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A rapid, reproducible, on-the-fly orthogonal array optimization method for targeted protein quantification by LC/MS and its application for accurate and sensitive quantification of carbonyl reductases in human liver

Jin Cao[†,§,‡,#], **Vanessa Gonzalez-Covarrubias**[†,#], **Robert M. Straubinger**[†,§], **Hao Wang**[†,§], **Xiaotao Duan**[†,§], **Haoying Yu**[†,§], **Jun Qu**[†,§,*], and **Javier G. Blanco**[†,§]

[†]The Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst, NY 14260

[§]New York State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, NY 14203

Abstract

Liquid chromatography (LC)/mass spectrometry (MS) in selected-reactions-monitoring (SRM) mode provides a powerful tool for targeted protein quantification. However, efficient, high-throughput strategies for proper selection of signature peptides (SP) for protein quantification and accurate optimization of their SRM conditions remain elusive. Here we describe an on-the-fly, orthogonal array optimization (OAO) approach that enables rapid, comprehensive, and reproducible SRM optimization of a large number of candidate peptides in a single nanoflow-LC/MS run. With the optimized conditions, many peptide candidates can be evaluated in biological matrices for selection of the final SP. The OAO strategy employs a systematic experimental design that strategically varies product ions, de-clustering energy and collision energy in a cycle of 25 consecutive SRM trials, which accurately reveals the effects of these factors on the single-to-noise ratio of a candidate peptide, and optimizes each. As proof of concept, we developed a highly sensitive, accurate, and reproducible method for the quantification of carbonyl reductases CBR1 and CBR3 in human liver. Candidate peptides were identified by nano-LC/LTQ/Orbitrap, filtered using a stringent set of criteria, and subjected to OAO. After evaluating both sensitivity and stability of the candidates, two SP were selected for quantification of each protein. As a result of the accurate OAO of assay conditions, sensitivities of 80 and 110 amol were achieved for CBR1 and CBR3, respectively. The method was validated and used to quantify the CBRs in 33 human liver samples. The mean level of CBR1 was 93.4±49.7 (range: 26.2–241) ppm of total protein, and for CBR3 was 7.69±4.38 (range: 1.26–17.9) ppm. Key observations of this study are that: i) evaluation of peptide stability in the target matrix is essential for final selection of the SP; ii) utilization of two unique SP contributes to high reliability of target protein quantification; and iii) it is beneficial to construct calibration curves using standard proteins of verified concentrations to avoid severe biases that may result if synthesized peptides alone are used. Overall, the OAO method is versatile and adaptable to high-throughput quantification of validated biomarkers identified by proteomic discovery experiments.

[*]Corresponding Author: Jun Qu, Ph.D., The Department of Pharmaceutical Sciences, 531 Cooke Hall, University at Buffalo, State University of New York, Buffalo, NY 14260-1200, Phone: (716) 645-2844 x283, Fax: (716) 645-3693, junqu@Buffalo.edu.
[#]Authors contributed equally to this work
[‡]Current address: Research Centre of Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing, 100091, China.

INTRODUCTION

Liquid chromatography/mass spectrometry (LC/MS) based methods represent promising analytical techniques for protein quantification in complex biological matrices because they provide high specificity, sensitivity, and multiplexing capability [1–2]. LC/MS techniques permit both proteome comparison (i.e. discovery-based protein quantification) and quantification of specific proteins of interest (i.e. targeted protein quantification). Whereas discovery-based strategies provide relative quantification of proteins on a proteomic scale [3–11], targeted quantitative strategies enable absolute quantification of a more limited number of proteins, usually at significantly higher sensitivity, dynamic range, and specificity [1–2, 12–13]. For the analysis regulatory proteins, which are usually low abundance, targeted quantification is most suitable.

Although LC/MS-based quantification of intact proteins has been reported [14–15], targeted methods usually quantify peptides generated by proteolytic digestion of the parent protein. Selected reactions monitoring (SRM) on triple-quadrupole instruments is preferable for the MS strategy because of its high sensitivity, selectivity, and wide dynamic range [13, 16–18]. Absolute quantification in biological samples can be accomplished by several approaches, including differential isotopic labeling *via* chemical or metabolic methods [12–13, 19–23]. More prevalent is isotope dilution using synthetic, stable-isotope-labeled internal standards (I.S.) that are chemically identical to the peptides derived from target proteins [17, 19, 24–30].

Although targeted protein quantification by LC/MS is practiced relatively widely, the development of such strategies faces several challenges. The first critical issue is selection of the tryptic peptide for quantification (the *signature peptide, SP*), which will determine the sensitivity, specificity, and accuracy achievable [16, 26]. Selection of randomly-predicted, proteolytic peptides as the SP carries a significant risk of failure because a suitable SP for quantification must meet several criteria: i) it must be unique to the target protein and target proteome; ii) ionization/fragmentation efficiencies of the peptide must be high to ensure sensitive quantification; iii) interference from the biological matrix should be relatively low and thus provide a good single-to-noise ratio (S/N); iv) the peptide must be sufficiently stable in the digested biological sample. Because digestion procedures, chromatographic separation, and MS ionization/fragmentation can exhibit perplexing preferences [13], accurate prediction of optimal peptides that meet criteria *ii–iv* is difficult. Although *in silico* prediction programs have been developed recently [19, 31], successful selection of the SP often relies upon information obtained from other experimental approaches. For example, peptide(s) derived from the target protein that have been observed in prior proteomic profiling experiments can serve as potential candidates. However, because such peptides are observed in the data-dependent MS/MS mode, which under-samples the peptides of highly complex biological samples [16], the best peptide for quantification of a target protein may not have been observed during profiling.

