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Comparison of liquid chromatography–tandem mass spectrometry-based targeted proteomics and conventional analytical methods for the determination of P-glycoprotein in human breast cancer cells

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ABSTRACT

P-glycoprotein (P-gp) is the most frequently proposed factor for multi-drug resistance. It is traditionally measured using antibody-based methods. While these techniques can provide relative quantification values for P-gp levels, the important information that is usually missing is its amount in the biological system. In this study, a novel and advanced liquid chromatography–tandem mass spectrometry (LC/MS/MS)-based targeted proteomics assay was developed and validated for the determination of P-gp in the breast cancer drug sensitive cell line MCF-7/WT and the drug resistant cell line MCF-7/ADR. Three tryptic peptides (434STTVQLMQR442, 674GSQAQDR680 and 368IIDNKPSIDSYSK380) can specifically represent P-gp. Among these peptides, 434STTVQLMQR442 was selected as the surrogate analyte for quantification, and a stable isotope-labeled synthetic peptide with the same sequence was used as an internal standard. The calibration range was validated from 10 to 1000 ng/mL. The intra- and inter-day precisions were within 5.9% and 3.7%, respectively. The accuracy for the quality control (QC) samples was within 8.0%. Using this assay, the amounts of P-gp were accurately quantified as 3.53 fg/cell ($\sim 2.08 \times 10^{-2}$ amol/cell) in the MCF-7/WT cells and 34.5 fg/cell ($\sim 2.02 \times 10^{-1}$ amol/cell) in the MCF-7/ADR cells. This outcome was then compared with those obtained by conventional analytical methods including confocal microscopy, western blotting and flow cytometry. The comparative results show that not only is the LC/MS/MS-based targeted proteomics assay able to monitor the protein levels in a more accurate manner, but the large discrepancy observed between the other methods was most likely due to the lack of specificity and the semi-quantitative nature of the conventional assays

1. Introduction

Resistance to multiple chemotherapeutic drugs frequently develops during the treatment of various cancers, including breast cancer [1]. Thus far, a number of mechanisms have been suggested to explain the development of drug resistance [2]. The most frequently proposed mechanism is associated with an increased level of P-glycoprotein (P-gp) – the product of the MDR1 gene. As an energy-dependent drug efflux pump, P-gp can cause a deficient accumulation of drugs in cells [3]. In addition to the effects of multi-drug resistance, the altered expression of P-gp has also been correlated with a variety of biological processes, such as disease susceptibility [4], drug–drug interactions [5] and lipid transport [6]. However, the important information usually missing in the description of P-gp's role and function in the context of systems biology is its amount in the biological system.

The expression of P-gp has traditionally been measured using antibody-based methods, including western blot analysis. While these techniques provide valuable information on protein levels, they often lack the necessary specificity and reproducibility [7]. Other analytical approaches available for measuring P-gp, such as confocal (fluorescence) microscopy and conventional flow cytometry, also involve the same challenges that are inherent in the antibody-based probes. In addition, the results that are obtained using these assays are potentially complicated by the fact that the protein may exist in several forms [8]. More importantly, most of the assays that have been developed are qualitative or semi-quantitative, but not quantitative [9]. Thus, there is an incentive to develop an accurate and precise P-gp quantitative assay.

Mass spectrometry techniques, such as the "shotgun" strategy, have been used as powerful tools for the relative quantitative

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measurement of proteins on a proteomic scale [10,11]. However, these approaches are generally non-targeted. For example, in each measurement, they stochastically sample a fraction of the proteome that is usually biased toward the higher end of the abundance scale [12,13]. Since there is a growing interest in a relatively small number of proteins (instead of the whole proteome), targeted proteomics offering high sensitivity, selectivity and a wide dynamic range has recently increased in popularity [14]. In a targeted analysis, selected reaction monitoring (SRM or MRM) on a triple quadrupole instrument is generally employed for quantification, and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) assays are developed to detect fragment ion signals from proteolytic peptides representing the targeted protein [15]. To date, several LC/MS/MS-based targeted proteomics assays for the quantification of specific membrane transporters have been developed [16–19]. As reported, these assays have greater sensitivity than the immunoassays [17]. However, it is noteworthy that no standard exists that would allow objective validation and cross comparison among different studies (or different laboratories). Because of this knowledge gap, we examined the correlation between the P-gp expression level determined using LC/MS/MS-based targeted proteomics assay and those obtained with conventional analytical methods.

In this report, a novel and advanced LC/MS/MS-based targeted proteomics assay for the quantification of P-gp was developed and validated. A stable isotope-labeled synthetic peptide was used as an internal standard. Finally, this assay was applied to the quantitative analysis of P-gp in the breast cancer drug sensitive cell line MCF-7/WT and the drug resistant cell line MCF-7/ADR. The resulting values were compared to those obtained using confocal microscopy, western blotting and flow cytometry.

