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Optimizing the Conditions of a Multiple Reaction Monitoring Assay for Membrane Proteins: Quantification of Cytochrome P450 11A1 and Adrenodoxin Reductase in Bovine Adrenal Cortex and Retina

Wei-Li Liao[†,§], **Gun-Young Heo**[‡], **Nathan G. Dodder**[§,1], **Irina A. Pikuleva**[‡], and **Illarion V. Turko**[*,†,§]

Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, Maryland 20850, Department of Ophthalmology and Visual Sciences, Case Western Reserve University, Cleveland, Ohio 44106, and Analytical Chemistry Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

Abstract

Approximately 30% of naturally occurring proteins are predicted to be embedded in biological membranes. Nevertheless, this group of proteins is traditionally understudied due to limitations of the available analytical tools. To facilitate the analysis of membrane proteins, the analytical methods for their soluble counterparts must be optimized or modified. Multiple reaction monitoring (MRM) assays have proven successful for the absolute quantification of proteins and for profiling protein modifications in cell lysates and human plasma/serum, but have found little application in the analysis of membrane proteins. We report on the optimization of sample preparation conditions for the quantification of two membrane proteins, cytochrome P450 11A1 (CYP11A1) and adrenodoxin reductase (AdR). These conditions can be used for the analysis of other membrane proteins. We have demonstrated that membrane proteins that are tightly associated with the membrane, such as CYP11A1, can be quantified in the total tissue membrane pellet obtained after high-speed centrifugation, whereas proteins that are weakly associated with the membrane, such as AdR, must be quantified in the whole tissue homogenate. We have compared quantifications of CYP11A1 using two different detergents, *Rapi*Gest SP and sodium cholate, and two different trypsins, sequencing grade modified trypsin and trypsin, type IX-S from porcine pancreas. The measured concentrations in these experiments were similar and encouraged the use of either combination of detergent/trypsin for quantification of other membrane proteins. Overall, the CYP11A1 and AdR quantified in this work ranged from hundred pmol to ten fmol per mg of tissue protein.

Multiple reaction monitoring (MRM) assays use liquid chromatography/triple quadrupole mass spectrometry (LC-MS/MS) to measure the absolute concentration (for example, in mol analyte per mass tissue) of small molecules and, more recently, proteins in complex biological systems. The high specificity of MRM assays for proteins is achieved by monitoring multiple

[*]To whom correspondence should be addressed. Dr. Illarion V. Turko, Center for Advanced Research in Biotechnology, 9600 Gudelsky Dr., Rockville, MD 20850. Tel.: 240-314-6257. Fax: 240-314-6225. turko@umbi.umd.edu.
[†]Center for Advanced Research in Biotechnology.
[‡]Department of Ophthalmology and Visual Sciences, Case Western Reserve University.
[§]Analytical Chemistry Division, National Institute of Standards and Technology.
[1]Current address: Southern California Coastal Water Research Project, Costa Mesa, California 92626

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

transitions (precursor ion – product ion pairs) for each target peptide derived from the protein of interest. Quantification is performed by comparing the chromatographic peak area of a transition from the (unlabeled) native peptide to the corresponding transition from the internal standard. The internal standard can be either a synthetic stable isotope labeled peptide (usually ^{13}C labeled) or a ^{15}N labeled protein which has been digested in the course of the measurement procedure. This approach has been successfully used for biomarker validation and determination of protein modifications in cell lysates as well as in human plasma and serum.[1–11] However, the application of MRM assays to the direct measurement of membrane proteins is more recent [12] and remains a challenge.

Membrane proteins constitute approximately 30% of all cellular proteins.[13] Given the importance of membrane proteins in the various cellular processes and their potential as drug targets, it is imperative that the sample preparation portion of MRM assays be optimized to perform quantitative measurements on membrane proteins. The sample preparation steps that should be optimized are summarized below. First, high speed centrifugation of the whole tissue homogenate followed by the MRM assay of the resulting total membrane pellet can be an important enrichment procedure for the quantification of low abundance membrane proteins. However, this advantage will only be applicable to those membrane proteins that are tightly associated with the membrane. Second, to make membrane proteins more amenable to cleavage by trypsin, the sample should be dissolved in the buffer containing a detergent. This detergent should be trypsin compatible. It is also beneficial if the detergent can be removed after the digestion is complete because the detergent can interfere with the instrumental analysis. Third, the quantification of low abundance membrane proteins requires the biological sample to be scaled up, which requires an increase in the amount of trypsin. An inexpensive source of trypsin that can be used for large biological samples will advance the field of membrane protein quantification.

To address these general caveats we have focused on the absolute quantification of two monotopic membrane proteins, cytochrome P450 11A1 (CYP11A1) and NADPH-adrenodoxin reductase (AdR). CYP11A1 resides at the matrix side of the inner mitochondrial membrane and catalyses the first step in the overall steroid hormone biosynthesis, the conversion of cholesterol to pregnenolone. This enzyme is part of the electron transfer system, which also includes the small soluble protein adrenodoxin and membrane-bound AdR. AdR is reduced by NADPH and in turn reduces adrenodoxin. Adrenodoxin then reduces CYP11A1. CYP11A1 and AdR are highly abundant in the mitochondria of steroidogenic tissues.[14] There is also evidence for the presence of CYP11A1 in the brain,[15] retina,[16–18] and retinal pigment epithelium.[19] In the present study, we first used bovine adrenal cortex that highly expresses CYP11A1 and AdR to optimize the conditions of the MRM assay. The optimized conditions were then applied to the measurement of CYP11A1 and AdR in bovine retina at sub-pmol per mg tissue protein levels.

