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# LC–MS/MS-based quantification of clinically relevant intestinal uptake and efflux transporter proteins

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ABSTRACT

Multidrug transporter proteins are crucial determinants in the pharmacokinetics of many drugs. To evaluate their impact on intestinal drug absorption, we developed and validated quantification methods for 10 uptake transporters (OATP1A2, OATP2B1, PEPT1, ASBT, OCT1, OCT3) and efflux transporters (ABCB1, ABCC2, ABCC3, ABCG2) that have been reported to be expressed and to be of clinical relevance in the human intestine. Quantification was performed by targeted liquid chromatography with tandem mass spectrometry (LC–MS/MS)-based quantification of proteospecific peptides after tryptic digestion using stable isotope labeled internal standard peptides. The chromatography of the respective peptides was performed by gradient elution using a reversed phase (C18) column (Kinetex®, 100 × 3.0 mm, 2.6 μm) and 0.1% formic acid (FA) and acetonitrile with 0.1% FA as mobile phases at a flow rate of 0.5 ml/min. The MS/MS detection was done in the positive multiple reaction monitoring (MRM) mode by monitoring in each case three mass transitions for the transporter-derived peptides and the internal standard peptides. The assays were validated with respect to specificity, linearity (0.1–25 nM), within-day and between-day accuracy and precision as well as stability according to current bioanalytical guidelines. Finally, the developed methods were used to determine the transporter protein content in human intestinal tissue (jejunum and ileum). The methods were shown to possess sufficient specificity, sensitivity, accuracy, precision and stability to measure transporter proteins in the human intestine.



## 1. Introduction

The oral bioavailability of many drugs is significantly influenced by several intestinal transporter proteins [1]. With reference to this, ABC (ATP binding cassette) transporters such as ABCB1 (P-glycoprotein), ABCC2 (MRP2) and ABCG2 (BCRP) act as efflux transporters thereby limiting the intestinal absorption of many compounds by pumping them back to gut lumen. On the other side, several uptake SLC transporters have been reported to facilitate the intestinal uptake of many endogenous compounds (e.g. bile acids, amino acids) and drugs [1,2]. Thus, it is not surprising that co-administration of inducers or inhibitors and substrates of these proteins cause clinically relevant drug–drug interactions (DDIs) [3–5].

In order to estimate or even to predict the impact of the aforementioned transporters on oral drug absorption, quantitative information on intestinal transporter expression is needed. However, the majority of so far available information is based on mRNA expression data which is not necessarily correlated with the respective protein levels [6–9]. The methods of choice for protein quantifications so far were immunological methods such as Western blotting or quantitative immunohistochemistry [3,5,10]. However, these methods are of poor reproducibility and accuracy, low sample throughput and require antibodies that are often expensive and of uncertain specificity.

A novel and promising approach for the determination of transporter proteins appears to be liquid chromatography-tandem mass spectrometry (LC–MS/MS) based targeted proteomics [11]. Here, amino acid sequence-dependent mass transitions of protein specific peptides generated by tryptic digestion are monitored for quantification. Because the tryptic digestion of the respective protein is supposed to be complete, the molarity of the measured peptide acts as a surrogate for the respective protein expression.

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**Table 1**
Overview of tryptic proteospecific peptides and their respective ions and mass transitions used for transporter quantification (*isotope-labeled amino acid, *z* = charge state). The labeling of Arg (R), Lys (K) and Ser (S) was done by introducing C-13 and N-15.