2. Materials and methods

2.1. Chemicals and reagents

The synthetic proteotypic peptide and its corresponding stable isotope-labeled internal standard were developed by ChinaPeptides Co., Ltd. (Shanghai, China). Human serum albumin (HSA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium bicarbonate (NH_4HCO_3) was obtained from Qiangshun Chemical Reagent Co., Ltd. (Shanghai, China). DL-dithiothreitol (DTT), iodoacetamide (IAA), Tris–HCl and Triton X-114 were supplied by Sigma–Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Phosphate buffered saline (PBS) was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Acetonitrile (ACN) and methanol were obtained from Tedia Company, Inc. (Fairfield, OH, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were provided by Aladdin Chemistry Co., Ltd. (Shanghai, China) and Xilong Chemical Industrial Factory Co., Ltd. (Shantou, China), respectively. Dulbecco's Modified Eagle Media (DMEM) and fetal bovine serum were obtained from Invitrogen (Burlington, ON, Canada). Penicillin was supplied by CSPC Zhongnuo Pharmaceutical Co., Ltd (Shijiazhuang, China). Streptomycin was obtained from Merro Pharmaceutical Co., Ltd (Dalian, China). Trypan blue and sodium dodecyl sulfate (SDS) were obtained from Generay Biotech Co., Ltd (Shanghai, China). Water was purified and deionized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA).

2.2. Preparation of stock solutions, calibration standards and quality controls (QCs)

A 1 mg/mL stock solution was prepared by accurately weighing the peptide and dissolving it in deionized water. The solution was stored at −20 °C in a brown glass tube to protect it from light. An isotope-labeled synthetic peptide was used as an internal standard. Details about the selection of the internal standard are described below. The internal standard was also weighed, and a 5 μg/mL stock solution was prepared in deionized water. A 250 ng/mL internal standard solution was prepared by diluting the stock solution with an ACN:water mixture (50:50, v/v) containing 0.1% FA.

Since matrix complexity is one of the significant obstacles in the quantification of endogenous analyte in which a true blank is not available, two kinds of surrogate matrices (i.e., 5% HSA in PBS [18] and P-gp depleted membrane fraction [20]) were employed here. The experimental detail of immuno-depleted matrix preparation is given in the supplementary material. The calibration standards were prepared by serial dilution of the stock solution. The concentrations of the calibration standards were 10, 25, 50, 100, 250, 400, 700 and 1000 ng/mL. The QC standards for lower limit of quantification (LLOQ), low QC, mid QC and high QC were prepared at 10, 30, 200 and 800 ng/mL, respectively, in the same matrix and frozen prior to use.

2.3. Cell culture and membrane protein extraction

The MCF-7/WT (ATTC, Manassas, VA) and MCF-7/ADR (Keygen Biotech, Nanjing, China) cells were cultured in a DMEM media supplemented with 10% fetal bovine serum, 80 U/mL penicillin and 80 μg/mL streptomycin at 37 °C and 5% CO_2. The cells were split every 5–7 days by lifting cells with 0.25% trypsin, and feeding between splits was accomplished through the addition of fresh medium. To maintain a highly drug-resistant cell population, MCF-7/ADR cells were periodically reselected by growing them in the presence of 1000 ng/mL DOX [21]. Experiments were performed using the cells incubated without DOX for 48 h and cells were counted with a hemocytometer (Qiujing, Shanghai, China). Cell viability was assessed by trypan blue (0.4%) exclusion, which was completed by mixing the cell suspension, trypan blue and 1× PBS in a 2:5:3 ratio and counting the percentage of viable cells following a 5 min incubation at 37 °C.

As one of the most powerful tools for preparing membrane proteins [22], Triton X-114 was applied to extract the P-gp. The extraction of membrane proteins in Triton X-114 has been described previously [22]. Briefly, cells were pelleted at 1480 × *g* for 10 min. Then, they were resuspended in 500 μL of the 1% Triton X-114 extraction buffer (1 mM DTT, 2 mM EDTA in 50 mM Tris/HCl, pH 7.4) containing a protease inhibitor cocktail. After an incubation period of 30 min on ice and 10 min at 37 °C, the samples were spinned at 10,000 × *g* for 3 min to separate the detergent and aqueous phases. To achieve a complete extraction, 500 μL of the 1% Triton X-114 extraction buffer and 500 μL of a 0.06% Triton X-114 wash buffer (1 mM DTT, 2 mM EDTA in 50 mM Tris/HCl, pH 7.4) were added to the aqueous phase and the detergent phase, respectively. The incubation and centrifugation steps were repeated. The detergent phases from the consecutive extractions were pooled and the proteins were precipitated in cold acetone. After precipitation, all protein pellets were dissolved in a 1% SDS solution. Protein concentrations of the obtained membrane fraction were determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.4. SDS–PAGE and in-gel tryptic digestion

The membrane fraction was separated using a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The protein band corresponding to the migration size for P-gp (170 kDa) was cut out and dissected into 1–2 mm pieces. After the gel pieces were dehydrated twice with ACN, they were reduced using 10 mM DTT for 20 min at 60 °C, followed by 50 mM IAA for 6 h at room

temperature. The pieces were then washed with 50 mM NH_4HCO_3 for 10 min and dehydrated again with ACN for approximately 5 min. Finally, the gel pieces were dried in a Savant SpeedVac and were incubated with sequencing grade trypsin at 37 °C for 24 h. The reaction was stopped by adding 10 μL of 0.1% TFA. The proteins in the gel pieces digested by trypsin were extracted with a solution containing 5% formic acid in water, followed by 5% formic acid in water/ACN (50:50). The peptide extract was stored at −20 °C before analysis.

