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LC-MS/MS-based quantification of efflux transporter proteins at the BBB

David Gomez-Zepeda^{a,b,c,*}, Meryam Taghi^{a,b,c}, Maria Smirnova^{a,b,c}, Philippe Sergent^d, Wang-Qing Liu^{b,e}, Cerina Chhuon^f, Michel Vidal^{b,e,g}, Martin Picard^{b,h,1}, Elizabeth Thioulouseⁱ, Isabelle Broutin^{b,h}, Ida-Chiara Guerrera^f, Jean-Michel Scherrmann^{a,b,c}, Yannick Parmentier^d, Xavier Declèves^{a,b,c,g}, Marie-Claude Menet^{a,b,c,j,*}

^a Inserm, UMR-S 1144, Response Variability to Psychotropics, Paris, France

^b Université Paris Descartes, Paris, France

^c Université Paris Diderot, Paris, France

^d Technologie Servier, Département de recherche biopharmaceutique, Orléans, France

^e CNRS, UMR 8638, Chimie Organique, Médicinale et Extractive et Toxicologie Expérimentale, Paris, France

^f Plateforme Protéomique 3P5-Necker, SFR Necker, US24, Université Paris Descartes, Paris, France

^g UF Biologie du médicament et toxicologie, Hôpital Cochin, AP HP, Paris, France

^h CNRS, UMR 8015, Laboratoire de cristallographie et RMN biologiques, France

ⁱ Service de biochimie, Hôpital Armand Trousseau, APHP, Paris, France

^j UF Hormonologie, Hôpital Cochin, AP HP, Paris, France

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ABSTRACT

Targeted protein quantification using tandem mass spectrometry coupled to high performance chromatography (LC-MS/MS) has been used to quantify proteins involved in the absorption, distribution, metabolism and excretion (ADME) of xenobiotics to better understand these processes. At the blood-brain barrier (BBB), these proteins are particularly important for the maintenance of brain homeostasis, but also regulate the distribution of therapeutic drugs. Absolute quantification (AQUA) is achieved by using stable isotope labeled surrogate peptides specific to the target protein and analyzing the digested proteins in a triple-quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode to achieve a high specificity, sensitivity, accuracy and reproducibility. The main objective in this work was to develop and validate an UHPLC-MS/MS method for quantification of the ATP-binding cassette (ABC) transporter proteins Bcrp and P-gp and Na⁺/K⁺ + ATPase pump at the BBB. Three isoforms of the α -subunit from this pump (Atp1a 1, 2 and 3) were quantified to evaluate the presence of non-endothelial cells in the BBB using one common and three isoform-specific peptides; while Bcrp and P-gp were quantified using 2 and 3 peptides, respectively, to improve the confidence on their quantification. The protein digestion was optimized, and the analytical method was comprehensively validated according to the American Food and Drug Administration Bioanalytical Method Validation Guidance published in 2018. Linearity across four magnitude orders (0.125 to 510 pmol·mL⁻¹) sub-pmol·mL⁻¹ LOD and LOQ, accuracy and precision (deviation < 15% and CV < 15%) were proven for most of the peptides by analyzing calibration curves and four levels of quality controls in both a pure solution and a complex matrix of digested yeast proteins, to mimic the matrix effect. In addition, digestion performance and stability of the peptides was shown using standard peptides spiked in a yeast digest or mouse kidney plasma membrane proteins as a study case. The validated method was used to characterize mouse kidney plasma membrane proteins, mouse brain cortical vessels and rat brain cortical microvessels. Most of the results agree with previously reported values, although some differences are seen due to different sample treatment, heterogeneity of the sample or peptide used. Importantly, the use of three peptides

Abbreviations: ADME, absorption, distribution, metabolism and excretion; BBB, blood-brain; MRM, multiple reaction monitoring; SIL, stable isotope labeled.

* Corresponding authors at: Inserm, UMR-S 1144, Response Variability to Psychotropics, Paris, France.

E-mail addresses: david.gomez.zepeda@cinvestav.mx (D. Gomez-Zepeda), marie-claude.menet@parisdescartes.fr (M.-C. Menet).

¹ Current address: Laboratoire de Biologie Physico-Chimique des Protéines Membranaires, CNRS UMR 7099, Institut de Biologie Physico-Chimique (IBPC), Paris, France.

allowed the quantification of P-gp in mouse kidney plasma membrane proteins which was below the limit of quantification of the previously NTTGALTR peptide. The different levels obtained for each peptide highlight the importance and difficulty of choosing surrogate peptides for protein quantification. In addition, using isoform-specific peptides for the quantification of the Na⁺/K⁺ ATPase pump, we evaluated the presence of neuronal and glial cells on rat and mouse brain cortical vessels in addition to endothelial cells. In mouse liver and kidney, only the alpha-1 isoform was detected.

1. Introduction

Absolute quantification of proteins by mass spectrometry (MS) is used in many fields of biology to characterize cells and tissues as well as their biological processes and protein level changes. These methods have gained popularity because of its high sensitivity, accuracy, reproducibility and the possibility of analyzing multiple proteins in a single analysis. In biopharmaceutical research, there is a particular interest in the use of these methods to quantify proteins involved in the absorption, distribution, metabolism and excretion (ADME) of xenobiotics in order to better understand these processes. Therefore, targeted absolute protein quantification has been used to characterize the expression of transporters, receptors, tight junction proteins and drug metabolizing enzymes in different study models (*in vivo* [1,2] and *in vitro* [3]), but also in human samples [4–7].

The ATP-binding (ABC) transporters family is particularly important for the ADME of nutrients and exogenous substances. They are mostly located at the plasma membrane in the tissue interfaces such as endothelia and blood-tissue barriers where they efflux a high variety of substrates. They are found in the intestine, liver, kidney, heart, lungs, brain, placenta and testis. The ABC transporters are thus fundamental for the maintenance of several physiological functions such as protection from toxic substances, transport of important metabolites and cell signaling. At the endothelial cells of the blood brain barrier (BBB), these transporters are particularly important to maintain the brain homeostasis by regulating the penetration of dangerous substances but also of therapeutic drugs into the brain; and thus, impact their pharmacology effects [8]. Therefore, the study of their function and expression in the endothelial and epithelial barriers is necessary to better understand the ADME of drugs and their metabolites. The quantification of ABC transporters at the BBB is generally performed on samples of micro-vessels extracted using a protocol well described by Dauchy et al. [9]. It allows to obtain a sample enriched in endothelial cells but polluted by different cells or cellular fractions such as pericytes, astrocytes and neurons [10]. The determination of certain markers, such as ATPase (a1, a2 and a3) enables to evaluate the proportion of endothelial cells in the sample and better quantify the transporters [11]. MS-based absolute quantification of proteins is usually performed by a bottom-up approach; which implies several preparation steps where care must be taken in order to minimize variability and ensure reproducibility. Protein extraction and fractionation is often followed by cleaning steps before protein denaturation and enzymatic hydrolysis (most frequently with trypsin). The samples are then analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in multiplexed selected reaction monitoring (SRM / MRM) to quantify specific target peptides [1,12] as a surrogate of the protein. In order to achieve an absolute quantification in non-arbitrary units (e.g pmol·mg⁻¹ of proteins), stable isotope labeled (SIL) internal standards are added to the sample after digestion in the form of synthetic homologous peptides (AQUA strategy [12]).

One of the major issues of MS-based protein quantification is the lack of universal sample treatment because of the high variety of

physicochemical properties of proteins. In addition, protocols often consist of multiple steps, which increase the risk for inter- or intra-assay variability. Therefore, it is important to carefully evaluate and validate all the steps of the analysis to achieve the desired accuracy and precision [13].

The quantification of membrane proteins as ATP-binding cassette (ABC) transporters at the BBB and other barriers is already described in the literature. The Table S-1 summarizes the major analytical features of the several available methods in the field on P-gp and Bcrp. Kamiie et al. [1] developed an AQUA method for the quantification of membrane transporter proteins, including the selection of candidate peptide probes and the sensitivity and accuracy of the multi-channel MRM analyses. Nevertheless, they did not perform an analytical validation of the dosage method according to the FDA guidelines [13] as Prasad et al. [6] did for the determination of BCRP in the liver and, independently, for P-gp in the same tissue [5]. Groer et al. [7] and Harwood et al. [14] also validated analytically the quantification of Pgp and BCRP in human intestine in accordance with these guidelines. However, these authors did not validate any membrane marker proteins (such as ATPase).

Each study described in Table S-1 used only one peptide per protein for the quantification and did not take into account that the digestion efficiency may vary depending on the peptide as discussed by Prasad et al. [15], which implies that digestion protocol must be proven. In these studies, the authors used digestion protocols already described in the literature [1,5,6,14,16], controlled the digestion through the CV value of a QC (<5%) [16] or optimized the protocol by themselves [7]. Zhang et al. [17] suggested controlling the P-gp digestion through the use of a surrogate digestion peptide in the QC samples and Harwood et al. [14] through the QconCAT technique.

Gröer et al. [7] and Zhang et al. [17] were the only authors discussing the matrix effect. The first did not study it because they supposed that matrix effects are of minor importance because they used a long gradient elution and high-resolution chromatography. Zhang et al. [17] discussed the role of the internal standard in the minimization of the potential inconsistency in the ionization by mass spectrometer of analytes in complex biological matrixes. However, most of the authors made standards and QC samples in a complex matrix. To minimize a potential matrix effect on the quantification, these solutions should be made in a matrix identical to that of the samples to be assayed. But the latter are membrane extracts that usually come from human or animal tissues or cells that are not always available in large quantities. To mimic the matrix of the samples the authors used the buffer of the membrane extraction kit [5,6], or solutions of peptides resulting from the digestion of BSA [7,17] or, more specifically for QC, true samples (membrane extraction of MDCK or HEK293 cells) [16,17] for which they knew the membrane protein concentration and that they spiked with peptides to be assayed at known concentrations. None of the previous works compared the matrix effect that may occur on the detection of the peptides contained in these solutions (more or less complex) versus the same peptides in aqueous solution.