EXPERIMENTAL SECTION

Materials

Ammonium chloride (^{15}N, 99 %) was purchased from Cambridge Isotope Laboratories (Andover, MA). *Rapi*Gest™ SF was from Waters (Milford, MA) and the *DC* Protein Assay kit was from Bio-Rad Laboratories (Hercules, CA). Sequencing grade modified trypsin was from Promega Corp. (Madison, WI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Bovine tissues were obtained from a local slaughterhouse. Adrenal glands were trimmed to remove fat and cut in half longitudinally. The central medulla and a minor portion of the adjacent zone reticularis were scraped and discarded. Then the cortex was scraped and used

for analysis. To isolate the retina, the eyes were dissected and the neural retina was separated from the retinal pigment epithelium.

Protein Standards

Recombinant bovine CYP11A1 and AdR were expressed as described.[20, 21] For expression of ^{15}N-labeled CYP11A1 (^{15}N-CYP11A1) and ^{15}N-labeled AdR (^{15}N-AdR), *Escherichia coli* GC5 cells were co-transformed with expression constructs for CYP11A1 and AdR, and the Gro7 vector containing chaperones GroEL and GroES. Subsequent expression of ^{15}N-labeled proteins followed the procedure in Marley et al.[22] using M9 minimal salt solution containing 1 g/L of $^{15}NH_4Cl$ (^{15}N, 99%) as the sole nitrogen source. Details of the expression and purification can be found in Supporting Information. CYP11A1 and ^{15}N-CYP11A1 concentrations were calculated from the CO-reduced difference spectra using an absorption coefficient of 91 $mM^{-1}cm^{-1}$ for the absorbance difference between 450 and 490 nm.[23] AdR and ^{15}N-AdR concentrations were measured based on the flavin moiety using an absorption coefficient of 11 $mM^{-1}cm^{-1}$ at 450 nm.[24]

Sample Preparation

The bovine adrenal cortex and retina were manually homogenized in 25 mM NH_4HCO_3 using a grinder with glass pestle and then sonicated at 30 W using three 10 s continuous cycles (Sonicator 3000, Misonix Inc., Farmingdale, NY). The total protein concentration was measured in the presence of 2 % SDS using the *DC* protein Assay kit and bovine serum albumin as a standard. The whole homogenate was then aliquoted into 1 mg portions of the total tissue protein *per* tube. One set of tubes was frozen at −80 °C while another set of tubes was first centrifuged at 153,000 *g* for 30 min to generate the total membrane pellet and then frozen at −80 °C. Consequently, the measurements of CYP11A1 and AdR were later performed on two different samples: the whole tissue homogenate and the total membrane pellet. In both cases, the samples were placed in the 0.2% *Rapi*Gest SF surfactant or 0.2% sodium cholate in 25 mM NH_4HCO_3 and each sample was supplemented with an exact amount of the ^{15}N-labeled internal standards ^{15}N-CYP11A1 and/or ^{15}N-AdR. The samples were heated at 90 °C for 5 min, cooled down to room temperature, and treated with either sequencing grade modified trypsin (Promega) or with trypsin, type IX-S from porcine pancreas (Sigma-Aldrich, catalog number T0303) for 15 hrs at 37 °C. The substrate/trypsin ratio was 50/1 (w/w). After hydrolysis, the samples were centrifuged at 153,000 *g* for 30 min and the supernatant was transferred to new tubes. The supernatant was then treated with 0.5% TFA for 30 min at 37 °C and centrifuged again at 153,000 *g* for 30 min. Supernatant from the second centrifugation was transferred to new tubes, mixed with equal volume of acetonitrile, and dried using a Vacufuge (Eppendorf AG, Hamburg, Germany).

LC-MS/MS Analysis

Instrumental analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP, ABI/MDS-Sciex). Peptide separations were performed on an Eksigent nanoLC-2D system (Dublin, CA) with a PicoFrit (75 μm ID/10 μm tip ID, New Objective) column self-packed to a bed length of 12 cm with Reprosil-Pur 120 C18-AQ, 3 μm resin (Dr. Maisch GmbH, Germany). Peptides were eluted over a 42 min gradient from 13 % to 31 % acetonitrile, containing 0.1 % formic acid and at a flow rate of 300 nL/min. The column effluent was continuously directed into the nanospray source of the mass spectrometer. All acquisition methods used the following parameters: an ion spray voltage of 2100 V, curtain gas of 30 psi, source gas of 9 psi, interface heating temperature of 170 °C, declustering potential of 76 V for +2 precursor ions and 65 V for +3 precursor ions, collision energy of 30 V for +2 precursor ions and 22 V for +3 precursor ions, and collision cell exit potential of 16 V for +2 precursor ions and 13V for +3 precursor ions. The dwell time for all transitions was 40 ms.