2.5. In-solution tryptic digestion

A quantity of 100 μL of each sample (calibration standards, QCs and cell membrane fraction, ~5 mg) was mixed with 50 μL of 50 mM NH_4HCO_3. Denaturation was performed at 95 °C for 8 min. Subsequently, the protein was reduced by an addition of 50 mM DTT until a final concentration of 10 mM was achieved. The sample was then incubated at 60 °C for 20 min. The sample was then alkylated by adding 400 mM IAA to obtain a final concentration of 50 mM and incubated at room temperature for 6 h in the dark. Finally, sequencing grade trypsin was added, and the sample was incubated at 37 °C for 24 h. The reaction was stopped by adding 10 μL of 0.1% TFA. Then, 100 μL of the internal standard solution (250 ng/mL) was added to the tryptic peptide mixture before transferring it into an Oasis HLB cartridge (60 mg/3 mL; Waters, Milford, MA, USA) that was preconditioned with 3 mL ACN and 3 mL deionized water. After the sample was loaded, the cartridge was washed with 2 mL of water and 2 mL of ACN:water (50:50, v/v) and eluted with 1 mL of 100% ACN. Finally, the eluent was evaporated to dryness, and the sample was resuspended in 100 μL of ACN:water (50:50, v/v) containing 0.1% FA.

2.6. Stable isotope-labeled peptide internal standard

The peptide extract obtained from in-gel digestion was assessed using matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The most intense tryptic peptides identified in the MALDI-TOF mass spectrum were selected to verify its specificity for P-gp. Their sequences were searched for exact matches using BLASTP against the genome-derived human Ensembl peptides in Ensembl Blastview (www.ensembl.org/Homo_sapiens/blastview) [11]. The sequence unique to P-gp was used to design the stable isotope-labeled internal standard. The stable isotope-labeled amino acid was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

2.7. LC/MS/MS

An Agilent Series 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) and a 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) were used for the LC/MS/MS studies.

The liquid chromatography separations were performed on a hypersil gold column (3 μm, 20 mm × 2.1 mm; Thermo Fisher Scientific, USA) at room temperature. The mobile phase consisted of solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol). A linear gradient with a flow rate of 0.3 mL/min was applied in the following manner: B 10% (0 min) → 10% (1 min) → 90% (4 min) → 90% (8 min) → 10% (9 min). The injection volume was 10 μL.

The mass spectrometer was interfaced with an electrospray ion source and operated in the positive MRM mode. Q1 and Q3 were both set at unit resolution. The flow of the drying gas was 10 L/min and the drying gas temperature was held at 350 °C. The electrospray capillary voltage was optimized to 4000 V. The nebulizer pressure was set to 45 psi. The data were collected and processed using the Agilent MassHunter Workstation Software (version B.01.04).

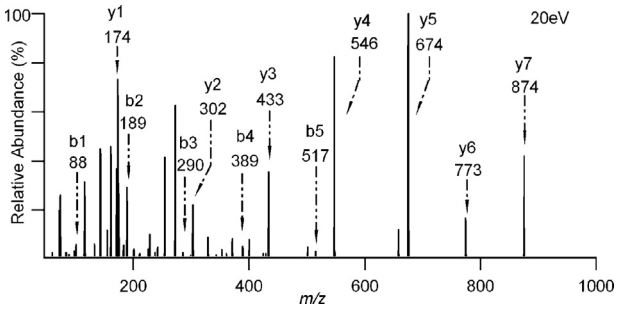

Fig. 1. The product ion spectrum of 434STTVQLMQR442.

2.8. Method validation

Method validation involves evaluating the linear range, accuracy, precision, limit of quantification (LOQ) and recovery. The detailed procedures and the acceptance criteria used to validate the assay have been described in a number of publications [23].

2.9. Conventional analytical methods

For the experimental details of confocal microscopy, western blotting and flow cytometry, please see the supplementary material.