Therefore, the main objective in this work is to develop and validate a LC-MS/MS method according to the FDA guideline (released

in May 2018 [13]) for absolute quantification of the mouse and rat membrane proteins Bcrp and P-gp, in addition to the membrane marker Na^+/K^+ ATPase using isoform-specific peptides as cell-markers. The method was used to determine the expression of these proteins at the brain cortical vessels of rat and mouse, and plasma membrane proteins of mouse kidney and liver. We have studied more particularly three critical points which can modify the quality of the quantification. First, we verified that the digestion protocol was optimized for digestion of membrane proteins after precipitation with methanol-chloroform-water system. We also studied the absence of impact from a high complex matrix on the quantification performance, the quantification linearity, accuracy, precision, LOD and LOQ determinations. And finally, we studied the peptide stability for the whole sample treatment. Importantly, to improve the quantification, we employed two different peptides for Bcrp, three for P-gp and isoform-specific peptides for Atp1a, in addition to a previously used multi-isoform probe peptide [1].

2. Material and methods

2.1. Chemicals and reagents

Bovine serum albumin (BSA) and dextran (molecular weight 70,000) were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). Protease Inhibitor Cocktail cOMplete Mini[®] was provided by Roche (Bâle, Switzerland). Sequencing grade Modified Trypsin, MS-grade rLys-C and ProteaseMAX surfactant were purchased at Promega (Charbonnières-les-Bains). Some of the peptides were provided by Pepscan (Lelystad, The Netherlands). All Fmoc protected amino acids, preloaded Wang resin and peptide synthesis reagents were provided by Novabiochem[®] (Merck Millipore, Darmstadt, Germany). ¹⁵N and ¹³C labeled (with 98% of isotopic enrichment) Fmoc-protected amino acids came from Sigma-Aldrich. DMF (dimethylformamide) and piperidine were supplied by Carlo Erba Reagents (Val de Reuil, France).

2.2. Peptide synthesis

The synthesis of peptides was performed in solid phase by using Fmoc chemistry and Fmoc-amino acid preloaded Wang resin (0.1 mmol, 0.6 mmol·g⁻¹) on microwave assisted CEM-Liberty 1 synthesizer, with DIC/Oxymapure as coupling reagents. Fmoc deprotection was achieved by 20% (V/V) piperidine with 0.1 mol·L⁻¹ Oxymapure in DMF. After synthesis, peptides were cleaved from resin with a simultaneous removal of side chain protections through a treatment with a 10 mL solution of TFA containing 2.5% water and 2.5% triisopropylsilane (V/V) (TIPS). The resin was then filtered off and the filtrate was concentrated, precipitated into cold diethyl ester and collected by centrifugation. Peptides were then purified on Shimadzu semi-preparative HPLC system by using a GRACE Vydac Protein and Peptide 218 TP column (10 × 250 mm) and analyzed on a Shimadzu Prominence LC-20AD HPLC by using a GRACE Vydac Protein and Peptide 218 TP column (4.6 × 250 mm), within a linear A–B gradient (A: 0.1% (V/V) TFA aqueous; B: 0.09% (V/V) TFA in 70% (V/V) acetonitrile aqueous) at a flow rate of 2 mL min⁻¹ for purification and 1 mL min⁻¹ for analysis. Purity was above 99% for all the peptides synthesized, except for AAVPDAV[+6]GK (98.7%). The molecular weight of peptides was characterized by high resolution MALDI-TOF mass spectrometer. The peptide concentration in standard solutions was determined by Amino Acid Analysis (AAA) after total acid hydrolysis.

2.3. Biological samples

2.3.1. Yeast microsomal fraction

Yeast microsomal fraction was obtained by lysing the cells with an Ultraturrax[®] (IKA[®]-Werke GmbH & Co. KG, Staufen, Germany) in a 250 mmol·L⁻¹ sucrose buffer (with 20 mmol·L⁻¹ Tris pH 7.4 and 5.4 mmol·L⁻¹ EDTA). After clarifying the sample by centrifugation (15 min. at 10,000 g, 4 °C), the microsomal fraction was pelleted by ultracentrifugation (1 h at 100,000 g, 4 °C) and recovered in 250 mmol·L⁻¹ sucrose buffer.

2.3.2. Animals

Male C57BL/6 (10–12 weeks old) mice and male Sprague–Dawley rats (5–7 weeks old), both provided by Janvier Labs (Le Genest-Saint-Isle, France) were handled in accordance with the European Communities Council Directive. The animals were housed in a standard (non-enriched) environment on a 12/12-h light-dark cycle in a temperature-controlled room (22 ± 1 °C). Food and water were provided *ad libitum*. We endeavored to minimize the number of animals used and their discomfort. The animals were anesthetized with Ketamine/Xylazine, exsanguinated with physiological serum and euthanized by decapitation. The mouse kidneys and brains were flash frozen in liquid nitrogen, while rat brains were treated freshly.

2.3.3. Plasma Membrane Protein (PMP) fractions of mouse kidney and mouse liver

Plasma Membrane Protein (PMP) fractions of kidney and liver were obtained as previously described [18] with minor modifications, by using an Ultraturrax[®] for homogenization and differential centrifugation with a 38% (W/V) sucrose cushion for PMP enrichment.

2.3.4. Mouse brain cortical vessels

Mouse brain cortical vessels were obtained as previously described [9,19] with some modifications, while keeping the samples at 4 °C. After thawing the brains, the cortexes were dissected and cleaned of white matter and the meninges. 5 brain cortexes were pooled and chopped in buffer (HBSS and 10 mmol·L⁻¹ HEPES). The suspension was centrifuged (5 min. at 600 g, 4 °C) and the pellet was suspended in the same buffer supplemented by an enzymatic mixture. The samples were incubated at 37 °C and pelleted by centrifugation (15 min. at 5000 g, 4 °C). The pellet was suspended in suspension buffer (HBSS with 10 mmol·L⁻¹ HEPES) with 17.5% (w/w) dextran and centrifuged (30 min. at 4500 g) to separate myelin and other contaminants from the brain vessels. The supernatant was eliminated, and the pellet suspended in suspension buffer with 1% (w/w) BSA, before filtration by using a 10 μm nylon mesh. The retained vessels were recovered in the same buffer and centrifuged (5 min. at 600 g, 4 °C). The pellet was suspended in suspension buffer without BSA and centrifuged again (5 min. at 600 g). After eliminating the supernatant, the vessels were collected in a hypotonic buffer (10 mmol·L⁻¹ Tris pH 7.4, 10 mmol·L⁻¹ NaCl, 1.5 mmol·L⁻¹ MgCl₂) supplemented with protease inhibitor cocktail, incubated for 15 min and sonicated in Bioruptor[®] in high mode for 5 min with on/off cycles of 30 s. Samples were centrifuged (10 min. at 10,000 g, 4 °C) and the supernatant was recovered.

2.3.5. Rat brain cortical microvessels

Rat brain cortical microvessels were isolated from fresh tissue as previously described [19], by using a mechanical homogenization. The procedure was similar to the mouse vessels, but the incubation with enzymes was replaced by mechanical disruption. The minced samples were homogenized in a Potter–Thomas homogenizer (Kontes Glass, Vineland, NJ, USA) (0.25 mm clearance) by using 15 to 20 up-and-down strokes at 400 rpm. Microvessels were

isolated by filtering the samples through a 100 μm nylon mesh followed by a second filter of 10 μm . Proteins were extracted by suspending the final pellet in the hypotonic buffer supplemented with protease inhibitor cocktail, incubated for 15 min and sonicated in Bioruptor® in high mode for 5 min with on / off cycles of 30 s. Samples were centrifuged (10 min. at 10,000 g, 4 °C) and the supernatant was recovered.

2.4. Protein digestion

The total protein determination was performed by using the BCA or micro-BCA Protein Assay Kits, following the recommendations of the supplier (Thermo Scientific, Illkirch, France). The proteins in samples were digested in solution as previously described [1,2] with some modifications. Briefly, proteins were denatured in denaturing buffer (7 mol·L⁻¹ guanidine hydrochloride, 10 mmol·L⁻¹ EDTA, 500 mmol·L⁻¹ Tris pH 8.5), reduced by DTT (1.4 mmol·L⁻¹) and alkylated by iodoacetamide (2.9 mmol·L⁻¹). Proteins were then precipitated by using a methanol-chloroform-water system. The pellets were resuspended in 6 mol·L⁻¹ urea and 0.2% (W/V) ProteaseMax™ detergent. After a 10-minute incubation and agitation at room temperature, the samples were diluted with 0.1 mol·L⁻¹ Tris buffer (pH 8.5) to a final urea concentration of 1.4 mol·L⁻¹ and 0.05% (W/V) of ProteaseMax™ before sonicating for a complete resuspension. rLysC endoprotease was added to the samples in an enzyme-protein mass ratio of 1:50 and digested at room temperature for 3 h. Proteins were then digested with trypsin (enzyme-protein mass ratio = 1:100) by overnight incubation (16 h) at 37 °C. The stable isotope-labeled (SIL) peptide mixture was added before stopping the digestion by adding formic acid. The samples were dried in a centrifugal vacuum concentrator (Maxi-Dry Lyo, Heto Lab Equipment, Denmark), stored at -80 °C and solubilized just before analysis, by using a mixture of 10% (V/V) acetonitrile, 90% (v/v) water plus 0.1% (V/V) formic acid.

2.5. Analysis by ultrahigh performance liquid chromatography on line with tandem mass spectrometry

2.5.1. UHPLC MS/MS

All the Mass Spectrometry analyses were performed on an ACQUITY UPLC H-Class® System on line with a Waters Xevo® TQ-S mass spectrometer (Waters, Manchester, UK). 5 μL of a solution at 1 g L⁻¹ of proteins (before tryptic digestion) were injected on the column. Peptides were separated by using an ACQUITY UPLC BEH® C18 column (Peptide BEH® C18 Column, 300 Å, 1.7 μm , 2.1 mm X 100 mm; Guyancourt, France) in a 34 min gradient going from 100% aqueous mobile phase (water and 0.1% formic acid (V/V)) to 35% of organic mobile phase (ACN with 0.1% formic acid (V/V)) in aqueous mobile phase, at a flow-rate of 0.5 mL/min, at 30 °C.

