Development of an LC-MRM method for quantification of P-glycoprotein

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Objectives

- To develop a quantification assay for P-glycoprotein (P-gp) using LC-MRM methodology.
- To correlate the expression level of P-gp with corresponding transcript and functional data when comparing measurements obtained from 10 *vs* 29 days of culture period of Caco-2 cells.

Introduction

P-gp belongs to the superfamily of ABC-transporters (ATP binding cassette), which utilise ATP hydrolysis to actively pump their substrates across membranes. Active transport of drugs across membranes has increasingly been recognized as an important function for drug uptake, disposition and excretion [1]. Several different in-vitro methods have been developed in order to study the role and function of drug transporters. However, the current models only indicate qualitatively whether the studied drug is a substrate or inhibitor of a specific transporter. Methods to predict the quantitative importance of the individual transporter and scaling of in-vitro studies to in-vivo is highly warranted. Recently, Li *et al.* published an LC-MRM based methodology for quantification of multidrug resistance-associated protein 2(MRP2/ABCC2) [2]. Quantification of the expression level of transporters in the in-vitro system and the corresponding expression level in the organ will enable scaling of in-vitro data and hereby facilitate PK prediction in humans.

Materials and Methods

Cell culture

Caco-2 cells were purchased from ATCC (Rockville, MD, USA) at passage 18. The cells were routinely sub-cultivated by trypsination using trypsin (0.05%) - EDTA (0.02%) solution and seeded at a density of 2.0 x 10^6 cells per 175-cm^2 flask. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% heat inactivated foetal calf bovine serum, 1% non-essential amino acids and 1.5% L-glutamine, in an atmosphere of 95% air and 5% CO_2 at 37°C. The cells were seeded at passage 39 with an initial density of 2.5 x 10^5 cells per 1.13 cm^2 filter with a pore size of 0.4 µm (Transwell®). Functional study and mRNA was isolated from Caco-2 cells after 10 and 29 days in culture on filters.

Extraction of membrane fraction

The cell pellets (~20 million cells) were resuspended in 10 volumes of ice-cold 10mM $NaHCO_3$ pH 8 followed by adding a protease inhibitor cocktail according to the instructions of the manufacturer (Roche). The cells were allowed to swell for 10 minutes. Then the cells were subjected to lysis using a glass Dounce homogenizer (25 strokes). Nuclei, unbroken cells and mitochondria were pelleted by centrifugation (10000xg, 10 min, 4 ºC). The post-nuclear supernatant, containing the membranes of interest, was collected and incubated with 100mM Na_2CO_3 for 20 min while disrupting and stirring the membranes by bath sonication in order to disrupt bonds between the membranes and the proteins associated to them by electrostatic interactions. After the carbonate wash the membrane material was transferred to a centrifuge tube and pelleted by ultracentrifugation (120.000xg, 1.5 h, 4 ºC). The supernatant was discarded and the membrane pellet was resuspended in 50mM AMBIC, using tip-sonication to properly disperse the sample. The membrane samples were stored at -80 ºC for future analysis. Total protein concentrations of the various membrane fractions were determined with the DC protein assay kit (Bio-Rad).

Schematic illustration of the LC-MRM method development for P-gp quantification

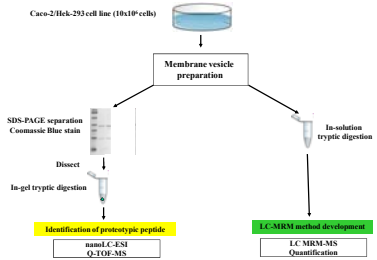

Figure 1. SDS-page separation of the membrane vesicle preparation was subsequently followed by nano LC-ESI MS/MS to identify the proteotypic peptide. Synthetic proteotypic peptide and its corresponding stable isotope labelled variant was purchased and employed for the optimization of LC and MS (MRM) parameters. The Agilent MassHunter Optimizer Software for peptides (B.03.0014) was used for optimization of the fragmentor voltages and collision energies. With the establishment and optimization of the MRM assay, the membrane fraction of the biological sample is subjected to LC-MS/MS analysis on a QQQ instrument after in-solution tryptic digestion.

LC-MRM set up

Figure 2. An Agilent 1290 Infinity LC system was coupled to an Agilent 6460 (Jet Stream) Triple Quadrupole instrument.