3. Results and discussion

3.1. Electrospray response of tryptic peptides

The most critical step in the experimental design and assay establishment is the selection of tryptic peptides that represent a candidate protein and could provide specificity and an adequate response. Because ionization suppression may be caused by other co-eluting peptides, it is difficult to predict the electrospray ionization efficiency of a peptide in a complex mixture. Therefore, a full scan LC/MS/MS analysis was performed to identify the tryptic peptide with the greatest abundance. The most intense peptides were the doubly charged ion of 434STTVQLMQR442 and 674GSQAQDR680, and the triply charged ion of 368IIDNKPSIDSYSK380. The presence of tryptic peptides was also confirmed in the MALDI mass spectrum (data not shown). In addition, these sequences were found to be unique to P-gp (accession no. P08183 (MDR1_HUMAN), gi: 2506118) using a BLAST search, suggesting that they could be used to specifically quantify P-gp. The sequences were also consistent with the previous evidence (http://www.peptideatlas.org) [24].

Notably, the retention of the peptide in reversed phase chromatography is generally proportional to how many hydrophobic residues it contains [25]. Because 674GSQAQDR680 contains a majority of hydrophilic amino acids (retention time ~0.4 min), it was not employed in quantification here. In addition, as the selected peptides of 434STTVQLMQR442 and 368IIDNKPSIDSYSK380 can achieve similar selectivity and sensitivity in LC/MS/MS, only the data for 434STTVQLMQR442 is presented in this study.

The product ion spectrum of 434STTVQLMQR442 is shown in Fig. 1. The characteristic sequence-specific b ions (*m/z* 88(b1), *m/z* 189(b2), *m/z* 290(b3), *m/z* 389(b4) and *m/z* 517(b5)) and y ions (*m/z* 174(y1), *m/z* 302(y2), *m/z* 433(y3), *m/z* 546(y4), *m/z* 674(y5), *m/z* 773(y6) and *m/z* 874(y7)) were indicative of this peptide. Therefore, a synthetic stable isotope-labeled peptide, STTV*QLMQR, was prepared. A stable isotope-labeled valine with an added mass of 8 Da from deuterium was coupled to the peptide sequence at positions 4 to yield a molecular mass shift of 8 Da from the non-labeled peptide and a monoisotopic molecular mass of 1071 Da. In addition, its

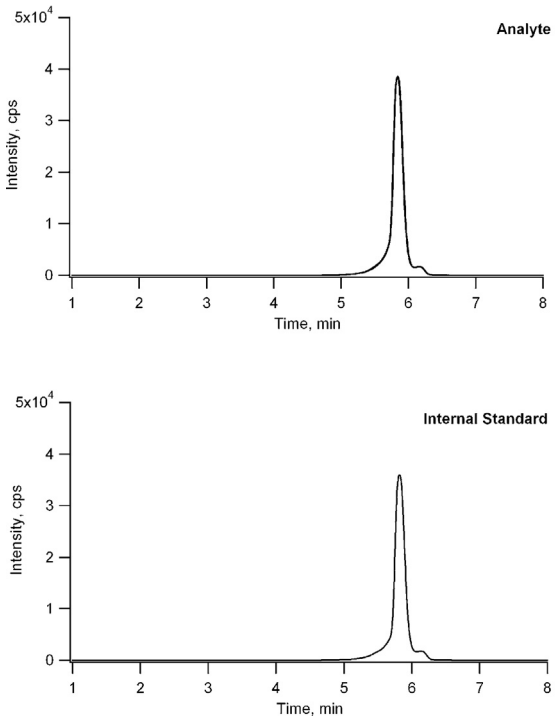

Fig. 2. The LC/MS/MS chromatograms of 434STTVQLMQR442 and the stable isotope-labeled internal standard. The estimated amount of peptides on column is 5.0 μg.

product ion spectrum was acquired and validated (data not shown). The retention times for STTVQLMQR and its isotope-labeled peptide were identical (~5.6 min), implying that the D isotopic effect on the retention time was negligible (Fig. 2).

We also found that the three most abundant peptides are all located in the cytoplasmic domain of P-gp. As previously described, P-gp is organized as two homologous halves, each of which is composed of six transmembrane segments and a cytosolic nucleotide-binding domain (NBD) [24]. The NBD was shown to bind ATP and analogues, as well as a number of potent modulators of drug resistance. Thus, it is not difficult to predict the abundance of peptides in the NBD after trypsin-digestive action due to its exposure to cytosol.

3.2. Membrane protein extraction

Ideally, a pure target protein is used as a reference standard for protein quantification. However, it is usually limited by the commercial availability of purified proteins (e.g., P-gp) [18,26]. Thus, the recovery of membrane extraction and the completeness of trypsin digestion must be carefully assessed using the proteolytic peptide as the surrogate of the target protein.