The mass spectrometer was operated in MRM mode by using positive electrospray ionization (ESI) with ion spray capillary voltage at 2.80 kV. The resolution of the quadrupoles was 0.75 Da (FWHM) after calibration using ortho-phosphoric acid (0.1%) and resolution verification with Sodium Iodide (0.1 mg mL⁻¹) and Cesium Iodide (2.5 μg mL⁻¹) in positive mode (m/z 102.1300, 772.4610, 1372.0379 and 1971.6149), as recommended by the manufacturer (ref. 700005471, Waters, Manchester, UK). After manual optimization, drying gas flow-rate was set to 1000 L/h with a temperature of 650 °C. The AQUA approach [12] was used for the targeted quantification of selected peptides. Method development is detailed in Fig. S-1. Skyline [20] software (version 3.1.0.7382) was used for the MRM method development, including the collision energy optimization, and the peak integration. MassLynx v4.1 (Waters, Manchester, UK) was used to pilot the mass spectrometer and initial inspection of chromatograms. Collision energies were optimized both manually and with the help of Skyline as previously

described [21]. Briefly, for manual optimization a standard solution of each peptide at 100 pmol·mL⁻¹ was analyzed by direct infusion while changing the CE until highest signal intensity was obtained. In addition, a mix of standard peptides at 25 pmol·mL⁻¹ was analyzed using a MRM method created by Skyline with 5 different values of CE including the predicted optimal CE, -6, -3, +3 and +6 eV. Results were imported to Skyline and the CE giving the highest peak area for most of the transitions of a peptide was selected; obtaining similar results to manual optimization (Table 1).

2.5.2. Data treatment

All the chromatograms were evaluated with Skyline software by using the internal standard method and peak-area ratio for calculation. QuaSAR [22], integrated as a plugin in Skyline, was used to calculate the limit of detection (LOD), limit of quantification (LOQ) and linear regression equations from the calibration curves for each transition surveyed (Table 2). This plugin uses the AuDIT [23] algorithm to evaluate the presence of interferences by calculating an adjusted p -value of the probability of signal interferences for each transition according to the relative ratios between light and heavy transitions. A transition is marked as “bad” when the combined p -value is inferior to a threshold (10^{-5}) or the %CV of the light-to-heavy ratio between the injection replicates is higher than the accepted value (20%). Transitions are considered “good” when none of these conditions are satisfied, making them suitable for quantification. The LOD is calculated by using the light-to-heavy ratio and takes into account the estimated average response (light-to-heavy ratio) values of blank samples, the standard deviation of blank and low concentration points and the number of replicates [23]. LLOQ was then estimated as 3 x LOD. The calibration equations are obtained from a robust linear fit by using least median of squares regression. These calibration equations were used to calculate the LOD and LOQ values in pmol·mL⁻¹ units.

The peptide abundance in samples and quality controls (QCs) were calculated for each transition of the target peptides by using the calibration equations and home-developed R scripts and then transformed into fmol per μg of total protein. Finally, home-developed R scripts were used for all subsequent statistical evaluations. Reported expression values correspond to the mean from 3 or 4 transitions; except for AAVPDAVGK which was quantified by using 2 transitions. The reported accuracy (DEV%) and precision (%CV) were calculated by using the peptide abundance from the technical replicates of sample treatment for biological samples or peptide mixing and dilution for the calibration curves and QCs; therefore, the variability between the transitions of a same peptide is not considered.

2.6. Development of the absolute protein quantification (AQUA) method

2.6.1. Peptide selection for AQUA method

The absolute protein quantification (AQUA) [12] of analyte proteins (Bcrp, P-gp and Na⁺/K⁺ ATPase) was performed by combining an *in silico* peptide selection with an experimental validation (Fig. S-1). Briefly, the possible proteotypic peptides were selected *in silico* by using criteria suggested previously [1,24] (Table S-2), with the help of bioinformatics tools (Table S-3). The Protein Information Resource (PIR) peptide search (<http://pir.georgetown.edu/>) [25] was particularly useful to verify peptide specificity as it performs a quick search for the sequences *versus* the UniProtKB entries. The peptides selected are specific to the corresponding protein homologues in mouse, rat and human proteome; except for peptide VGTQFIR from Bcrp, which is not present in the rat protein. Finally, the target peptides for the control protein and interest proteins were chosen by analyzing protein digests by LC-MS/MS which uses an unscheduled MRM method. At least 2 peptides were

Table 1
Target peptides, MRM parameters used in the UHPLC-MS/MS analysis.

Protein	Peptide		Light		Heavy		Fragment Ion	CV (V)	CE (eV)
	N°	Sequence (light/heavy)	Precursor m/z	Product m/z	Precursor m/z	Product			
P-gp (a)	1	NTTGALTR/NTTGALTR[+10]	467.8	719.4	472.8	729.4	y7	35	16
				618.4		628.4	y6		
				561.3		571.3	y5		
				490.3		500.3	y4		
				816.4		822.4	y8		
2	LANDAAQVK/LANDAAQV[+6]K	465.3	745.4	468.3	751.4	y7	35	16	
			631.3		637.4	y6*			
			516.3		522.3	y5			
			979.5		983.5	y8			
			749.4		753.4	y6			
			678.4		678.4	y5			
			757.5		761.5	y7			
P-gp (a/b)	3	IATEAIENFR/IATEA[+4]IENFR	582.3	749.4	584.3	753.4	y6	35	21
				678.4		678.4	y5		
				757.5		761.5	y7		
Bcrp	1	SSLLDVLAAR/SSLLDVLA[+4]AR	522.8	644.4	524.8	648.4	y6	35	18
				529.3		533.4	y5		
				430.3		434.3	y4		
				721.4		731.4	y6		
	2	VGTQFIR/VGTQFIR[+10]	410.7	664.4	415.7	674.4	y5	35	14
				563.3		573.3	y4		
				435.3		445.3	y3		
Na ⁺ /K ⁺ ATPase Atp1a1/2/3		AAVPDAVGK/AAVPDAV[+6]GK	414.2	756.4	417.2	762.4	y8*	35	14
				685.4		691.4	y7		
				586.3		592.3	y6		
				489.3		495.3	y5*		
				1049.5		1057.5	y9		
Na ⁺ /K ⁺ ATPase Atp1a1		IVEIPFNSTNK/IVEIPFNSTNK[+8]	631.3	920.5	635.4	928.5	y8	35	22
				807.4		815.4	y7		
				710.3		718.4	y6*		
				807.4		815.4	y7		
Na ⁺ /K ⁺ ATPase Atp1a2		GVIATGDR/GVIATGDR[+10]	451.3	731.4	456.3	741.4	y7	35	16
				632.3		642.3	y6		
				519.3		529.3	y5		
				448.2		458.2	y4		
				717.4		721.4	y7		
Na ⁺ /K ⁺ ATPase Atp1a3		GVVATGDR/GVVVA[+4]TGDR	437.2	618.3	439.2	622.3	y6	35	15
				519.3		523.3	y5		
				448.2		448.2	y4		

selected and used for the quantification of each protein including some sequences previously used for their quantification [1,5,6] and some novel sequences (Table 1).

2.6.2. Protein digestion optimization

The protein digestion efficiency was investigated by using kidney tissue (available in higher quantity than microvessels) which was digested for 4 h (protocol A), 16 h (protocol B) and 24 h (protocol C) at 37 °C with the reagent quantities described in Section 2.5; for 16 h at 37 °C without rLysC and trypsin with enzyme-protein mass ratio = 1:100 (protocol D), for 16 h at 37 °C with rLysC and trypsin at very low concentration (1:1000 instead of 1:100) (protocol E) or a higher concentration (1:50 instead of 1:100) (protocol F). Protein linearization by heating at 95 °C before the reduction step and room temperature (25 °C) trypsin digestion were also tested (protocol G and H, respectively). In each case 50 µg of proteins were digested in triplicate and the peptide abundance were determined for P-gp (a), P-gp (a/b), Bcrp and Na⁺/K⁺ ATPases.

2.7. Validation of the absolute protein quantification (AQUA) method

The method validation was performed based on the recommendations of the Food and Drugs Administration Bioanalytical Method Validation Guidance for Industry [13].

2.7.1. Preparation of calibration curves and quality controls (QCs)

Calibration curves in pure solution were prepared in three dilution replicates at three different days with three different batches of peptides in a mixture of 10% (V/V) acetonitrile, 90% (V/V) water plus 0.1% (V/V) formic acid by successive dilution

before adding a constant amount of stable isotope-labeled peptides. Light peptide concentrations covered a range from 0.125 to 125 pmol·mL⁻¹ (0.125, 0.25, 1.25, 2.5, 12.5, 25, 62.5 and 125 pmol·mL⁻¹). Two extra points were included for the Na⁺/K⁺ ATPase peptides (300 and 510 pmol·mL⁻¹). **Calibration curve in a complex matrix** was prepared as a surrogate of the sample blank matrix. The calibration points were prepared in triplicate, using the same three batches of stock standard peptide mixture and internal standards as in the pure solution but in a complex background matrix consisting of a digest of microsomal-yeast proteins (*S. cerevisiae*) at a concentration of 1 µg of hydrolysate per µL (mimicking the concentration of samples). The final concentrations were calculated to cover a range from 0.195 to 25 pmol·mL⁻¹ (0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25 pmol·mL⁻¹). **QC samples** were prepared by using a digest of yeast microsomal proteins, as explained above, in four concentrations: near to LLOQ (below three times the LLOQ, see Tables 3 and S-4), low, medium and high (10, 20 and 60 pmol·mL⁻¹, respectively). Three batches were prepared in different days.

2.7.2. Peptide purity, standard selectivity

Purity and concentration of reference standards was assessed for all the peptides synthesized in our laboratory as mentioned in section 2.1.

