Reliability and Robustness of Simultaneous Absolute Quantification of Drug Transporters, Cytochrome P450 Enzymes, and Udp-glucuronosyltransferases in Human Liver Tissue by Multiplexed MRM/Selected Reaction Monitoring Mode Tandem Mass Spectrometry with Nano-Liquid Chromatography

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ABSTRACT: Mass spectrometry (MS)-based multiplexed multiple reaction monitoring quantification of proteins has recently evolved as a versatile tool for accurate, absolute quantification of proteins. The purpose of this study was to examine the validity of the present method with regard to standard bioanalytical criteria for drug transporters, cytochrome P450 (CYP) enzymes and uridine 5′-diphospho-glucuronosyltransferases (UGTs). Membrane preparations from human liver tissue were used for target protein quantification. As a result, the determination coefficients (r^2) of all targets were greater than 0.986. In the absence of matrix, inaccuracy values (expressed as % deviation) were −8.1% to 20.3%, whereas imprecision values (expressed as % coefficient of variation) were within 15.9%. In the presence of matrix, which consisted of digested plasma membrane fraction for transporters and digested microsomal membrane fraction for CYP enzymes and UGTs, respectively, the inaccuracy was −15.3%–8.1%, and the imprecision were within 18.9%. Sufficient sample stability of membrane fraction was shown for three freeze–thaw cycles, 32 days at −20°C, and in processed samples for 7 days at 10°C. In conclusion, this study demonstrated, for the first time, that the MS-based assay with nano-liquid chromatography provides adequate reliability and robustness for the quantification of selected drug transporters, P450 enzymes and UGTs. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:4037–4043, 2011

Keywords: multiplexed MRM quantification of proteins; transporters; membrane transporter; cytochrome P450; UDP-glucuronosyltransferases; nano-LC; LC–MS/MS; mass spectrometry; proteomics

INTRODUCTION

Drug transporters, cytochrome P450 (CYP) enzymes and uridine 5′-diphospho-glucuronosyltransferase (UGT) enzymes, play an important role in the absorption, distribution, metabolism, and excretion (ADME) profile of most drugs, xenobiotics, and endogenous compounds.[1–5] Absolute quantitative information on the expression levels of these proteins in various tissues and organs (e.g., liver and small intestine), in humans and in animal models, and *in vitro* systems can help to improve understanding and prediction of the pharmacokinetic and safety profile of new drugs.

Abbreviations used: BCRP, breast cancer resistance protein; CV, coefficient of variation; CYP, cytochrome P450; γ-gtp, gamma-glutamyl transpeptidase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; MRM, multiple reaction monitoring; NTCP, Na^+/taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; QC, quality control; UGTs, UDP-glucuronosyltransferases

Additional Supporting Information may be found in the online version of this article. Supporting Information

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Quantitative studies on these proteins applying liquid chromatography–mass spectrometry (LC–MS) technology and regulatory quality standards for bioanalytical assays have not been performed in the past, despite the significant progress in the field of drug transporter and drug metabolism research. Instead, relative mRNA expression has been widely used as a surrogate for protein expression level. Several studies, however, demonstrated only poor correlation between mRNA and protein expression levels.[6–8] On the contrary, quantitative immunological approaches such as enzyme-linked immunosorbent assay (ELISA) are capable of highly sensitive quantification, but development of specific antibodies can be time consuming (>1 year) or may fail, for example, for protein classes showing high homology in amino acid sequence.[9,10] Recently, several quantitative proteomics methods based on MS have been developed as alternatives to the conventional immunobased methods.[11–13] Kamiie et al.[14] first described a highly sensitive and simultaneous quantification method for drug transporters by LC coupled to tandem MS (LC–MS/MS). Kawakami et al.[15] reported a quantification method for CYP enzymes and simultaneously quantified expression of several CYP enzymes in human liver microsomes. Ito et al.[16] and Uchida et al.[17] quantified drug transporters, receptors, and junctional proteins in monkey and human blood–brain barrier.

The principle of this method is based on the quantification of unique peptide fragments by means of multiple reaction monitoring (MRM), which are released from the target proteins by previous trypsin digestion. Synthetic peptides and stable isotope-labeled peptides of identical amino acid sequence serve as calibrators and as internal standard, respectively. Nanoflow LC (nano-LC) combined with MRM analysis allows for the sensitive and selective quantification of multiple target peptides within the same sample and a single chromatographic run.