Quantitative Analysis and Validation

Protein concentrations were calculated from the ratio of the light and heavy MRM peak areas multiplied by the known amount of ^{15}N-labeled protein internal standard spiked into the sample prior to digestion. The mass spectrometer monitored three transitions per peptide. The identities of the measured peptides were confirmed using two criteria. Both the retention time of the three MRM peaks from a given peptide and the ratios among the three MRM peaks must match the retention time and ratios obtained when running standards under the same conditions. Selection of the MRM transitions is described in detail below. The three transitions from each peptide were treated as independent measurements, each resulting in a concentration value for the corresponding protein. These three values show some experimental variation. Quantitative results were expressed as pmol of quantified protein per mg of total tissue protein. The mean and standard deviation of the protein concentration were calculated by treating the three transitions for each of the different target peptides and the three experimental replicates all as independent measurements.

RESULTS AND DISCUSSION

^{15}N-labeled Internal Standards

The majority of MRM approaches for protein quantification use stable isotope labeled synthetic peptides as the internal standards. However, this approach can lead to a quantification error that originates from incomplete protein hydrolysis or missed cleavages in a complex biological sample. This can be prevented by using stable isotope-labeled intact proteins.[7, 8, 25, 26]

The isotopic incorporation in the recombinant protein was measured to ensure that the isotopic distribution of the heavy peptides did not overlap with that of the light peptides. Purified ^{15}N-CYP11A1 and ^{15}N-AdR were digested in-solution with trypsin and experimental MALDI spectra of the digests were acquired on a 4700 MALDI-TOF/TOF (Applied Biosystems, Framingham, MA) operated in the single stage MS mode. During acquisition of the experimental mass spectra, the laser intensity was set so that the peak intensity was no greater than 25,000 cps to avoid saturating the detector and distorting the observed isotopic distribution. Multiple peptides from each protein were analyzed for their ^{15}N incorporation. Upon first inspection, the incorporation was close to 100%. Therefore, we simulated isotopic distributions with 97%, 98%, and 99% ^{15}N incorporation for each peptide, using the OrgMassSpecR computer program (http://orgmassspecr.r-forge.r-project.org and Supporting Information). These simulations were compared to the experimental spectra to determine the best fit. The same labeling efficiencies were observed in multiple peptides from each protein. Figure 1 shows experimental spectra for representative peptides and their closest simulated isotopic distribution. This comparison shows that the ^{15}N-labeling efficiency was approximately 99% for both recombinant proteins, ^{15}N-CYP11A1 and ^{15}N-AdR.

Selection of MRM Transitions

The target peptide(s) and optimum transitions were obtained by using purified recombinant bovine CYP11A1 and AdR. The OrgMassSpecR program was used to calculate the theoretical peptide sequences, precursor m/z values, and fragment ion m/z values in the following procedure. Based on the protein sequence, a list of theoretical tryptic peptides with zero missed cleavages and molecular masses between 700 Da and 2500 Da was generated. Methionine or cysteine containing peptides were omitted due to the existence of various oxidation entities in nature, which could introduce variations in the quantification. The abundance of these peptides in the digest was determined by setting the LC-MS/MS to select for all +1,+2 and +3 precursor ions in the list (Q1MI mode: quadrupole 1, multiple ion). The uniqueness of the observed peptides was verified by performing a tblastn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The optimum (intense and selective) MRM transitions for the observed peptides were then

determined by generating a table of the observed +2 charge precursor ions with the corresponding +1 charge *b* and *y* fragment ions, and the +3 charge precursor ions with the corresponding +1 and +2 charge *b* and *y* fragment ions. Only ions with *m/z* values greater than the precursor ion *m/z* were included in the table, because these fragments tend to be more intense and there is less noise in this region of the spectrum. The LC-MS/MS was set to select all the MRM transitions in the table, at periods within the chromatographic run based on their observed retention times in the previous experiment. The transitions for CYP11A1 and AdR were monitored in separate runs. The intensities of the MRM transitions were recorded and the three most intense transitions per peptide were selected. The identities of the MRM transitions were verified by collecting full scan fragmentation spectra of the target peptides by using the first mass filter to select the target peptide and operating the linear ion trap in scan mode. The fragmentation patterns matched those expected for the target peptides, and the relative peak intensities in the fragmentation spectra matched those in the MRM experiments. The optimum MRM transitions were then monitored in a digested bovine tissue sample to verify that they could be observed in a complex matrix. The target peptides and optimum MRM transitions are shown in Table 1. There is potential for interference when the MRM assays are performed in complex biological samples. The specific sources of interference and non-linearity in response curves are broadly discussed.[6–11] Here, we would like to emphasize that consistency of the sample extraction procedure, target protein digestion, and selection of transitions which maximize specificity and minimize interferences from species that fall within the mass windows of the mass filters is critical for MRM assay development.

The linearity of the optimum transitions were verified by spiking five adrenal cortex samples with varying amounts of the internal standards. Figure 2 shows response curves for the LHPISVTLQR peptide derived from ^{15}N-CYP11A1 and the FGVAPDHPEVK peptide derived from ^{15}N-AdR. The peak area ratios were determined from the extracted ion chromatograms of the transitions. The response curves show linearity and low scatter over the 2 orders of magnitude concentration range tested. Had it been observed, deviation from linearity could be caused by detector saturation at higher concentrations, a change in the efficiency of trypsin towards the digestion of the internal standard as increasing amounts if the internal standard was present, or the presence of a compound with interfering MRM transitions. Significant scatter within the response curve for a transition could be caused by inconsistency in the sample preparation or electrospray conditions. These response curves are representative of the other target peptides used for the quantification of CYP11A1 and AdR.