Membrane proteins constitute approximately 30% of the proteome, and more than two-thirds of the known proteins are important pharmacological targets [27]. However, they have often escaped a systematic analysis and quantification in biological systems due to their hydrophobic, proteolytically resistant nature and low expression levels [28]. Therefore, those methods that give high yields of desired proteins are usually a prerequisite step in membrane protein investigation. To date, great efforts have been made to solve this problem [29]. In this study, Triton X-114 was selected as the extraction detergent. Triton X-114 cannot only solubilize membrane proteins, but also has the ability to separate them from hydrophilic proteins *via* phase partitioning at a physiological temperature [30]. To determine the recovery of P-gp, the concentration

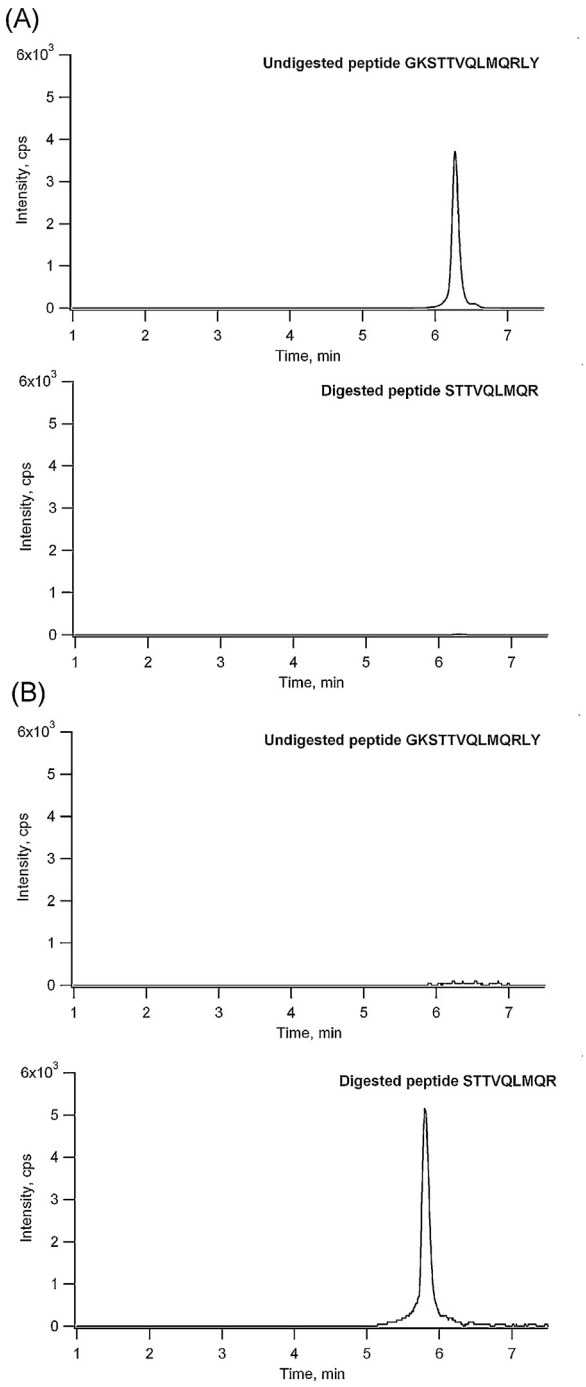

Fig. 3. LC/MS/MS chromatograms of 434GKSTTVQLMQRLY442 before (A) and after (B) the tryptic digestion. The estimated amount of peptides on column is 0.6 μg.

of the surrogate peptide in the total cell pellet was taken as a 100%, and its concentration in the membrane protein extract was given as a percentile. Consequently, the recovery of P-gp was approximately 92.1%.

We also evaluated whether membrane proteins with surrogate peptides on the extracellular side of the membrane might be digested efficiently without having to solubilize the membrane. Consequently, a larger yield was obtained for the digestion of the membrane extract than the digestion of an intact membrane. As previously reported, the differential membrane association of trypsin may affect the outcome of the proteolytic cleavage of membrane-bound proteins [31]. Insufficient digestion was

Table 1
Accuracy and precision for the QC samples using HSA (A) and P-gp depleted membrane fraction (B) as surrogate matrices.

Nominal concentration	10 ng/mL	30 ng/mL	200 ng/mL	800 ng/mL
(A)				
Mean	9.41	27.6	195	862
%Bias	−5.9	−8.0	−2.4	7.7
Intra-day precision (%CV)	3.0	5.9	3.8	0.6
Inter-day precision (%CV)	3.1	1.5	0.8	3.7
n	18	18	18	18
Number of runs	3	3	3	3
(B)				
Mean	9.33	29.0	200	770
%Bias	−6.6	−3.3	0.0	−3.8
Intra-day precision (%CV)	4.4	4.6	2.8	2.7
Inter-day precision (%CV)	9.4	4.1	3.1	1.0
n	18	18	18	18
Number of runs	3	3	3	3

a confounding factor in intact membrane digestion [7]. Thus, membrane protein extraction was performed in this study.