Conditions

Column:	Waters Acquity UPLC,1.0 x 50 µm, 1.7µm BEH C18
Mobile phase:	A= 0.1% FA in water, B= 0.1% FA in acetonitrile
Injection volume:	10 µL
Column temperature:	40 ºC
Flow rate:	0.3 mL/min
Gradient:	3% to 55% B in 10 min, 2 min washing, 2min re-equil.
Polarity:	Positive ion mode
Drying gas temp.:	300 ºC
Drying gas flow:	6 L/min
Sheath gas:	350 ºC
Capillary:	4500 V
Transitions:	635.4 → 971.6, 635.4 → 900.5, 635.4 → 771.5
Dwell time:	50 ms

Results

Identification of proteotypic peptide

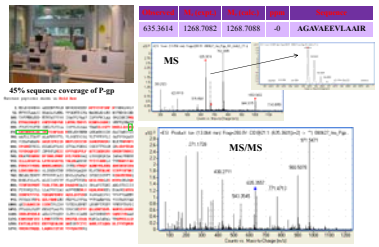

Figure 3. The tryptic fragment of AGAVAEEVLAAIR (m/z 635.4 for doubly charge) was selected as the proteotypic peptide for P-gp and was identified on an Agilent 6520 chip QTOF. The MS spectrum and the corresponding product ion mass spectrum showing the fragment ions are illustrated in the spectra above.

Standard calibration curve

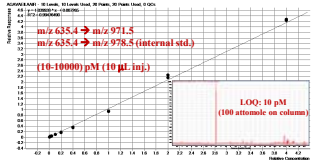

Figure 4. The standard calibration curve was prepared at concentrations of 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, and 10000 pM of the proteotypic peptide with fixed concentration (2500 pM) of the corresponding stable isotopic labelled peptide that was used as an internal standard. Each standard point was measured in duplicate at the front and back of each run to bracket the unknowns. The insert illustrates the extracted MRM transition of 635.4 → 971.6 at the lowest point of the standard curve (10 pM).

Quantification of P-gp using LC-MRM

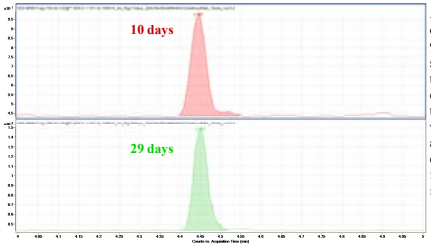

Figure 4. Extracted ion chromatograms of MRM monitoring at m/z 635.4 → 971.6 transition for the tryptic digested sample from the membrane extract of the Caco-2 cells at 10 and 29 days of cell growth, respectively. By applying the LC-MRM method the P-gp conc. was determined to ~1080 pM (10 days) and ~4190 pM (29 days). The coefficient of variation was below 5% for both measurements (triplicate injections).

P-gp protein quantification correlated with mRNA expression and digoxin efflux (P-gp functionality) in Caco-2 cells grown for 10 and 29 days, respectively

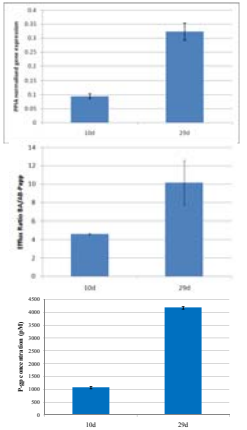

Figure 5. P-gp (ABCB1) mRNA expression in Caco-2 cell monolayers grown on polycarbonate filters for 10 respective 29days. Relative expression is normalised against reference gene PPIA. Expression levels of ABCB1 mRNA increased 3.4-fold between 10 and 29 days in culture.

Figure 6. Efflux of the P-gp-specific probe substrate digoxin in Caco-2 cell monolayers grown on polycarbonate filters for 10 respective 29 days. Efflux is expressed as ratio between vectorial permeability over the cell monolayer in BA-direction (supported by the transporter) and in AB-direction (against the transporter). Efflux of P-gp increased 2.2 fold between 10 and 29 days in culture.

Figure 7. P-gp protein expression in Caco-2 cell monolayers grown on polycarbonate filters grown for 10 respective 29days. P-gp expression is normalised against internal standard (stable isotope labelled variant of the proteotypic peptide). Expression levels of P-gp increased 3.9-fold between 10 and 29 days in culture.

References

1. N. Mizuno, *et al.*, Pharmacol. Rev. 55 (2003) 425-461.
2. N. Li, *et al.*, Anal. Biochem., 380 (2008) 211-222.

Conclusions

- Successful implementation of LC-MRM methodology for P-gp quantification.
- P-gp protein expression correlated with mRNA expression and digoxin efflux (P-gp functionality).
- Quantitative data of transporters will be a key tool for performing scaling from preclinical studies to man.

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