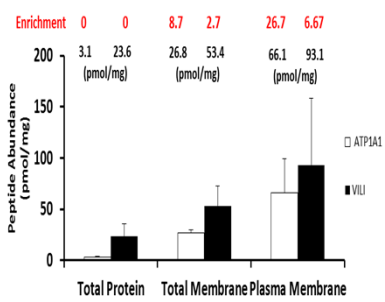

Fig 2. Abundance of ATPA1 and VILI in Caco-2 fractions (n=4). The mean abundances are given (black text, values above bars in pmol/mg). The fold enrichment in abundances relative to the TP fraction are provided (red text, values above bars) for each peptide.

Eq. 2. $$\text{Actual Enrichment (PM)} = \frac{\text{Protein Abundance (PM)}}{\text{Protein Abundance (TP)}}$$

C. Recovery Factors (RF's)

To correct for losses of protein during centrifugation, a RF (**Table 1**) is determined as the ratio of the expected and actual enrichments for a transporter isoform (**Eq. 3**) and is used to correct the abundances generated in the membrane fraction (**Eq. 4** & **Fig. 3**).

Eq. 3. $$\text{Recovery Factor (RF)} = \frac{\text{Expected Enrichment}}{\text{Actual Enrichment}}$$

Table 1. Recovery factors are generated (Eq. 3) for each sample and peptide.

	ATP1A1	VILI
Sample	**Recovery Factor**	**Recovery Factor**
Caco-2 -10d (sample 1)	5.16	26.65
Caco-2 -10d (sample 2)	3.24	9.41
Caco-2 -16d	8.45	40.55
Caco-2 -29d	5.34	64.24
Mean	**5.55**	**35.21**
SD	**2.16**	**23.17**

Eq. 4. $$\text{Corrected Abundance (PM Fraction)} = \text{PM Abundance} \cdot \text{RF}$$

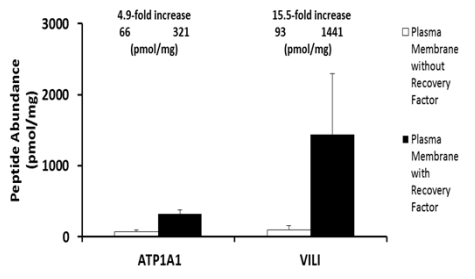

Fig. 3. The corrected abundances for ATPA1 and VILI from Caco-2 fractions (n=4) is given after applying the corresponding RF (**Eq. 4**). The fold enrichment in abundance is provided after RF application with the corrected abundances (values above bars).

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 RF's.
- 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, 2-28
2. Balogh *et al.*, 2012, Proteomics & Bioinformatics, 54, 1-5