3.3. Digestion efficiency

As mentioned above, the tryptic digestion must be complete to accurately quantify the protein of interest in a biological sample. This phenomenon is especially true in the absence of pure target protein and in cases of detecting the proteolytic peptides as an alternative to the target protein. Generally, substrate peptides containing the same sequence as the surrogate peptides (to mimic a piece of the targeted protein) were employed to investigate the digestion efficiency. In this study, a substrate peptide 432GKSTTVQLMQRLY444 containing 434STTVQLMQR442 was synthesized. The substrate peptide was spiked into 5% HSA and digested using the same digestion protocol as for P-gp as described in the Experimental section. Both the undigested substrate peptide and the peptides after digestion were monitored using LC/MS/MS. Fig. 3 shows the absence of the starting substrate peptide 432GKSTTVQLMQRLY444 and the presence of the product tryptic fragment 434STTVQLMQR442. The recovery of 434STTVQLMQR442 was calculated by comparing the response ratios of the tryptic peptide after digestion and the equimolar surrogate synthetic peptide standard in the digestion. Consequently, the estimated recovery was 99.3%.

However, from our point of view, this process does not take the steric hindrance of enzymatic digestion into account. The cleavage site by trypsin may be buried within a large protein structure. Therefore, we applied another strategy to estimate the digestion efficiency in addition to the synthesized peptide approach. The details of the strategy have been described previously [32]. Briefly, peptide mapping of the P-gp yielded the 434STTVQLMQR442 corresponding molecular ion at *m/z* 1064.2 and larger peptide fragments with 1–6 missed cleavages (i.e., *m/z* 2651.0, *m/z* 2941.3, *m/z* 3176.7, *m/z* 4528.1, *m/z* 3525.0, *m/z* 3531.2, *m/z* 5053.7, *m/z* 5111.8, *m/z* 3941.5, *m/z* 5408.2, *m/z* 5637.4, *m/z* 5528.3, *m/z* 5991.9, *m/z* 6053.9 and *m/z* 6408.4, Table 1S). Their multiply charged ions were also monitored. By assuming that the mass response of each peptide fragment was the same, the peptide-bond cleavage for the generation of 434STTVQLMQR442 was 97.1% complete.

3.4. LC/MS/MS assay development and validation

As previously reported, an important factor needed to achieve good quantification of proteins is to generate high-quality MRM [33]. The MRM assays are generally developed on a triple quadrupole instrument to detect fragment ion signals arising from unique surrogate peptides. The y-series fragment ions are usually employed to establish the MRM transitions for quantification [34]. However, the transition that had the best signal-to-noise and LOQ for P-gp in this study was afforded by the immonium product ions. This type of ion, with a mass that is 27 u lower than that of the corresponding amino acid residue, has been suggested as a good indicator of the presence or absence of the particular amino acid in the peptide sequence [6,30]. For precursor ions of the surrogate peptides, 434STTVQLMQR442 and 368IIDNKPSIDSYSK380, the most intense immonium ions were at *m/z* 56.1 and *m/z* 70.0, which correspond to glutamine residue and proline residue, respectively. This characteristic mass pattern was also observed in the stable isotope-labeled internal standard. The enhanced intensity of the glutamine-related immonium ions could be due to the presence of two glutamine residues in the surrogate peptide, whereas the high intensity of proline product ion could be attributable to the terminal effect [35] and the propensity of the peptide bond to preferentially cleave adjacent to proline residues [36]. In fact, other sensitive peptide analyses have been previously reported in our laboratory. For example, we demonstrated that structurally distinctive immonium ions are formed in high abundance [17,32,37].

To examine the specificity of immonium product ions for quantification, a second product ion was evaluated here to serve as a confirmation [28,38]. The alternative product ions for 434STTVQLMQR442 and 368IIDNKPSIDSYSK380 were y5 *m/z* 674.7 and y8 *m/z* 896.4, respectively. Their calibration curves are shown in Fig. 2S. The values determined for both pairs of product ions (*m/z* 56.1/*m/z* 674.7 and *m/z* 86.1/*m/z* 896.4) were consistent throughout the calibration range (Table 2S).

Using the transitions of *m/z* 532.2 → 56.1 and *m/z* 536.1 → 56.1 (internal standard), a P-gp LC/MS/MS assay was developed and validated. Solid phase extraction was selected as the technique of choice for sample cleanup and enrichment in this study because, as previously reported, it has shown great promise for sample preparation [32]. The calibration curve was constructed using a weighted linear regression model with a weighting factor of $1/x^2$. The relative peak area ratio of the analyte, 434STTVQLMQR442, and the stable isotope-labeled internal standard was plotted against concentration. A representative calibration curve using 5% HSA as the matrix is shown in Fig. 1S. The LOQ was 10 ng/mL. A representative chromatogram of the LLOQ is shown in Fig. 4A. No significant interfering peak was found at the retention time of P-gp in the chromatogram of the blank matrix (Fig. 4B), in agreement with that the analyte response at the LLOQ was at least 5 times the response compared to blank response [39]. Since the matrix is preferred to represent the regular membrane protein component in the biological samples, we also employed P-gp depleted membrane fraction as surrogate matrix in this study. No detectable P-gp was observed in immuno-depleted matrix using western blotting and LC/MS/MS-based targeted proteomics assay (Fig. 3S).