The second challenge in the development of targeted quantification methods is that even with candidate peptides identified by proteomic experiments, the optimization of SRM conditions that is required for evaluation of their eligibility as SP and for carrying out sensitive quantification is not straightforward. The MS platform used for proteomic profiling experiments usually differs from that employed for SRM-based quantification. The optimal MS conditions and peptide fragmentation patterns, which are platform-dependent, seldom can be transferred from one system to another. Therefore, optimal SRM conditions for SP candidates must be obtained experimentally. Optimization often is carried out using synthesized candidate peptides [13, 16], but without some degree of assurance that a given candidate is suitable for quantification, the synthesis of numerous peptide standards can be prohibitive in cost, especially when the quantification of multiple proteins is of interest.

To address these fundamental challenges, especially for quantification of low abundance proteins, we developed a strategy to enable straightforward, rapid, and reliable method development, termed *on-the-fly orthogonal array optimization* (OAO). For each candidate peptide derived from the target protein, a series of short (20–50 ms), consecutive SRM experiments (referred to as "trials") would be performed in the target matrix, each employing different product ions, and critical SRM parameters would be varied according to an L_{25} (3^5) orthogonal array design [32–33]. By statistical analysis of the S/N generated in the series of trials, the optimal transitions and SRM conditions for maximum sensitivity could be obtained readily. Through this rapid and accurate optimization, it would be feasible to establish the optimal SRM conditions for a relatively large number of candidate peptides, which could be evaluated subsequently in a high-throughput manner against additional criteria bearing upon their suitability for protein quantification (*e.g*. adequate stability in the target matrix, the presence of serious chemical interference in the biological sample, *etc*.). Therefore, the proposed strategy would lend itself to facile, rapid evaluation and selection of the SP from candidate peptides.

As proof of concept, we applied the OAO strategy to develop an accurate, sensitive method for the quantification of carbonyl reductases 1 (CBR1) and 3 (CBR3) in human liver cytosol. These CBRs are monomeric short-chain dehydrogenases that play a prominent role in hepatic metabolism of clinically important drugs such as haloperidol, doxorubicin/daunorubicin, dolasteron, and pentoxifylline. Recent studies suggest that the substrate specificity of CBR1 and CBR3 overlaps only partially [34–35] and investigation of substrate metabolism patterns would necessitate their quantification. CBR1 levels in human liver have been estimated by Western blotting [36–37], whereas the expression of CBR3 has been semi-quantified only at the mRNA level [36, 38]. Therefore, accurate and sensitive quantification of CBR1 and CBR3 in a clinical research set of human liver samples was the objective to which we applied the OAO approach.

EXPERIMENTAL

Materials

HPLC grade methanol, acetonitrile, acetone, and water were from B&J (Muskegon, MI). LC/MS grade formic acid was from Fluka (Buchs, Switzerland). Tris(2-carboxyethyl) phosphine (TCEP), Tris, iodoacetamide (IAA), and phosphate-buffered saline were obtained from Sigma-Aldrich (St. Louis, MO). All peptides were synthesized by Sigma-Aldrich (St. Louis, MO), including ^{13}C-coded peptides used as I.S.. Purity was verified by quantitative amino acid analysis. Sequencing-grade trypsin was from Promega (Madison WI). Protease, phosphatase, and kinase inhibitor cocktail tablets were from Roche (Basel, Switzerland). Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). Recombinant CBR1 and CBR3 protein standards were expressed in *E. coli* and purified using size exclusion- and strong cation exchange chromatography and their integrity was confirmed by SDS-PAGE. Their purities were 97.2% and 98.5%, respectively, as determined by quantitative amino acid analysis.

Human liver samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Human liver tissues from 33 cadaveric donors were procured through Dr. Mary Relling's laboratory at St. Jude Children's Research Hospital and processed through the Liver Tissue Cell Distribution System, which is funded by Contract #N01-DK-7-0004/HHSN267200700004C from the U.S. National Institute of Health. Liver cytosol was isolated and processed using a precipitation/on-pellet-digestion strategy we developed previously [39] and describe in detail in the Supplemental Material.