| Protein | Peptide | Mass | Q1 | Ion/*z* | Q3.1 | Ion/*z* | Q3.2 | Ion/*z* | Q3.3 | Ion/*z* |
|---|---|---|---|---|---|---|---|---|---|---|
| ABCB1 | AGAVAEEVLAAIR | 1269.7 | 635.7 | *y*13/2+ | 972.1 | *y*9/1+ | 900.9 | *y*8/1+ | 771.7 | *y*7/1+ |
| | AGAVAEEVLAAIR* | 1278.0 | 640.7 | *y*13/2+ | 981.9 | *y*9/1+ | 911.0 | *y*8/1+ | 782.0 | *y*7/1+ |
| ABCC2 | LTIIPQDPILFSGSLR | 1770.0 | 885.8 | *y*16/2+ | 1330.2 | *y*12/1+ | 665.6 | *y*12/2+ | 989.8 | *y*9/1+ |
| | LTIIPQDPILFS*GS*LR | 1778.0 | 889.6 | *y*16/2+ | 1337.1 | *y*12/1+ | 669.2 | *y*12/2+ | 997.1 | *y*9/1+ |
| ABCC3 | IDGLNVADIGLHDLR | 1620.9 | 540.6 | *y*15/3+ | 697.2 | *y*13/2+ | 755.1 | *y*14/2+ | 612.1 | *y*11/2+ |
| | IDGLNVADIGLHDLR* | 1630.0 | 544.5 | *y*15/3+ | 702.6 | *y*13/2+ | 759.8 | *y*14/2+ | 617.1 | *y*11/2+ |
| ABCG2 | SSLLDVLAAR | 1044.6 | 523.0 | *y*10/2+ | 644.6 | *y*6/1+ | 757.8 | *y*7/1+ | 430.6 | *y*4/1+ |
| | SSLLDVLAAR* | 1054.6 | 528.0 | *y*10/2+ | 654.6 | *y*6/1+ | 767.5 | *y*7/1+ | 440.6 | *y*4/1+ |
| OATP1A2 | EGLETNADIIK | 1202.6 | 602.1 | *y*11/2+ | 774.7 | *y*7/1+ | 673.8 | *y*6/1+ | 903.8 | *y*8/1+ |
| | EGLETNADIIK* | 1210.0 | 606.2 | *y*11/2+ | 783.3 | *y*7/1+ | 681.7 | *y*6/1+ | 911.9 | *y*8/1+ |
| OATP2B1 | SSPAVEQQLLVSGPGK | 1596.9 | 799.1 | *y*16/2+ | 712.2 | *y*14/2+ | 1156.1 | *y*11/1+ | 445.5 | *y*5/1+ |
| | SSPAVEQQLLVSGPGK* | 1606.9 | 803.3 | *y*16/2+ | 716.2 | *y*14/2+ | 1163.7 | *y*11/1+ | 453.5 | *y*5/1+ |
| OCT1 | ENTIYLK | 880.5 | 441.0 | *y*7/2+ | 423.6 | *y*3/1+ | 637.7 | *y*5/1+ | 536.6 | *y*4/1+ |
| | ENTIYLK* | 887.0 | 445.1 | *y*7/2+ | 431.6 | *y*3/1+ | 645.8 | *y*5/1+ | 544.5 | *y*4/1+ |
| OCT3 | GIALPETVDDVEK | 1385.7 | 693.5 | *y*13/2+ | 1031.9 | *y*9/1+ | 805.7 | *y*7/1+ | 934.6 | *y*8/1+ |
| | GIALPETVDDVEK* | 1394.0 | 697.7 | *y*13/2+ | 1040.0 | *y*9/1+ | 813.9 | *y*7/1+ | 943.0 | *y*8/1+ |
| PEPT1 | GNEVQIK | 787.4 | 394.3 | *y*7/2+ | 616.6 | *y*5/1+ | 487.9 | *y*4/1+ | 388.6 | *y*3/1+ |
| | GNEVQIK* | 794.0 | 398.4 | *y*7/2+ | 624.8 | *y*5/1+ | 495.8 | *y*4/1+ | 396.2 | *y*3/1+ |
| ASBT | ENGTEPESSFYK | 1387.6 | 695.1 | *y*12/2+ | 857.7 | *y*7/1+ | 1145.3 | *y*10/1+ | 987.0 | i8/1+ |
| | ENGTEPESSFYK* | 1395.0 | 699.1 | *y*12/2+ | 866.0 | *y*7/1+ | 1153.0 | *y*10/1+ | 995.0 | *y*8/1+ |

Therefore, the absolute protein levels can be assessed by calibration of the quantitative assays using synthetic peptide standards and isotope labeled internal standards. These methods have been successfully used to quantify ABCB1, ABCC2, ABCG2, OATP1B1, OATP2B1 and OATP1B3 in human tissue (liver, brain and kidney), human cells (hepatocytes, platelets) and transfected cell lines [12–22].