The relative ratios of the three transitions monitored in the buffer for ^{15}N-LHPISVTLQR, derived from ^{15}N-CYP11A1, show the ideal ratios of the transitions in the absence of any interference, i.e. buffer (Figures 3A and 3D). Figures 3B and 3E show that the ratios obtained for this peptide by spiking ^{15}N-CYP11A1 into a total adrenal cortex membrane pellet agree well with those observed in the buffer. In addition, the MRM transition ratios of this native peptide from adrenal CYP11A1 (Figures 3C and 3F) are also similar to those observed for ^{15}N-LHPISVTLQR in buffer. Taking together, this data demonstrate no significant interference for the quantification of CYP11A1 from the adrenal cortex sample using the selected transitions from the unlabeled and labeled forms of the LHPISVTLQR peptide. The data for NFIPLLNPVSQDFVSLLHK and YTEIFYQDLR peptides from CYP11A1 are deposited to the Supporting Information and also show no significant interference.

Figure 4 shows the same set of data for the three transitions of peptide FGVAPDHPEVK used for the quantification of AdR. The ratios for ^{15}N- FGVAPDHPEVK in the buffer and spiked into the whole adrenal cortex homogenate, and for native FGVAPDHPEVK from adrenal AdR, are essentially the same. These results show the lack of significant interference for the quantification of AdR in adrenal cortex samples using the selected transitions from the unlabeled and labeled forms of the FGVAPDHPEVK peptide. The data for the other two

peptides from AdR, TATEKPGVEEAAR and SPQQVLPSPDGR, are in the Supporting Information and also show no significant interference.

Optimized sample preparation conditions for absolute quantification of membrane proteins

The absolute quantification of membrane proteins requires several steps of sample processing before LC-MS/MS analysis. To optimize sample processing, we first used adrenal cortex, a tissue with a high level of CYP11A1 and AdR expression. High-speed centrifugation of the whole tissue homogenate yielded the total membrane pellet. Isolation of this fraction enriches the membrane proteins and increases assay sensitivity. However, this advantage is applicable only to those membrane proteins that are tightly associated with the membrane. The measured concentrations for CYP11A1 based on three different peptides were in the range of 91 to 103 pmol/mg tissue protein in the whole adrenal cortex homogenate, and in the range of 89 to 101 pmol/mg tissue protein in the total adrenal cortex membrane pellet (Table 2). CYP11A1 was not detected in the high-speed supernatant, indicating that it is strongly bound to the membrane. The measured concentrations of AdR were substantially different: approximately 87 to 93 pmol AdR/mg tissue protein in the whole homogenate and only 38 to 46 pmol/mg tissue protein in the total membrane pellet (Table 2). The remainder of the AdR was observed in the high-speed supernatant. These results clearly demonstrate that homogenization and sonication during sample processing re-distributes the weakly membrane-associated AdR between the soluble (supernatant) and membrane (pellet) fractions. Therefore, the MRM assay for AdR should be performed on the whole tissue homogenate.

There are three general types of protein association with membranes. The first type is irreversibly associated proteins, including integral transmembrane proteins, integral monotopic proteins, and some water-soluble proteins that can form transmembrane channels (pore-forming proteins). For this type, the MRM measurements can be performed on the total membrane pellet. The second type is weakly associated peripheral membrane proteins, for which the measurements must be performed on the whole tissue homogenate. The third type includes monotopic proteins, whose association with the membrane relies on amphipathic a-helixes, hydrophobic loops, covalently bound membrane lipids and electrostatic or ionic interactions with membrane lipids. A choice of sample processing for this type of protein is not immediately clear. If it has been previously shown that detergent is required to solubilize a target protein of this type (for example CYP11A1 in the present study), the generalization would be that measurements on the total membrane pellet are appropriate. Otherwise, (for example AdR in the present study), the decision can be made based on experimental data only.

Digestion of the total membrane pellet requires a trypsin-compatible detergent to make the membrane proteins more amenable to trypsinolysis. In addition, the measurement of low abundance proteins requires a scale-up of the initial biological sample and therefore an excessive amount of trypsin to hydrolyze the sample. We measured CYP11A1 in the total adrenal membrane pellet using two different detergents and two different trypsins. The data is summarized in Table 3. *Rapi*Gest SF usually enhances the enzymatic digestion of proteins.[27] This detergent is also easily removed, preventing interference of the LC and mass spectrometry analyses of digested samples. Sodium cholate is a trypsin-compatible,[28] water soluble ionic detergent with a very low aggregation number (average 2–4) and very high critical micelle concentration (9–14 mM in H_2O). Treatment of the digested sample with 0.5 % TFA leads to precipitation of the free cholic acid and efficient detergent removal. These features make both detergents a good choice for MRM assays of membrane proteins. Depending on the CYP11A1 peptide, detergent, and trypsin used, the mean measured concentrations and standard deviations (Table 3) were very similar. The consensus of the three peptides shows that both detergents, *Rapi*Gest SF and sodium cholate, as well as both trypsins, sequencing grade modified trypsin (Promega) and trypsin, type IX-S from porcine pancreas (Sigma-Aldrich) can be used for

quantification of CYP11A1. Sodium cholate detergent and trypsin type IX-S from porcine pancreas are currently more economical. Based on these observations, the reasonable generalization would be that these two detergents and trypsins can be used for the quantification of other membrane proteins as well.