Cross-interference between labeled and unlabeled standards was tested by analyzing zero calibrators; meaning the mix of heavy-labeled standard peptides without light peptides, both in pure and complex matrix.

Table 2
Target peptides, MRM parameters used in the UHPLC-MS/MS analysis, calibration equations, R² and matrix effect comparison of slopes.

Protein: Peptide Sequence (light/heavy)	Fragment Ion	Calibration Equation						SlopeMatrix effect
		Low Complexity			High complexity			
		Slope	Y intercept	R ²	Slope	Y intercept	R ²	
P-gp (a): NTTGALTR/NTTGALTR[+10]	y7	0.0612	0.0008	0.9983	0.0587	0.0303	0.9973	-4.0%
	y6	0.0622	-0.0001	0.9988	0.0581	0.0001	0.9965	-6.5%
	y5	0.0618	0.0006	0.9990	0.0593	-0.0044	0.9916	-4.0%
	y4	0.0625	-0.0031	0.9995	0.0584	-0.0004	0.9969	-6.5%
	y8	0.0600	0.0014	0.9759	0.0578	-0.0014	0.9968	-3.6%
P-gp (a): LANDAAQVK/LANDAAQV[+6]K	y7	0.0574	0.0023	0.9931	0.0553	0.0008	0.9954	-3.5%
	y6*	0.0596	-0.0001	0.9968	0.0581	0.0043	0.9944	-2.5%
	y5	0.0586	0.0004	0.9939	0.0570	0.0018	0.9970	-2.8%
	y8	0.0594	0.0014	0.9994	0.0590	0.0013	0.9978	-0.8%
P-gp (a/b): IATEAIENFR/IATEA[+4]IENFR	y6	0.0614	0.0021	0.9992	0.0615	0.0016	0.9979	0.1%
	y5	0.0607	0.0024	0.9992	0.0589	0.0032	0.9972	-3.0%
	y7	0.0709	0.0030	0.9982	0.0687	0.0040	0.9976	-3.1%
	y6	0.0710	0.0026	0.9985	0.0687	0.0014	0.9968	-3.3%
Bcrp: SSLLDVLAAR/SSLLDVLA[+4]AR	y5	0.0713	0.0027	0.9989	0.0692	0.0019	0.9961	-3.0%
	y4	0.0709	0.0042	0.9989	0.0701	0.0024	0.9984	-1.0%
	y6	0.0597	0.0008	0.9991	0.0540	0.0020	0.9969	-9.5%
	y5	0.0598	0.0003	0.9988	0.0562	0.0009	0.9951	-6.2%
Bcrp: VGTQFIR/VGTQFIR[+10]	y4	0.0589	0.0021	0.9992	0.0560	0.0011	0.9973	-5.0%
	y3	0.0589	0.0006	0.9989	0.0564	0.0001	0.9972	-4.3%
	y8*	0.1100	-0.0066	0.9934	0.1383	-0.4152	0.9691	25.7%
	y7	0.1099	0.0026	0.9990	0.1097	-0.0026	0.9938	-0.2%
Atp1a1/2/3: AAVPDAVGK/AAVPDAV[+6]GK	y6	0.1098	0.0049	0.9989	0.1097	0.0012	0.9962	-0.1%
	y5*	0.1097	-0.0011	0.9890	0.1004	0.1065	0.9810	-8.5%
	y9	0.0555	0.0022	0.9948	0.0532	-0.0005	0.9982	-4.0%
	y8	0.0565	0.0031	0.9940	0.0539	0.0002	0.9957	-4.6%
Atp1a1: IVEIPFNSTNK/IVEIPFNSTNK[+8]	y7	0.0568	0.0019	0.9971	0.0530	0.0000	0.9977	-6.7%
	y6*	0.0541	0.0040	0.9863	0.0535	-0.0026	0.9908	-1.0%
	y7	0.0770	0.0009	0.9996	0.0723	-0.0001	0.9964	-6.1%
	y6	0.0796	0.0007	0.9997	0.0739	-0.0012	0.9986	-7.2%
Atp1a2: GIVIATGDR/GIVIATGDR[+10]	y5	0.0800	0.0008	0.9996	0.0745	-0.0022	0.9974	-6.8%
	y4	0.0776	0.0019	0.9991	0.0757	0.0003	0.9969	-2.4%
	y7	0.0606	0.0025	0.9987	0.0584	0.0037	0.9956	-3.6%
	y6	0.0617	0.0028	0.9981	0.0611	-0.0001	0.9986	-1.0%
Atp1a3: GVVATGDR/GVVVA[+4]TGDR	y5	0.0633	0.0028	0.9986	0.0605	0.0028	0.9971	-4.3%
	y4	0.0600	0.0025	0.9942	0.0593	0.0048	0.9953	-1.2%

Calibration equations and R² were obtained from the analysis of a calibration curve prepared in triplicate (each replicate was injected once). All the peptides are common between the mouse and rat proteins, except for VGTQFIR which is not present in rat Bcrp. Heavy isotope labeled residues are followed by their mass shift in brackets (rounded to the nearest integer; e.g. [+10]) between brackets (e.g. K[+8]). CV = Cone Voltage; CE = Collision Energy; Isotope Type: L = Light, H = Heavy. The matrix effect (ME) on slope was calculated using the following equation: ME = (S_{HC} - S_{LC})/S_{LC}; where S_{HC} = slope in high complexity matrix and S_{LC} = slope in low complexity matrix. *: these ions were not used for quantification due to possible interferences.

Table 3
Lower limit of detection (LOD) and lower limit of quantification (LOQ) as determined in low and high complexity matrices. Values are in pmol mL⁻¹.

Protein	Peptide		Conc. in LLOQ QCs	Low complexity matrix		High complexity matrix		Ratio LOQ High / Low
	N°	Sequence		LOD	LOQ	LOD	LOQ	
P-gp (a)	p1	NTTGALTR	3	0.125	0.275	0.205	1.65	6.0
P-gp (a)	p2	LANDAAQVK	2	0.085	0.334	0.292	1.02	3.1
P-gp (a/b)	p3	IATEAIENFR	2	0.053	0.207	0.214	0.752	3.6
Bcrp	p1	SSLLDVLAAR	1.5	0.117	0.468	0.227	0.751	1.6
Bcrp	p2	VGTQFIR	1	0.086	0.285	0.164	0.567	2.0
Na ⁺ /K ⁺ ATPase								
Atp1a1/2/3		AAVPDAVGK	8	1.07	3.31	0.318	0.976	0.3
Atp1a1		IVEIPFNSTNK	1.5	0.598	1.86	0.270	0.817	0.4
Atp1a2		GIVIATGDR	1.5	0.047	0.158	0.261	0.750	4.8
Atp1a3		GVVATGDR	2	0.078	0.316	0.290	0.961	3.0

2.7.3. Calibration curve linearity, limit of detection (LOD) and limit of quantification (LOQ)

The linear equation, limit of detection (LOD) and limit of quantification (LOQ) were obtained from the analysis of three batches of calibration curves in pure solution and complex matrix analyzed separately (single injection). The values were obtained using QuaSAR [29] as explained in section 2.5.

2.7.4. Accuracy and precision

Accuracy is expressed as the deviation to the nominal value (%DEV) and precision as the percent of coefficient of variation (%CV).

Accuracy and precision of the calibration curves were evaluated both in pure and complex matrices. Within-run accuracy and precision were calculated by analyzing in triplicate the four levels of QCs in complex matrix and one batch of calibration curve points in pure solution. Between run accuracy and precision was assessed by analyzing the three batches of low, medium and high QC samples on three different days.

2.7.5. Matrix effect

The absence of impact from the complex matrix on the quantification performance was evaluated by comparing the slope,

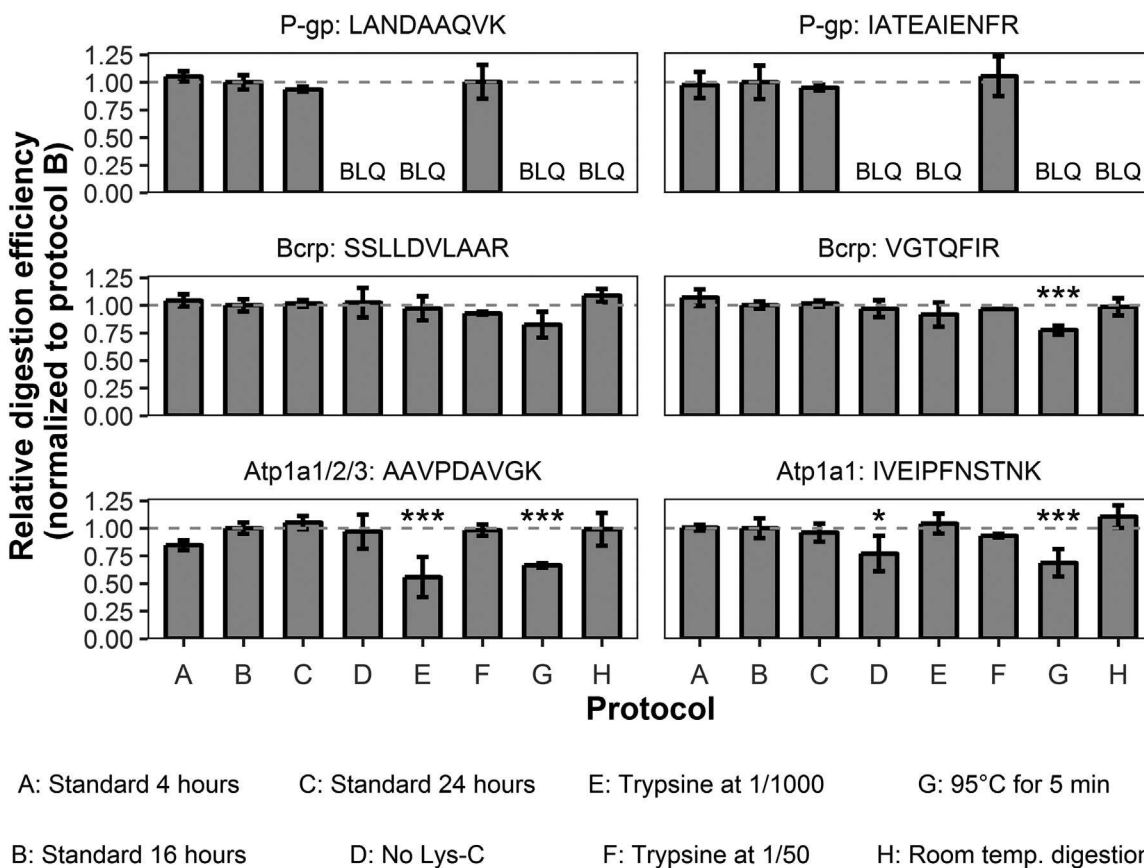


Fig. 1. Comparison of digestion protocols per peptide. Measured expression values were divided by the control protocol (standard protocol) with 16 h of digestion) to obtain a relative digestion efficiency. The gray dashed-line indicates an efficiency of 1 (the standard protocol). BLQ = below the limit of quantification. Significant differences marked by asterisks for p-value lower than 0.05 (*), 0.01 (**), and 0.001 (***) were studied using log₁₀ abundance values to fit to a normal distribution (t-test with Bonferroni's correction).

accuracy and precision between the pure and complex matrices. The values from the yeast hydrolysate should better represent the limits of the analytical method in the biological samples.