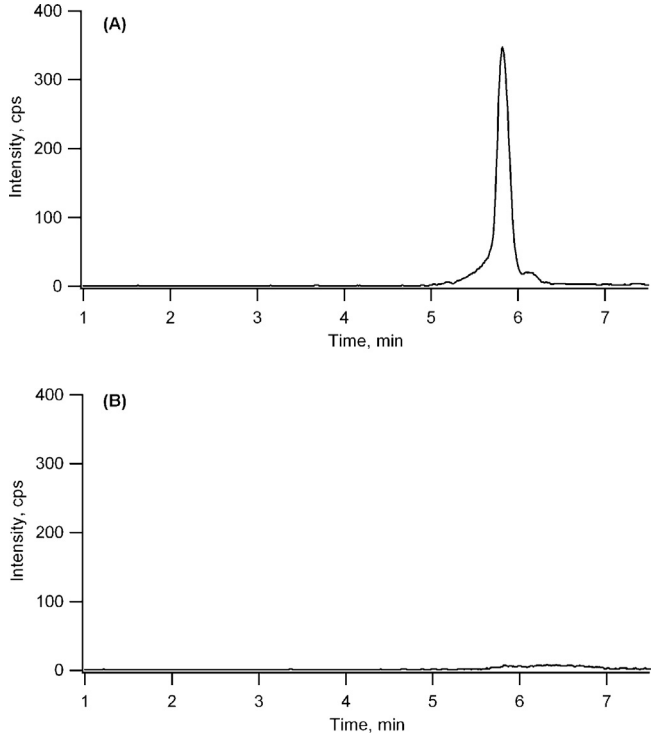

Fig. 4. The LC/MS/MS chromatograms for the LLOQ (A) and the blank matrix (B). The internal standard is omitted for clarity.

The precision and accuracy of the assay were assessed by observing the response of the QC samples with four different concentrations of P-gp in three validation runs. The intra- and inter-day precisions were expressed as the percent coefficient of variation (%CV). The accuracy was obtained by comparing the averaged calculated concentrations to their nominal values (%bias). The results are listed in Table 1. Both accuracy and precision were $\leq \pm 15\%$ (LLOQ, $\leq \pm 20\%$) [39]. The QC data indicated that the current method using either HSA or P-gp depleted membrane fraction as matrix has acceptable accuracy and precision for the determination of P-gp. Furthermore, a direct comparison of the peak areas of the internal standard in standards and QCs demonstrated that the matrix effect on the ionization of 434STTVQLMQR442 was minimal or consistent across HSA and immuno-depleted matrix (Fig. 4S). Their difference is not statistically significant at a 95% confidence level. Therefore, HSA can be used as a matrix instead of P-gp depleted membrane fraction to simplify the sample preparation process in the study.

3.5. Quantification of P-gp in breast cancer cells

As described previously, the level of P-gp is conventionally determined using molecular biology approaches, including immunoreaction and, in some cases, mRNA expression. To evaluate the LC/MS/MS performance, the amounts of P-gp in the two cell lines were also examined using confocal microscopy, western blotting and flow cytometry. The results are presented in Fig. 5 and Table 2. As shown, the expression levels of P-gp were accurately quantified to be 3.53 fg/cell ($\sim 2.08 \times 10^{-2}$ amol/cell, MW 170 kDa [40]) in MCF-7/WT and 34.5 fg/cell ($\sim 2.02 \times 10^{-1}$ amol/cell) in MCF-7/ADR cells using the LC/MS/MS-based targeted proteomics assay, whereas the other methods provided only the ratios of P-gp amounts in these cell lines. In addition, even though all the analytical approaches gave the difference in P-gp levels between the MCF-7/WT and MCF-7/ADR cells, the discrepancy between the results of LC/MS/MS and the other assays was significant. The