To our knowledge, validated methods for quantification of these transporters in human intestine were not described so far. Moreover, adequate assays are also not available for ABCC3, OATP1A2, PEPT1, ASBT, OCT1 and OCT3 whose role in intestinal drug absorption is of growing interest. Finally, details on method validation and quality control are rather scarce in the literature such as data on digestion efficiency, analyte stability and within-day as well as between-day accuracy and precision of the protein assays [12,15,16,19,20,23].

Therefore, it was the aim of this study to develop and validate LC–MS/MS assays for the absolute quantification of ABCB1, ABCC2, ABCC3, ABCG2, OATP1A2, OATP2B1, PEPT1, ASBT, OCT1 and OCT3 by measuring protein specific peptides. We applied these assays to comprehensively analyze the expression of clinically relevant uptake and efflux transporter proteins in human intestinal tissue.

## 2. Materials and methods

### 2.1. Materials/reagents

The ProteoExtract® Native Membrane Protein Extraction Kit and human serum albumin (HSA) were purchased from Calbiochem (San Diego, CA, USA). All peptide standards and their respective stable isotope-labelled internal standards were of analytical grade (purity >95%) and were synthesized and exactly quantified via amino acid analysis by New England Peptide (Boston, MA, USA). Sequencing Grade Modified Trypsin and ProteaseMAX™ surfactant were purchased from Promega (Madison, WI, USA). Ammonium bicarbonate, dithiothreitol, iodoacetamide and formic acid (FA) were obtained from Sigma (St. Louis, MO, USA). LC–MS grade acetonitrile (ACN) and water were from Burdick & Jackson (Muskegon, MI, USA).

### 2.2. Identification of proteotypic peptides and optimal MRMs

Proteotypic peptides for ABCB1, ABCC2, ABCC3, ABCG2, PEPT1, OATP1A2, OATP2B1, OCT1, OCT3 and ASBT were determined using a combined approach of in silico predictions and experimental data. Briefly, the respective protein sequences (database: UniProtKB/Swiss-Prot) underwent an in silico trypsin digestion (www.expasy.org/tools) allowing a sequence length of 7–25 amino acids and excluding any missed cleavages sites. Furthermore, peptides with the following features were excluded: (1) containing cysteine, methionine, tryptophan or N-terminal glutamic acid, (2) transmembrane region, (3) genetic polymorphisms (allele frequency >1%) and (4) experimentally proven post-translational modifications.

In parallel, appropriate proteotypic peptide candidates were verified by shotgun LC–MS experiments performed on a API4000 QTRAP or API TripleTOF 5600 (AB Sciex, Foster City, CA, USA) using tryptic digests (procedure described below) of target protein-containing membranes (ABCB1, ABCC2, ABCC3 and ABCG2 vesicles; BD Biosciences, San Jose, CA) or ASBT-, OATP1A2-, OATP2B1-, OCT1-, OCT3- and PEPT1-overexpressing cell lines as recently described [12,13,24]. Protein specificity of each observed peptide was assured by NCBI protein BLAST search against the UniProtKB/Swiss-Prot database.

In order to create optimal multiple reaction monitoring (MRM) methods for the best observed peptides, appropriate mass transitions for each peptide were identified and optimized by manual infusion of synthetic peptides and their stable isotope-labeled counterparts to the API4000 (AB Sciex). For each peptide, the three mass transitions of highest intensity were chosen. Table 1 shows all selected peptides and their optimized mass transitions.

### 2.3. Sample preparation and digestion procedure

The membrane protein fraction from intestinal tissue was extracted using the ProteoExtract® Native Membrane Protein Extraction kit according to the manufacturer's protocol with slight modifications. In brief, crushed intestinal tissue samples (~50 mg) were suspended in 1.6 ml cell lysing extraction buffer I (containing 5 μl/ml protease inhibitor cocktail). To assure sufficient sample disruption, all samples were afterwards homogenized using a glass Dounce homogenizer (10 strokes on ice) before incubation for 15 min at 4 °C while shaking. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4 °C. The supernatant (cytosolic proteins) was discarded, and the respective pellets were resuspended in 500 μl extraction buffer II (containing 5 μl/ml protease inhibitor cocktail) containing 10 μl of ProteaseMAX™ surfactant (1%, m/v). After incubation for 60 min at 4 °C while shaking, the suspension