2.7.6. Peptide stability

Freeze-thaw stability was tested by using the stock solution containing the mix of standard peptides. Individual peptide stock solutions are all stored at -80°C in 100 μL aliquot. When calibration range was prepared, these solutions were thawed and mixed to obtain a stock solution containing each peptide at the concentration of 250 $\text{pmol}\cdot\text{mL}^{-1}$. This stock solution could be refrozen and thawed for further handling. Therefore, its stability was evaluated after 1, 2, 4 and 6 cycles of freezing and thawing with 3 days intervals.

The stability of the peptides after several cycles of thawing the samples has not been tested because, in this study, we never reinjected a sample after just one or several freeze/thaw cycles. Similarly, when internal standards or calibrators are prepared, they are aliquoted in single use volumes.

Sample processing (digestion) stability was evaluated by spiking the yeast microsomes fraction with light standard peptides either before or after overnight incubation with trypsin (16 h at 37°C) during the protein digestion procedure. The SIL peptides were added after the incubation and samples were dried and analyzed as explained above.

Autosampler stability during the analysis (*i.e.* at 4°C) was evaluated for 3 types of samples: calibration curve points, QCs and mouse kidney plasma membrane proteins. Two different batches of calibration curves were injected twice with an interval of approximately 30 h between the first and the second analysis. Each QC (3

levels from 3 different batches) or protein digest sample (in triplicate of digestion) was injected three times: at the beginning of the analysis series, 45 h later and 75 h later.

3. Results and discussion

3.1. Method development

3.1.1. Peptide selection for AQUA method and CE optimization

The selection of peptides for protein quantification was performed *in silico* following criteria previously suggested by Kamiie et al. [1] and enriched with suggestions from Ludwig and Aebersold [24] (Table S-2). The use of several bioinformatic tools (Table S-3) allowed us to select the surrogate peptides. The PIR Peptide Search was particularly useful for the selection of protein-specific peptides that are present in the homologues of P-gp, Bcrp and the $\text{Na}^{+}/\text{K}^{+}$ ATPase pump (Atp1a1, 2 and 3) from mouse, rat and human and other species of interest for pre-clinical analysis (Table S-5). Skyline [20] was used for chromatogram inspection and data treatment. This state-of-the-art software facilitated the development, validation and analysis of targeted proteomics experiments. Collision energy was optimized both manually and with the help of Skyline [21] as explained in section 2.6.1. The optimal CE corresponded to the one predicted by the software or 3 eV below for all the peptides as shown in Fig. S-2.

3.1.2. Protein digestion optimization

Fig. 1 shows the relative digestion efficiency calculated by normalizing against the standard protocol with 16 h of digestion

(protocol B). Atp1a2 and Atp1a3 peptides are not included because these protein isoforms are not expressed in mouse kidney [26] and thus were not detected. Significant differences were studied using log₁₀ abundance values to fit to a normal distribution (t-test with Bonferroni's correction). Three replicates of each protocol were evaluated and thus some apparent differences were not statistically significant; nevertheless, this experiment allowed the detection of critical parameters on the sample treatment. P-gp peptide NTTGALTR presents a high LLOQ and was BLQ in all the samples. P-gp peptides LANDAAQVK and IATEAIENFR were not detected when LysC was not used (protocol D), when trypsin was used at very low concentration (1:1000; protocol E), after sample heating at 95 °C for 5 min (protocol G) or room temperature (25 °C) digestion (protocol H). The differences in digestion efficiency were significant for AAVPDAVG (Atp1a 1,2,3) and IVEIPFNSTNK (Atp1a1) by using low concentration of trypsin (protocol E) or heating the proteins (protocol G), and for VGTQFIR (Bcrp) in this last protocol. There were no significant differences in the digestion of all the proteins by using protocols A, B and C with LysC and trypsin (enzyme-protein mass ratio = 1:100; 4 16 and 24 h digestion, respectively); neither with protocol F where trypsin-protein ratio was of 1:50. Although a trypsin-to-protein ratio of 1:50 to 1:20 is often used in protein digestion, our results indicated that the 1:100 ratio is enough for complete protein digestion after pre-digestion with LysC in accordance to previously reported protocols for targeted proteomics of ABC transporters [1] and other proteins [27]. The recovery of P-gp peptides was especially sensible to all the conditions possibly due to its low abundance in mouse kidney. In addition, ABC transporters and other membrane proteins such as the Na⁺/K⁺ ATPase pump are heat-sensitive as can be observed in the results from protocol G (specially for P-gp). Some protocols use a 95 °C incubation to help linearize the proteins for digestion, but this should be avoided when quantifying membrane proteins. Other protocols perform the trypsin digestion under room temperature to avoid degradation, but this has proven insufficient for P-gp enzymatic hydrolysis. Consequently, LysC and trypsin with enzyme-protein mass ratio = 1:100 are adequate and necessary to obtain a complete digestion of proteins at 37 °C for 16 h.

3.2. Method validation

Several national or international agencies have provided regulatory guides for the validation of LC-MS based methods for the quantification of drugs and metabolites in pharmacokinetics studies, but they do not directly deal with the quantification of endogenous proteins or biomarkers. Only the recently published Bioanalytical Method Validation Guidance for Industry from the American Food and Drug Administration (FDA) [13] mention that it can be extended to the evaluation of protein biomarker levels in biological matrices. Therefore, we used this guide for the AQUA method validation.

The FDA guide states that the calibration range and the QCs should be made in the same matrix as the samples. However, we do not have a protein sample from micro-vessels that does not contain the proteins to be assayed. Some authors have substituted the matrix by different solutions. Ji et al. [28] used the membrane protein fraction of wild-type HEK cells as the blank matrix to prepare standard solution and the membrane fraction of control mouse liver tissue for the QC samples; Zhang et al [23] and Gröer et al. [8], solutions of peptides resulting from the digestion of BSA for standard and QC. The preparation of standard solution and QC in a hydrolysate of brain micro-vessels proteins is impossible for ethical reasons because many animals would have to be sacrificed for the matrix preparation. In addition, the sample without the analyte proteins is not available. Therefore, we tested the linearity, accuracy, precision and stability for all target peptides not only in the

low complexity solution (calibration curves prepared in 10% (V/V) acetonitrile, 90% (V/V) water + 0.1% (V/V) Formic Acid) but also in yeast microsomal proteins to test the effect of a complex matrix in the quality of the quantitative method. We used yeast microsomal proteins because it contains a high variety of proteins and its complexity can be compared to our samples of interest. Moreover, yeast proteins can be obtained at a cheap price and the digestion of these proteins does not produce any peptides redundant with our target peptides.

3.2.1. Peptide purity, standard selectivity

The purity of all the peptides used was above 99%, except for AAVPDAV[+6]GK (98.7%) (Fig. S-3). The concentration of the stock solutions was determined by AAA (Table S-6). The isotope enrichment of heavy labeled standards used for the synthesis was above 98% to minimize cross-contamination to unlabeled peptides.

There is less than 1% of interference (ratio of 0.01) for almost all the peptides both in the pure and complex matrices, although higher interferences are observed in the samples prepared on Yeast hydrolysate. Peptides AAVPDAV[+6]GK (ATP1a1/2/3) and IVEIPFNSTNK[+8] (Atp1a1) show slightly higher interferences, but they are still below the FDA acceptance criteria of 5%. Thus, cross-interference between labeled and unlabeled peptides (isotopic effects) are negligible. In addition, the signals at the light transitions for peptides with a *m/z* difference > 2 do not co-elute with the heavy-standard signal (Figs. S-4 and S-5) and do not have the same relative intensity; thus, the signal observed could be due to background noise instead of cross-interference.

3.2.2. Calibration curve linearity

Each peptide was quantified by surveying four different fragments from the [M+2H]⁺⁺ precursor as MRM transitions (Table 1). Some transitions turned out to be noisy and were then used only for peptide identification regarding their coelution with the other transitions. Nevertheless, three to four transitions were used for the quantification of each peptide, except for Atp1a1/2/3 (AAVPDAVGK) with only two. In order to improve the quantification, the average peptide abundances were obtained through the average values obtained for its transitions, which had a %CV lower than 15 in most cases.

Almost all the transitions evaluated gave R² values above 0.99 both in the pure and complex matrices (Table 2), proving a correct linear fit; thereby showing that target peptides are suitable for quantification. Fig. 2b shows the chromatogram obtained after injection of standard solutions at 12.5 pmol·mL⁻¹ in a mixture of 10% (v/v) acetonitrile, 90% (v/v) water plus 0.1% (v/v) formic acid (Fig. 2a and b) and in yeast protein digest (Fig. 2e and f).