Quantification of CYP11A1 and AdR in bovine retina

The discovery that tissue types other than adrenal and gonad tissues could make their own steroids from cholesterol[15–19], stimulated the idea that these tissues may have neuroendocrine functions. CYP11A1 catalyses the first step in the overall steroid hormone biosynthesis, and mRNA and protein expression of CYP11A1 has been detected in retina.[16–18] Therefore, we quantified CYP11A1 and the associated electron-transfer protein AdR in bovine retina to establish the expression levels. Compared to adrenal cortex samples, it was necessary to take at least 100 times more retinal sample mass to obtain reliable MRM signal intensities. Due to the larger sample size, we used sodium cholate and trypsin type IX-S from porcine pancreas (Sigma-Aldrich) for the sample preparation. The measured concentration data is summarized in Table 4. In the retina, the concentrations of CYP11A1 and AdR are on the sub-pmol per mg tissue protein level, and the concentration of AdR is approximately 100 fold higher than that of CYP11A1. This stoichiometry is different from that observed in adrenal cortex (~1:1, M/M) suggesting that other mitochondrial P450(s) are present in the retina as well. Indeed, mitochondrial P450, CYP27A1, has recently been identified in the retina.[29] Comparing two organs, CYP11A1 is approximately 10^{-4} less abundant in the retina than in the adrenal cortex, indicating a much lower production of steroid hormones in the retina. The contribution of steroid hormones to the retinal physiology and the regulation of steroidogenesis in this organ are currently unknown. However, it is proposed that steroid hormones may play an important role in retinal degeneration, either as neurodegenerative or neuroprotective agents, and that their levels may fluctuate in response to insult.[30] Local concentrations of steroid hormones are determined by the levels of the enzymes that generate these hormones. Consequently, we need to develop analytical tools to precisely quantify these low abundance enzymes to gain insight into the role of steroidogenesis in the retina. This has been accomplished in the presented study.

CONCLUSIONS

An LC-MS/MS MRM assay for the quantification of low abundance membrane protein was developed. The sample preparation method was designed and optimized specifically for membrane protein analysis and can be applied to other target proteins in addition to the two measured in this study. The optimized sample preparation conditions and instrumental parameters allowed quantitative MRM measurements of membrane bound proteins at sub-pmol per mg tissue protein concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that the materials, instruments, or equipment identified is necessarily the best available for the purpose.

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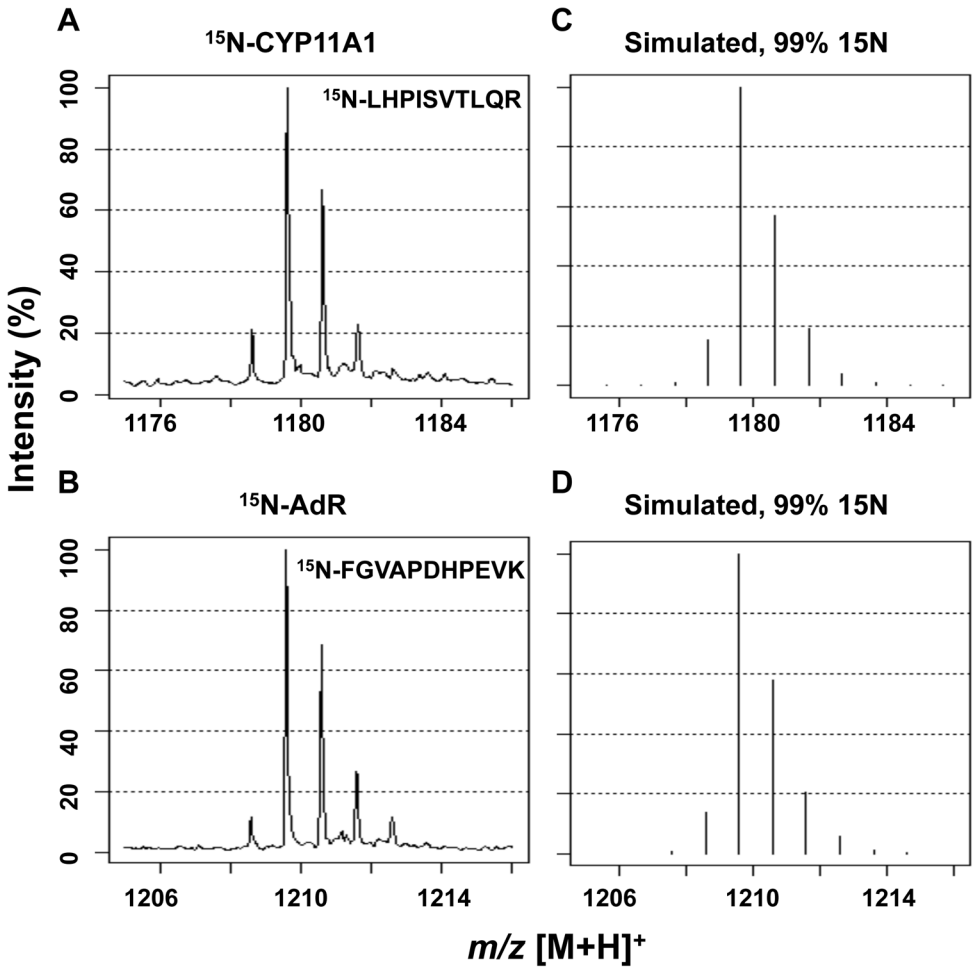

Figure 1.