So far, regulatory authorities such as US Food and Drug Administration (FDA) and European Medicines Agency provide only guidance on acceptance criteria for the quantification of either small molecules by LC–MS/MS or for the quantification of proteins by ligand-binding assays.[18,19] Currently, no guidance is given for the quantification of proteins by LC–MS/MS. Moreover, there are only sparse reports available in the literature evaluating assay performances of large molecules, such as proteins, by LC–MS/MS with regards to reliability and robustness. In the present study, we demonstrate reliability, such as linearity, reproducibility, and accuracy, of the simultaneous determination of eight drug transporters, eight CYP enzymes, three UGT enzymes, and two marker membrane proteins by nano-LC–MS/MS.

Table 1. Intra-assay Inaccuracy and Imprecision in Standard Solution

	Inaccuracy [Deviation (%)]			Imprecision [CV (%)]		
Protein	LQC	MQC	HQC	LQC	MQC	HQC
CYP1A2	7.9	4.9	3.2	4.4	4.5	2.9
CYP2B6	4.6	4.7	5.0	6.6	5.1	2.6
CYP2C8	−1.6	−2.1	−1.7	12.9	10.7	4.7
CYP2C9	9.5	4.1	2.5	4.8	6.0	3.9
CYP2C19	−5.5	−1.5	−4.1	5.3	5.1	3.1
CYP2D6	7.3	2.0	1.3	4.8	4.8	2.1
CYP3A4	11.5	3.7	5.7	6.8	4.9	2.9
CYP3A43	6.7	1.7	2.3	6.3	4.7	2.5
UGT1A1	5.0	2.4	2.9	5.7	5.2	3.5
UGT1A1_variant	3.6	1.4	−1.1	5.9	4.1	2.5
UGT2B7	19.0	−1.3	6.3	6.2	6.3	3.8
OATP1B1	−1.7	−0.6	−2.1	3.5	4.2	2.9
OATP1B3	11.8	6.4	7.0	5.5	4.3	3.3
OATP2B1	5.5	1.2	−1.2	5.2	6.0	2.9
MDR1	9.6	3.1	3.2	3.6	3.5	3.1
MRP2	10.3	1.0	2.0	2.2	3.9	3.1
BCRP	10.7	5.0	4.6	6.8	6.9	3.6
NTCP	−1.1	2.2	−0.6	6.5	5.2	3.5
OCT1	20.3	−8.1	0.4	4.3	6.4	4.2
Na^+/K^+ ATPase	−0.6	1.2	0.0	10.1	6.2	6.1
γ-gtp	12.6	4.2	6.5	3.7	5.1	2.7

The test samples for intra-assay variability contains three different standard peptide samples (LQC, MQC, and HQC); 2.5, 10, or 40 fmol of standard peptide and 20 fmol of internal standard peptide for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A43, OATP1B1, OATP1B3, OATP2B1, MDR1, BCRP, NTCP, and Na^+/K^+ ATPase; and 5, 20, or 80 fmol of standard peptide and 40 fmol of internal standard peptide for CYP2C8, UGT1A1, UGT1A1_variant, UGT2B7, MRP2, OCT1, and γ-gtp.

Each data point represents mean of one run ($n = 6$) in four MRM channels.

MATERIALS AND METHODS

Reagents

All peptides were chosen for synthesis based on the *in silico* selection criteria as described previously[15] and purchased from Thermo Fisher Scientific (Ulm, Germany). The amino acid sequences and the conditions for LC–MS/MS detection were listed in the Supplemental Table 1. Peptide purity (>95%) was provided by the manufacturer, using reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet detection (with a detection wavelength of 215 nm) and matrix-assisted laser desorption/ionization–time-of-flight MS analyses. Other chemicals were commercial products of analytical grade.

Mass Spectrometric Analysis

All samples were analyzed by the nano-LC system (Ultimate 3000; Dyonex, Amsterdam, the Netherlands), which was connected to an ESI-triple quadrupole mass spectrometer (QTRAP5500; AB Sciex, Foster City, California). The nano-LC system consisted of a trapping column (Pep Map C18, 5 μm