3.2.3. Limit of detection (LOD), limit of quantification (LOQ)

Limits of detection (LOD) and quantification (LOQ) for each transition (Table S-4) were obtained from calibration curves prepared either in pure solution or in yeast microsomal protein digest. The highest values were considered as the LOD and LOQ at the peptide level, which are presented in Table 3. It is well known that coeluting substances can have a major effect in the ionization and detection of target peptides; therefore, it is not surprising that the quantification limits are higher in the yeast digest matrix than the pure solution for most of the peptides. Furthermore, the difference between the high and low complexity matrix was different for each peptide, from a ratio of 0.3 (AAVPDAVGK) to 6 (NTTGALTR, P-gp (a)). This highlights that each peptide can be submitted to different matrix factors according to its retention time. Therefore, care should be taken when interpreting limits of quantification from calibration curves prepared in pure solution. Thus, the LOQ values obtained from the yeast digest were used in other experiments in order to consider an estimation of the background noise of a complex bio-

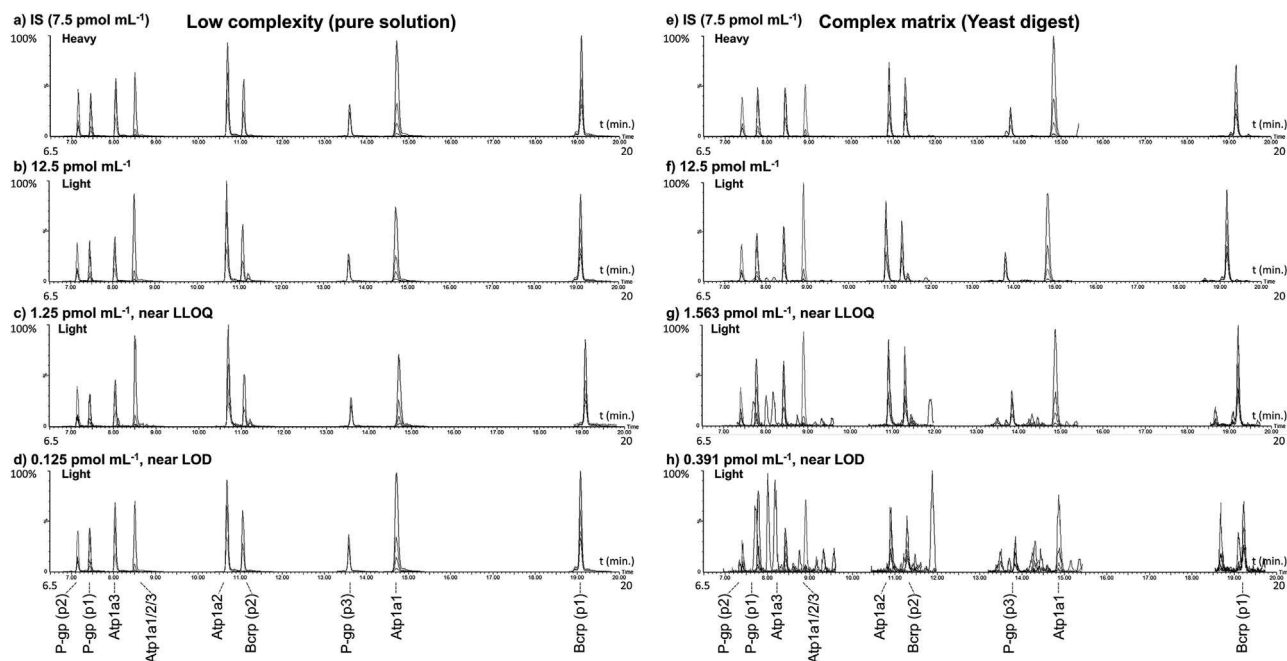


Fig. 2. Chromatograms obtained from the MRM analysis of standard solutions in low complexity matrix (left: a, b, c, d) and complex matrix (right: e, f, g, h). From top to bottom are shown: Internal standard heavy peptides example (a, e), unlabeled standards calibrators at concentration $12.5 \text{ pmol} \cdot \text{mL}^{-1}$ (b, f), near LLOQ (c, g) and near LOD (d, h).

logical sample. Fig. 2 shows examples of chromatograms from the injection of calibration points near to the LLOQ and LOD of most of the peptides from curves prepared in a mixture of 10% (V/V) acetonitrile, 90% (V/V) water plus 0.1% (V/V) formic acid (Fig. 2c and d) or in yeast protein digest (Fig. 2g and h). This study highlights the impact of matrix on the background noise, much higher in Fig. 2g and h than in Fig. 2c and d.

3.2.4. Accuracy and precision

According to the FDA guidance for bioanalytical method validation, the calculated concentration of QCs and calibration points of bioanalytical method should have a precision inferior to 15 %CV and accuracy should be within 15% of the nominal concentration, except for the LLOQ points where the threshold is set to 20%. At least 75% of the calibrator points and 67% of the QCs should satisfy these criteria for all the analytes [13].

In addition to the calibration curves, four levels of QCs were analyzed as proposed by the FDA guidance. The QCs close to LLOQ were prepared with specific concentrations of each peptide (see Table 3) within 3-fold the LLOQ in yeast; except for the Atp1a1/2/3 surrogate. This peptide has a considerably higher LLOQ in pure solution and the concentration was set to 8 because it is expected in high abundance levels in our samples.

Accuracy and precision of the calibration curves were evaluated both in pure and complex matrices. The estimated concentration in both matrices was calculated using the equations obtained from the pure calibration curve (Fig. 3a and b), but the values for the yeast calibrators were also obtained using their own equations (Fig. 3c) to compare the values as another measure of the matrix effect (Section 3.2.4). In our analysis, all calibration points above the LLOQ presented an error and precision below the threshold of 15%, both in the pure (Fig. 3a) and complex (Fig. 3c) matrices for all the target peptides.

Within-run accuracy and precision were evaluated using QCs from yeast hydrolysate. The Quality Control samples (QCs) (Fig. 4a) also presented a very low %CV, below 5% for all the peptides except for the Atp1a3 surrogate at high concentration levels, but they

were all below 15%. Nevertheless, a higher accuracy deviation was generally observed; probably as a result of the matrix effect on ionization or interferences (*i.e.* coeluting molecules). Almost all the peptides presented a deviation below the threshold of 15% and only the Low QCs of LANDAAQVK (P-gp) and AAVPDAVVK (Atp1a1/2/3) were between 15 and 25%. These results confirm that this analytical method allows peptide quantification with a satisfying accuracy and precision. In addition, as 3 different replicates of peptide mix and dilution were used, this indicates that there was a low inter-batch variability.

Between-runs accuracy and precision were calculated from the analysis three different days of three batches of low, medium and high QC samples. We observed a %CV and %Deviation below 15% for all the peptides (Fig. 4b).

3.2.5. Matrix effect

It is well known that other analytes in the sample can interfere with the quantification in the analytes due to different factors such as ionization competition or interference, which are summarized as the matrix effect. The FDA guidance mentions that there should not be a matrix effect in the quantification of biomarkers, but do not give clear instructions to measure this [13]. Considering that it is not possible to obtain enough micro-vessels samples to study this, we used yeast protein hydrolysate to mimic the matrix. The calibration points were prepared using the same three batches of stock standard peptide mixture and internal standards to avoid bias due to preparation. As detailed in Section 3.2.3, we generally observed a higher LOQ in the complex matrix. In addition, we compared the impact on the calibration equation slope (Table 2) and observed a general decrease in the complex matrix slope, going from 0 to -5% in 70% of the transitions. Similarly, the deviation of the concentration in the complex matrix calibrators estimated with the equation from pure calibrators (Fig. 3b) was wider than when the equation from the same matrix was used, although still within the limits of 15%. The precision values are more independent from the equation used and the values in Fig. 3b and c are similar and below 15% for the non LLOQ calibrators. Although the FDA guidance does not

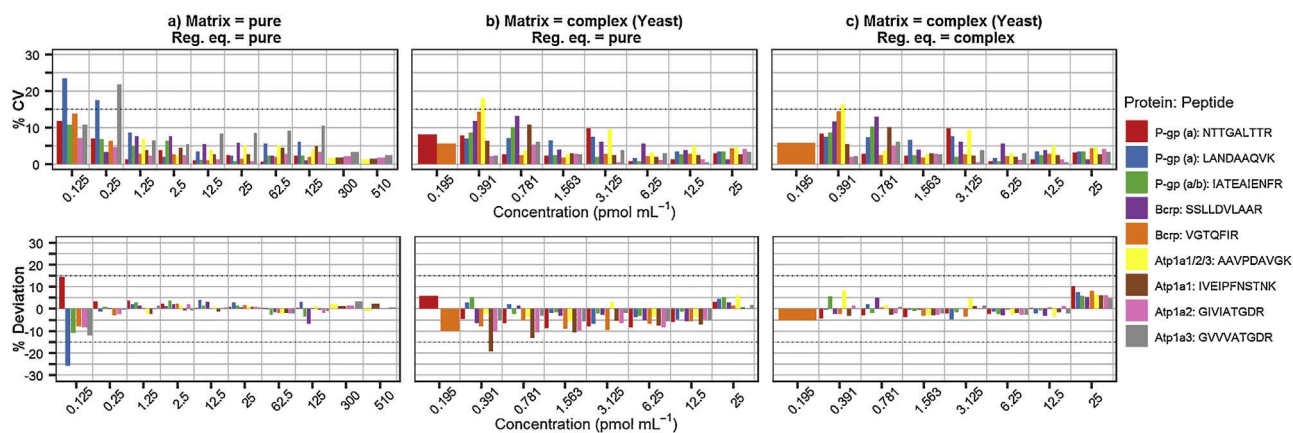


Fig. 3. Precision (%CV) and accuracy (%Deviation) for calibrators. Calibration curves were prepared in low complexity matrix (a) (90% water, 10% ACN + 0.1% formic acid) and high complexity matrix (b, c) (yeast digest). Concentration values were estimated using the equations from either the low complexity matrix (a, b) or the high complexity (c).