Experimental and simulated mass spectra of representative peptides from ^{15}N-CYP11A1 and ^{15}N-AdR. ^{15}N-LHPISVTLQR from ^{15}N-CYP11A1 (A); the labeling incorporation was determined to be 99% when compared to the simulated spectrum (C). Similarly, ^{15}N-FGVAPDHPEVK from ^{15}N-AdR (B); the labeling incorporation was determined to be 99% when compared to the simulated spectrum (D).

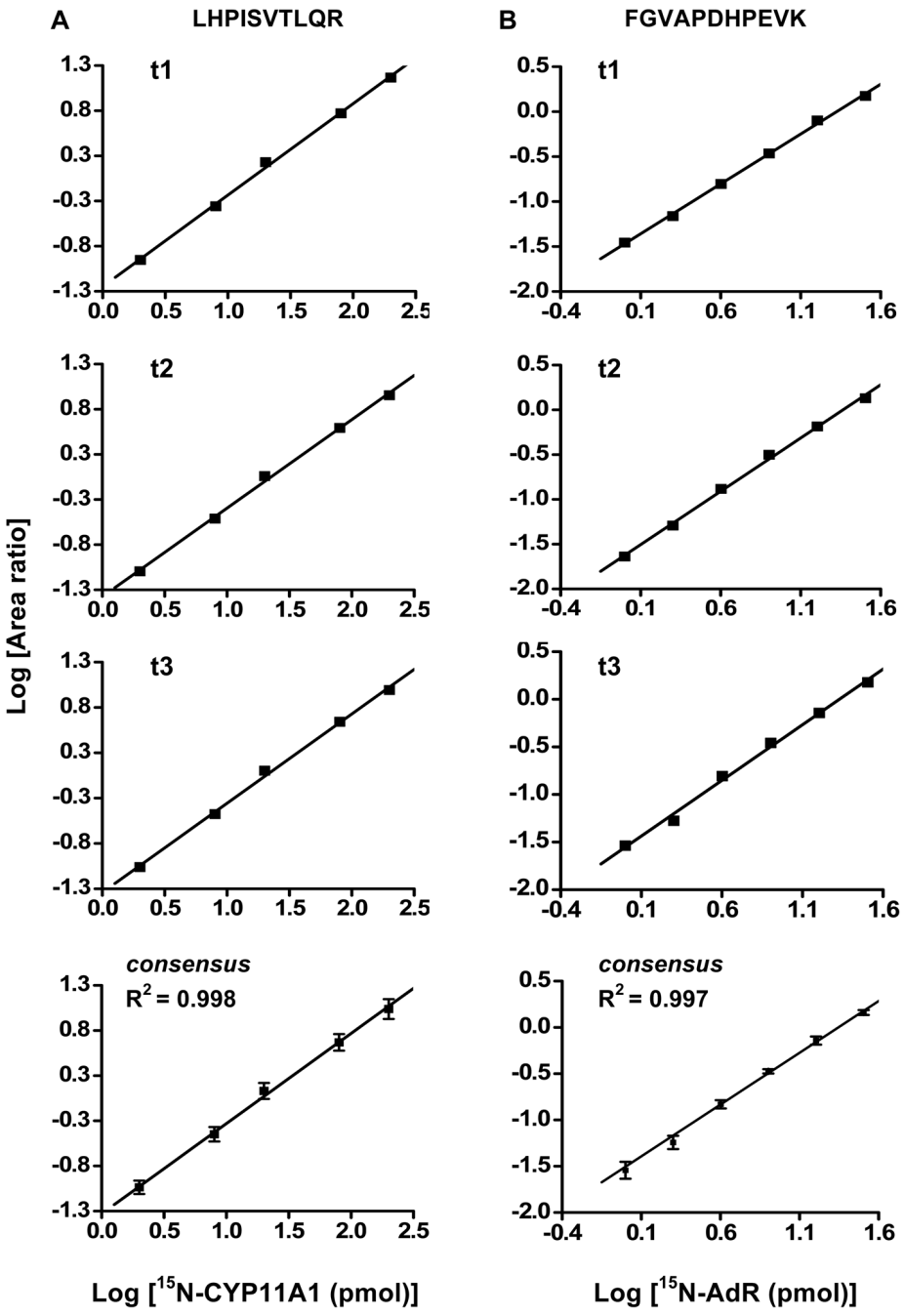

Figure 2.

Calibration curves for quantification of CYP11A1 and AdR in the whole adrenal cortex homogenate. The area ratio of a corresponding heavy peptide to light peptide was plotted *versus* protein concentration. (A) shows three individual transitions (t1–t3) and consensus of these transitions for LHPISVTLQR peptide from CYP11A1; t1: 388.6/548.3 and 393.9/555.3; t2: 388.6/517.3 and 393.9/525.3; t3: 388.6/416.3 and 393.9/423.2. (B) shows three individual transitions (t1-t3) and consensus of these transitions for FGVAPDHPEVK peptide from AdR; t1: 399.2/446.7 and 403.9/452.2; t2: 399.2/524.7 and 403.9/531.3; t3: 399.2/609.3 and 403.9/617.3. For *consensus*, the data for three individual transitions (t1–t3) were combined and presented as mean ± SD.