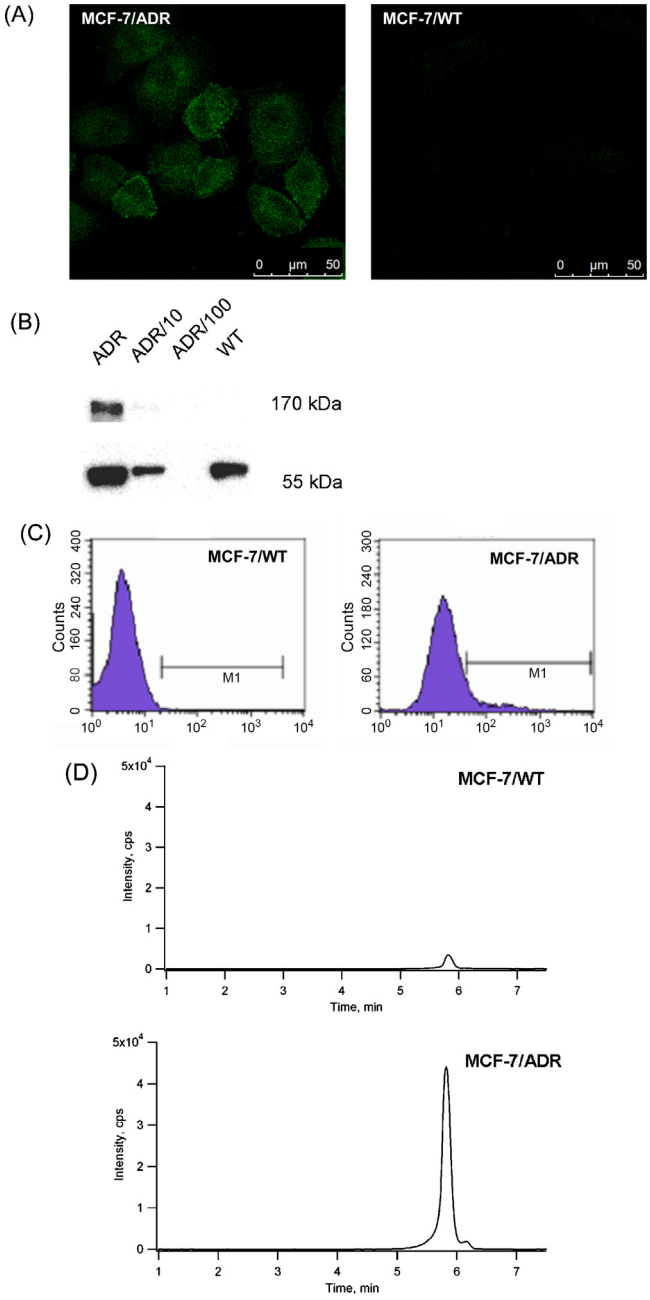

Fig. 5. Representative images of confocal microscopy, western blotting, flow cytometry and LC/MS/MS-based targeted proteomics. (A) Confocal images of P-gp after staining with the Alexa Flour 488 antibody. (B) Western blotting analysis of P-gp expression in the MCF-7/WT and MCF-7/ADR cells, normalized with β-tubulin. Lane 1: drug-resistant MCF-7/ADR cells; Lane 2: MCF-7/ADR cells with a 1:10 dilution; Lane 3: MCF-7/ADR cells with a 1:100 dilution; Lane 4: drug-sensitive MCF-7/WT cells. β-Tubulin was used as an internal control. (C) Expression of P-gp measured by flow cytometry. (D) LC/MS/MS chromatograms.

significant difference is most likely due to the lack of the specificity and the semi-quantitative nature of the latter methods. As mentioned previously, the antibodies obtained usually present a questionable specificity [41,42]. Furthermore, confocal microscopy and western blotting generally have a relatively poor detection limit and the deviation from linearity at low concentrations was often observed [43,44]. It is noteworthy that the distorted data using western blotting may result from the almost undetectable P-gp in the MCF-7/WT cells. The result of flow cytometry was closer to those measured by LC/MS/MS in this case. Flow cytometry can be quantitative to determine the number of antigens per cell,

Table 2
P-gp expression levels determined using confocal microscopy, western blotting, flow cytometry and LC/MS/MS-based targeted proteomics.

	MCF-7/WT	MCF-7/ADR	The ratio of P-gp amounts in MCF-7/WT and MCF-7/ADR cells
Confocal microscopy Mean density	4.40 ± 1.49	60.84 ± 8.98	13.8 ± 5.1
Western blotting Normalized IOD (Absolute integrated OD of each band)	(7.96 ± 1.34) × 10−3	1.25 ± 0.23	157.0 ± 39.2
Flow cytometry Mean fluorescence intensity	19.71 ± 2.21	182.60 ± 5.32	9.3 ± 1.1
LC/MS/MS Protein amount/cell (pg/cell)	(3.53 ± 0.36) × 10−3	(3.45 ± 0.28) × 10−2	9.8 ± 1.3

while calibration of instruments for quantification of fluorescence measurements is a trivial procedure [45]. Therefore, the results presented here suggest that the LC/MS/MS-based targeted proteomics assay can uniquely allow the accurate measurement of the P-gp expression level.

4. Conclusions

In this study, the P-gp expression levels in MCF-7/WT and MCF-7/ADR cells were determined using a simple, sensitive and validated LC/MS/MS-based targeted proteomics assay. The efficiency of the protein extraction and digestion was evaluated and the uniqueness of surrogate peptides was verified. Compared to the conventional analytical methods that provided only the ratios of the amounts of P-gp in the two cell lines, the LC/MS/MS technique is able to monitor the protein amounts in a more accurate manner. Thus, the approach proposed here is interesting for developing quantification methods of other proteins, which may result in greater refinement of our understanding of the roles of various proteins in development and disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.07.023.

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