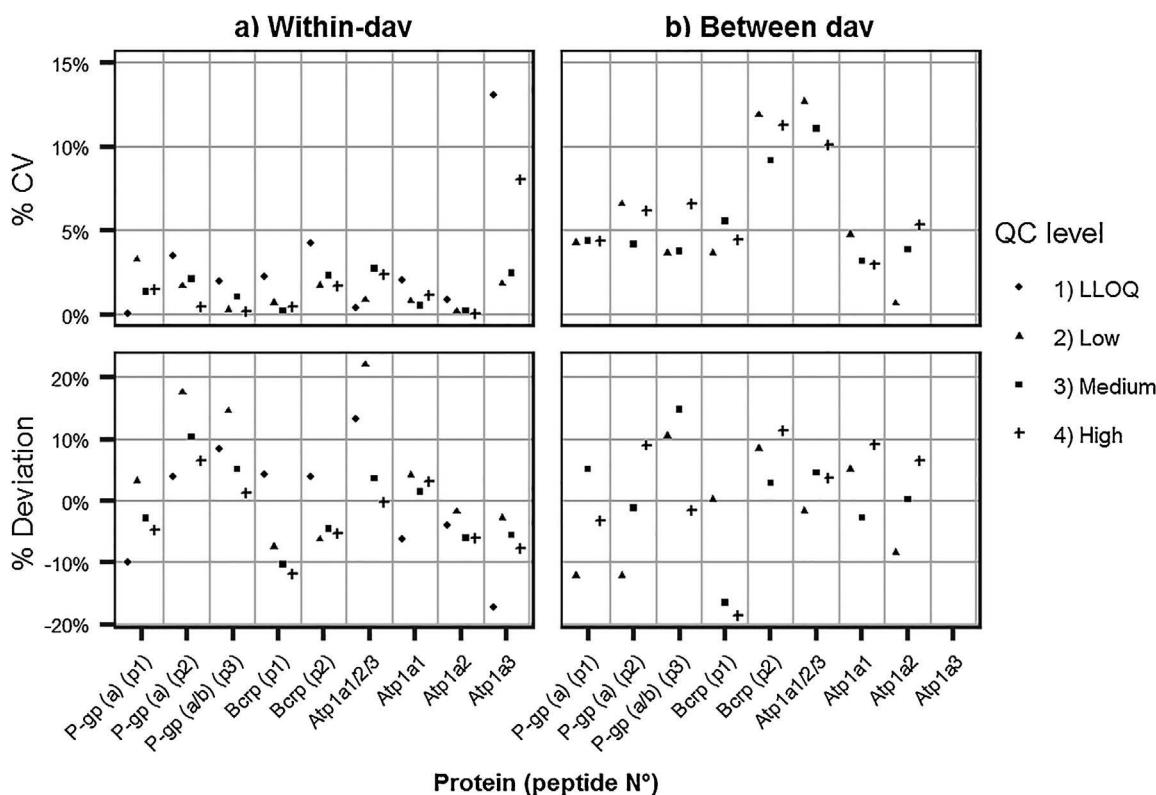


Fig. 4. Precision (%CV) and accuracy (%Deviation) for QCs. Three batches of quality control (QC) samples were prepared in high complexity matrix (yeast digest) in four concentrations: near to LLOQ (below 3-fold), 10, 20 and 60 pmol·mL⁻¹. The four QC levels were analyzed three times the same day for within-run evaluation (a) and in three separate days for between-run evaluation (b) (LLOQ-near was not included).

establish a tolerance threshold for matrix effect, we consider that values below 15% and the conservation of the accuracy also below this value should minimize the impact on the quantification. Therefore, we used the calibration curve prepared in pure solution for quantification of samples, to make the method simpler.

3.2.6. Peptide stability

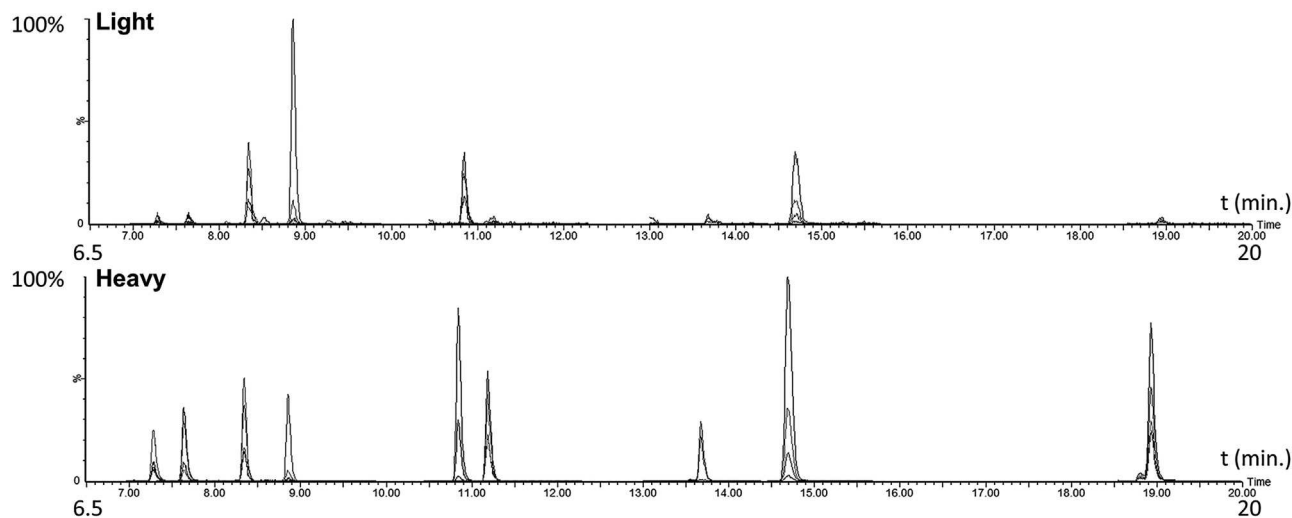
Freeze-thaw stability was proven for all the peptides, as they presented a recovery of 98.5–100% after six freezing/thawing cycles as shown in Table S-7.

Peptide stability during digestion was studied in yeast protein samples spiked with the standard light peptides before or after the digestion (Table S-8). Most of the peptides used in the present

study did not show a significantly different peptide amount (t-test on log₁₀ abundance values, *p*-value > 0.05). Only VGTQFIR (Bcrp) presented a significantly lower concentration (*p*-value = 0.0109) when the peptides followed the digestion incubation, representing a slight degradation (-9%). Nevertheless, these differences are within the accepted error margins (<20%) [13]. These results suggest that the target peptides selected are sufficiently stable in the trypsination conditions used (16 h at 37 °C), indicating thus that this quantification should not present bias due to peptide degradation at this step.

Stability in autosampler (4 °C) was evaluated by reinjecting all the calibration points 30 h after the first analysis and the QC levels after 45 and 75 h (Table S9 without observing any difference

a) Mouse cortical vessels



b) Rat brain microvessels

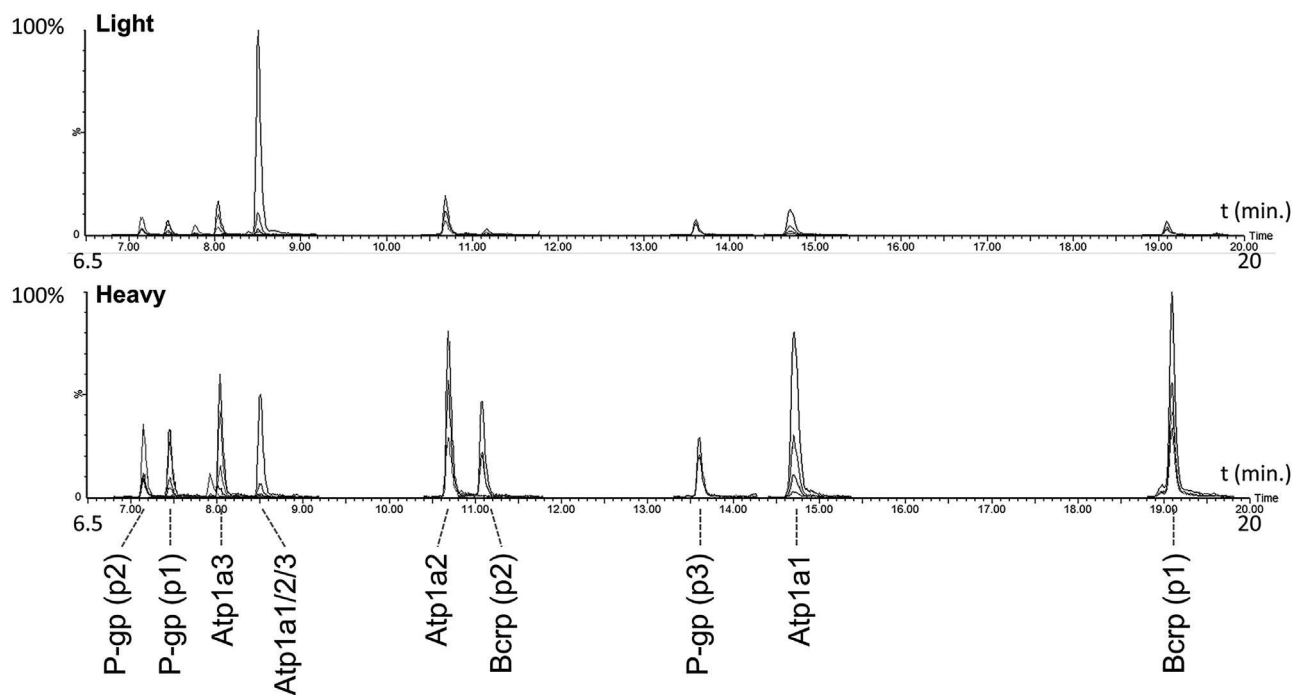


Fig. 5. Chromatograms obtained from the MRM of rat brain microvessels (a) and mouse microvessels (b).