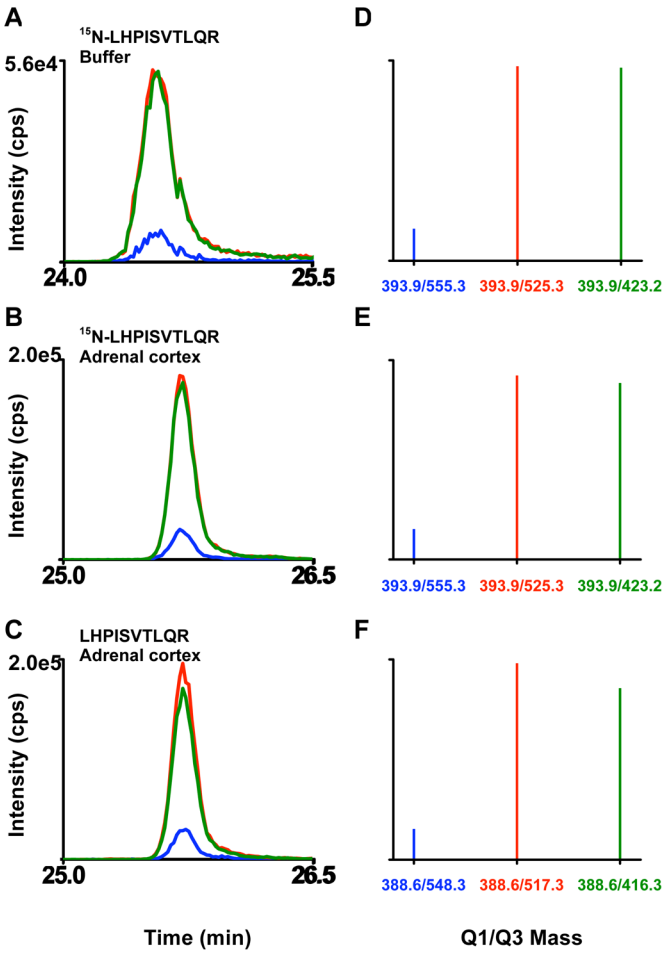

Figure 3.

Extracted ion chromatograms (A–C) and MRM spectra (D–F) of transitions monitored for ^{15}N-CYP11A1 in 25 mM NH_4HCO_3 (A and D) and in adrenal cortex (B and E) and for CYP11A1 in adrenal cortex (C and F). Data are presented for LHPISVTLQR peptide of CYP11A1. Overlaid extracted ion chromatograms and ions in the MRM/MS spectra are color-coordinated. *cps*, counts per second.

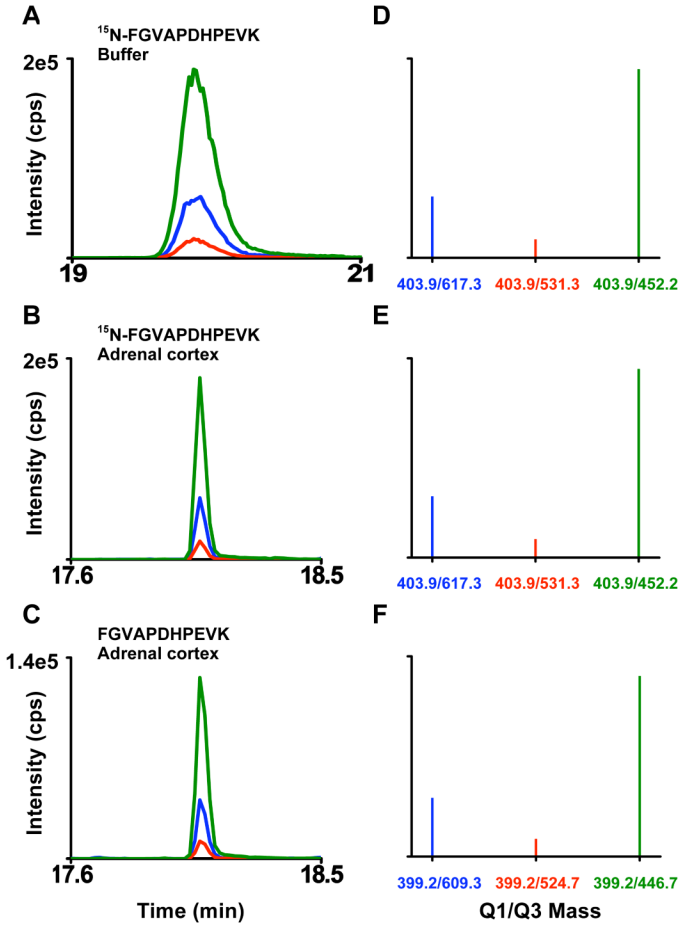

Figure 4.

Extracted ion chromatograms (A–C) and MRM spectra (D–F) of transitions monitored for ^{15}N-AdR in 25 mM NH_4HCO_3 (A and D) and in adrenal cortex (B and E) and for AdR in adrenal cortex (C and F). Data are presented for FGVAPDHPEVK peptide of AdR. Overlaid extracted ion chromatograms and ions in the MRM/MS spectra are color-coordinated. *cps*, counts per second.