Table 4

Targeted absolute quantification by LC-MS/MS (MRM) of the surrogate peptides from the endogenous proteins Bcrp, Pgp and Na⁺/K⁺ ATPase in mouse and rat tissues.

Protein	Peptide		Protein amount per peptide (fmol μg ⁻¹ ± s.d.)			
	N ^o	Sequence	Mouse-kidney (PMP)	Mouse Liver (PMP)	Mouse Cortical Vessels (WL)	Rat Cortical Microvessels (WL)
P-gp (a)	1	NTTGALTTR	BLQ (<1.64)	BLQ (<1.64)	BLQ (<1.64)	15.3 ± 0.21
P-gp (a)	2	LANDAAQVK	1.02 ± 0.02	BLQ (<1.02)	1.22 ± 0.10	23.8 ± 0.32
P-gp (a/b)	3	IATEAIENFR	1.59 ± 0.00	BLQ (<0.752)	1.04 ± 0.09	17.9 ± 0.07
Bcrp	1	SSLLDVLAAAR	30.6 ± 1.32	1.49 ± 0.11	BLQ (<0.751)	0.854 ± 0.00
Bcrp	2	VGTFQFIR	28.4 ± 1.33	1.35 ± 0.06	BLQ (<0.567)	BLQ (<0.567)
Atp1a1/2/3	0	AAVPDAVGK	150 ± 3.32	16.4 ± 0.01	32.8 ± 3.39	213 ± 2.90
Atp1a1	0	IVEIPFNSTNK	134 ± 3.82	11.1 ± 0.40	5.06 ± 0.34	32.9 ± 0.54
Atp1a2	0	GVIATGDR	BLQ (<0.75)	BLQ (<0.75)	3.49 ± 0.05	22.1 ± 1.13
Atp1a3	0	GVVVATGDR	BLQ (<0.961)	BLQ (<0.961)	11.3 ± 0.52	68.1 ± 3.07

The values presented were calculated as the mean (± s.d.) of two digestion replicates, each surveyed with 3 or 4 MRM transitions; except for AAVPDAVGK, that was quantified using only 2 transitions. Values below the limit of quantification (BLQ) were determined using the LOQs obtained from the yeast digest matrix (shown in parenthesis). VGTFQFIR is not present in the rat Bcrp sequence and was not detected (ND) in these samples. PMP = plasma membrane proteins, WL = whole lysate.

neither in the light-to-heavy peptide ratio level nor when quantification was performed. Similarly, mouse kidney PMP digests ($n=3$) spiked with the heavy peptides were injected three times to verify the peptide stability in sample after 45 and 75 h (Table S-10). No difference was seen in the light-to-heavy ratio or the determined abundance for any of the peptides quantified in mouse. Thus, our method allows the peptide quantification without significant bias for large sample sets. In any case, QCs should be injected at different times of the analysis to check the accuracy and precision.

3.3. Application of the method - AQUA quantification of P-gp, Bcrp and Na^+/K^+ ATPase

The developed and validated method was used to quantify P-gp, Bcrp and Na^+/K^+ ATPase α -subunit (Atp1a) isoforms in mouse kidney, mouse cortical vessels and rat cortical micro vessels. Fig. 5 show chromatograms obtained after 5 μL injection of samples. The results of the MRM peptide quantification in the samples are presented in Table 4 (values per transition are presented in Table S-11). The quantitative values were calculated as the mean (\pm s.d.) of 6 to 8 analytical measures from 3 to 4 MRM transitions (except for AAVPDAVGK, quantified with 2 transitions) detected in 2 digestion replicates; therefore they do not take into account biological variability. The variability between the transitions was low for each sample; presenting generally CVs below 20%. Noisy transitions that elevated the CV were not taken into account for the quantification of the sample (Table S-11).

In Sprague-Dawley (SD) rat cortical microvessels, the abundance of the IATEAIENFR and NTTGALTTR peptides were only slightly different, 17.9 ± 0.07 and $15.3 \pm 0.21 \text{ fmol} \cdot \mu\text{g}^{-1}$, respectively. These values are comparable to those previously obtained by Hoshi et al. [2], in SD and Wistar rat brain (19.0 ± 2.0 and $24.9 \pm 1.1 \text{ fmol} \cdot \mu\text{g}^{-1}$ respectively) using the NTTGALTTR peptide. Nevertheless, we observed a 1.5-fold higher abundance of LANDAAQVK ($23.8 \pm 0.32 \text{ fmol} \cdot \mu\text{g}^{-1}$), which could be due to differences in the digestion release of the peptide. The mouse cortical vessels presented a P-gp abundance of 1.22 ± 0.10 and 1.04 ± 0.09 with LANDAAQVK and IATEAIENFR, respectively, while NTTGALTTR was BLQ. The abundances were considerably lower than in rat cortical microvessels, probably because of the presence of differences in vessels isolation method. P-gp in plasma membrane of ddy mice renal cortex, renal medulla and liver was below the limits of quantification in the study of Kamiie et al. using the peptides NTTGALTTR and ATVSASHIIR. We also obtained BLQ values in C57BL/6 mice liver PMP with the three peptides, probably because it is expressed in very low levels [1]. In mouse kidney PMP we also obtained BLQ values using NTTGALTTR, but the more sensitive peptides LANDAAQVK and IATEAIENFR had abundances of 1.02 ± 0.02 and $1.59 \pm 0.00 \text{ fmol} \cdot \mu\text{g}^{-1}$, respectively.

These inter-peptide abundances differences observed could be due to isoforms or differences in digestion release. Indeed, IATEAIENFR is present in the two P-gp isoforms of P-gp (Abcb1a and b) expressed in rodents. In rat kidney and liver both isoforms are found [29] and it could be expected that the isoform specificity in mouse should be similar; thus the $0.57 \text{ fmol} \cdot \mu\text{g}^{-1}$ higher abundance in IATEAIENFR could represent the Mdr1b isoform. This does not explain the differences in rat cortical microvessels and mouse cortical vessels, as it has been found in rat cortical microvessels is almost exclusively the Abcb1a isoform [19]. Therefore, the 1.3-fold higher levels of LANDAAQVK than IATEAIENFR (in both animals) and 1.5-fold higher than NTTGALTTR (in rat) could be due to different digestion release of the peptides, as previously suggested [15].

Bcrp was BLQ in mouse cortical vessels, but in SD rat cortical microvessels the abundance of SLLDVLAAAR was $0.854 \text{ fmol} \cdot \mu\text{g}^{-1}$. This was lower than measured by Kamiie et al. [1] (using the

same peptide), which can be explained by a possible heterogeneity of the sample between the two studies. Indeed Bcrp unlike Pgp is expressed in different cells of the brain such as pericytes and astrocytes [30]. The analyzed samples in the two studies might not have the same cell composition and so overexpressed Bcrp in Kamiie et al. study. The abundance of SLLDVLAAAR and VGTQFIR in mouse kidney were 30.6 ± 1.32 and $28.4 \pm 1.33 \text{ fmol} \cdot \mu\text{g}^{-1}$, which is similar magnitudes to levels obtained by Kamiie et al. [1] (56.4 ± 1.82 and $25.9 \pm 1.35 \text{ fmol} \cdot \mu\text{g}^{-1}$ in renal cortex and medulla, respectively). In mouse liver we obtained abundances of 1.49 ± 0.11 and $1.35 \pm 0.06 \text{ fmol} \cdot \mu\text{g}^{-1}$. In these tissues, the levels of both Bcrp peptides were very similar; although the slightly lower value for VGTQFIR could be due to slight degradation, as shown in the digestion stability section. In a general way, P-gp was found in higher levels than Bcrp in the rodents cortical vessels while in mouse kidney it is the contrary as Bcrp is almost 30-fold more expressed than P-gp.

Na^+/K^+ ATPase α -subunit (Atp1a) was quantified as a marker of the plasma membrane. The previously reported peptide AAVPDAVGK [1] common to isoforms a1, a2 and a3 was used for the quantification as a multi-isoform probe, but we also selected peptides specific for each one of these isoforms. The a1-isoform peptide (IVEIPFNSTNK) was detected in all the tissues analysed, but the a2 and a3 peptides (GIVIATGDR and GVVVATGDR) were quantified only in brain cortical vessels. This is in accordance with previous studies which showed that rodent liver and kidney express almost exclusively the a1 isoform while in nervous tissues the a3 is the major isoform and a2 can also be found [26]. Interestingly, we observed that the a3 amount is 2-fold higher than a1 and 3-fold higher than a2 in mouse cortical vessels and rat cortical microvessels. In these samples, the total amount of atp1a, a2 and a3 peptides correspond to 60% of the amount of AAVPDAVGK; which could be due to different digestion release, but more studies should be performed to verify this hypothesis.

The abundance differences between peptides of a same protein that we observed point out the importance of the selection of surrogate peptides for LC-MS/MS protein quantification, as different probes may bias the results. Therefore, whenever possible, several peptide probes should be used for the quantification of each protein to increase the certainty on the measured values. In addition, it is advisable to use the same peptide probes when protein levels are compared between samples or experimental conditions.

4. Conclusion

We developed a method for the quantification of P-gp, Bcrp and Na^+/K^+ ATPase α -subunit isoforms at the BBB by LC-MS/MS (MRM) using the AQUA strategy with several peptides per protein. All the assays have been comprehensively validated in terms of linearity, accuracy, precision, digestion efficiency and peptide stability. This method was successfully applied to the determination of proteins in rat mouse kidney, mouse cortical vessels and rat cortical micro vessels. The different levels obtained for each peptide highlight the importance and difficulty of choosing surrogate peptides for protein quantification.

Author contributions

DGZ, PS, MV, MP, IB, ICG, JMS, YP, XD and MCM participated in research design. DGZ, MT, MS, WQ, CC, MP, ET and MCM performed experiments. DGZ developed the quantitative MS method. DGZ and MCM performed data analysis. The manuscript was written by DGZ and MCM with contributions and approbation from all authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jpba.2018.11.013>.

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