Table 1

Peptides and MRM transitions used for the quantification of CYP11A1 and AdR.

	MRM transitions (m/z)			
	Q1(charge)	Q3(type, charge)		
CYP11A1				
NFIPLLNPVSQDFVSLLHK	727.7(+3)	843.5(y^7, +1)	904.0(y^{16}, +2)	960.6(y^{17}, +2)
^{15}N-NFIPLLNPVSQDFVSLLHK	736.1(+3)	853.5(y^7, +1)	914.5(y^{16}, +2)	971.5(y^{17}, +2)
YTEIFYQDLR	674.3(+2)	694.3(y^5, +1)	841.4(y^6, +1)	1083.5(y^8, +1)
^{15}N-YTEIFYQDLR	681.3(+2)	703.3(y^5, +1)	851.4(y^6, +1)	1095.5(y^8, +1)
LHPISVTLQR	388.6(+3)	416.3(y^3, +1)	517.3(y^4, +1)	548.3(b^5, +1)
^{15}N-LHPISVTLQR	393.9(+3)	423.2(y^3, +1)	525.3(y^4, +1)	555.3(b^5, +1)
AdR				
FGVAPDHPEVK	399.2(+3)	446.7(y^8, +2)	524.7(y^{10}, +2)	609.3(y^5, +1)
^{15}N-FGVAPDHPEVK	403.9(+3)	452.2(y^8, +2)	531.3(y^{10}, +2)	617.3(y^5, +1)
TATEKPGVEEAAR	453.6(+3)	575.3(y^5, +1)	593.8(y^{11}, +2)	828.4(y^8, +1)
^{15}N-TATEKPGVEEAAR	459.2(+3)	583.3(y^5, +1)	601.3(y^{11}, +2)	839.4(y^8, +1)
SPQQVLPSPDGR	640.8(+2)	628.3(y^6, +1)	653.4(b^6, +1)	741.4(y^7, +1)
^{15}N-SPQQVLPSPDGR	649.3(+2)	637.3(y^6, +1)	661.3(b^6, +1)	751.4(y^7, +1)

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Table 2

Quantification of CYP11A1 and AdR in the bovine whole adrenal cortex homogenate, total membrane pellet, and high-speed supernatant[a]

	protein (pmol/mg tissue protein)[b]		
	homogenate	pellet	supernatant
CYP11A1			
NFIPLLNPVSQDFVSLLHK	93.1 ± 5.7	93.7 ± 9.0	ND
YTEIFYQDLR	103.2 ± 5.9	100.6 ± 6.4	ND
LHPISVTLQR	90.5 ± 12.1	88.5 ± 11.7	ND
AdR			
FGVAPDHPEVK	92.7 ± 7.8	37.9 ± 3.4	49.8 ± 5.2
TATEKPGVEEAAR	91.4 ± 15.3	45.6 ± 2.5	45.8 ± 8.1
SPQQVLPSPDGR	86.9 ± 10.6	44.7 ± 6.1	53.9 ± 5.6

[a] The whole adrenal cortex homogenate was centrifuged at 153,000 *g* for 30 min to generate the total membrane pellet and high-speed supernatant.

[b] The concentration was calculated for three experimental replicates by monitoring three transitions per individual peptide, with two different concentrations of ^{15}N-labeled internal standard, and presented as mean ± SD. The monitored transitions are summarized in Table 1.

ND, not detected. Experiments were performed using 0.2% *Rapi*Gest SF and sequencing grade modified trypsin (Promega).

Table 3

Quantification of CYP11A1 in the bovine adrenal cortex total membrane pellet

peptide	CYP11A1 (pmol/mg tissue protein)[a]			
	0.2% *Rapi*Gest SF		0.2% sodium cholate	
	trypsin 1[b]	trypsin 2[b]	trypsin 1	trypsin 2
NFIPLLNPVSQDFVSLLHK	91.0 ± 4.6	82.3 ± 6.0	118.7 ± 20.0	122.7 ± 10.4
YTEIFYQDLR	106.7 ± 10.5	109.7 ± 10.8	105.7 ± 6.1	111.0 ± 6.2
LHPISVTLQR	90.7 ± 7.1	93.3 ± 14.8	89.0 ± 5.3	85.7 ± 10.5
Consensus	96.1 ± 10.4	95.1 ± 15.3	104.4 ± 16.7	106.4 ± 18.2

[a] The concentration was calculated for three experimental replicates by monitoring three transitions per individual peptide and are presented as mean ± SD. For *consensus*, the data for three peptides were combined and presented as mean ± SD. The monitored transitions are summarized in Table 1.

[b] Trypsin 1, sequencing grade modified trypsin (Promega); trypsin 2, trypsin type IX-S from porcine pancreas (Sigma-Aldrich).

Table 4

Quantification of CYP11A1 and AdR in bovine retina

	protein (pmol/mg tissue protein)[a]
CYP11A1	
NFIPLLNPVSQDFVSLLHK	0.007 ± 0.002
AdR	
FGVAPDHPEVK	0.73 ± 0.15
SPQQVLPSPDGR	0.73 ± 0.12

[a] The concentration was calculated for three experimental replicates by monitoring three transitions per individual peptide and presented as mean ± SD. The monitored transitions are summarized in Table 1. Measurements for CYP11A1 and AdR were performed using total retina membrane pellet and whole retina homogenate, respectively.