Nano-LC/MS

A system consisting of a Spark Endurance autosampler (Emmen, Holland) and four Eksigent direct-flow capillary/nano-LC pumps (Dublin, CA) was used for all analyses. A direct-connection nano-LC/nanospray setup was employed that featured low void volume and high chromatographic reproducibility [39]. Two different MS instruments were employed: an LTQ/Orbitrap/ETD hybrid (ThermoFisher Scientific, San Jose, CA) was used for identification of candidates peptides, and a TSQ Quantum Ultra EMR triple quadrupole MS (ThermoFisher) was used for the OAO procedure, candidate peptide evaluation, and CBR quantification in liver samples. The nano-LC/nanospray system described above was used with both MS. Detailed nano-LC/MS conditions are provided in the Supplemental Material.

Identification of SP candidates from CBR1 and CBR3

Identification of peptides from tryptic digests of the protein standards was first performed using BioWorks 3.3.1 embedded with Sequest (ThermoFisher), searching against a FASTA database containing the sequences of human proteins derived from the Swiss-Prot database. The precursor mass tolerance was 10 ppm and the mass tolerance for CID fragments was 1.0 mass unit. A stringent set of score filters was employed. Correlation score (Xcorr) criteria were: ≥4 for quadruply-charged (4+) and higher charge states, ≥3 for 3+ ions, ≥2.2 for 2+ ions, and ≥1.7 for 1+ ions. The CID results were further analyzed using the Peptide Prophet algorithm embedded in Scaffold 2.5 proteome software (Portland, OR). A peptide probability of 95% or higher was required for identification of a peptide. The identified peptides were subsequently filtered by exclusion criteria described in the *RESULTS AND DISCUSSION* section, and the surviving peptides were designated "candidate peptides" (*cf.* SI Table 1). The retention time for each candidate was recorded for use in the OAO procedure (discussed below).

High-throughput orthogonal array optimization of candidates

The product ions, de-cluster energies (i.e. TL voltages), and CE were selected as the three factors for optimization because they can affect significantly the sensitivity achievable for a peptide. For each factor, five levels were evaluated. The experimental design was fit in an L_{25} (3^5) orthogonal array in order to provide a comprehensive evaluation of all factors based upon a minimum of SRM experiments (*cf.* Table 1). The five most abundant product ions obtained from the identification experiments were used as the five levels for product ions; levels for de-clustering energies and CE were predefined based on a pilot analysis of 103 unique tryptic peptides (data not shown). One set of 25 SRM trials was performed for each candidate peptide. To facilitate setup under high-throughput operation, five different MS method templates were developed, each designed for the optimization of a precursor within a certain m/z range, as defined in SI Table 2. For optimization of each peptide candidate, it was only necessary to input the precursor and product ion m/z values into the appropriate method template.

To conduct the on-the-fly OAO, CBR1 and CBR3 were each spiked into a pooled sample of human liver cytosol at 1 μg/mL and then processed and digested as described above. The digest mixture was separated using the same chromatographic system, gradient conditions, and nanospray interface as used for peptide identification. The TSQ was programmed to perform repetitively the 25 SRM trails dictated by the orthogonal array design within a 4-min chromatographic window that centered on the retention time of each candidate peptide. In the event that the elution windows of two or more candidates overlapped partially, an 8-min window that included all closely-eluting candidates was used, and the SRM trials for these candidates were performed sequentially within each MS scan cycle. The dwell times in each trial ranged from 20–50 ms, and were based on the precursor intensities obtained in the nano-LC/LTQ/Orbitrap experiment. For example, a dwell time of 20 ms was used for candidates having precursor intensities $>2\times10^7$ in the identification experiments, whereas a dwell time of

50 ms was used for those candidates having intensities $<1\times10^6$. All candidates were evaluated in a single nano-LC/MS run. The extracting ion currents (XIC) for each trial were extracted batch-wise using the MRMer software [18] (http://proteomics.fhcrc.org/CPL/MRMer.html) and the S/N of the XIC was calculated. The results were processed using the "fractional DOE" function of Minitab (State College, PA), which included analysis of variance and backward regression. Although effect curves for product ions and TL voltages were obtained directly from these analyses, the effect curves for CE were calculated individually for each product ion: for a given product ion, five SRM trials were performed with different levels of CE and TL voltage (Table 2). In order to isolate the effects of CE on sensitivity from those exerted by the TL voltages, the S/N obtained from these trials were normalized individually against the effects of the corresponding TL voltages. Thus the optimal CE for each candidate transition was obtained.

Candidate evaluation and SP selection

Before selection of the signature peptides, candidates were evaluated for both stability and the sensitivity achievable in human liver cytosol. CBR1 and CBR3 were spiked into a pooled liver cytosol sample at 5μg/mL each, digested as described above, and then the mixture was used for stability assessment. Peptide stabilities were evaluated by incubation under the conditions of i) tryptic digestion (37°C, pH=8.5) and (ii) storage in the autosampler (6°C, pH=2.8). For evaluation of stability under digestion conditions, the solution was sampled at 0, 1.5, 3, 6, 9, 12, and 24 h after the completion of digestion. At each time point, a 20 μL sample was taken, acidified with formic acid to a final concentration of 1% (v/v), and then analyzed immediately by nano-LC/MS using the optimized conditions obtained in the OAO procedure. For assessment of candidate stability under autosampler storage conditions, the digestion mixture was acidified immediately upon completion of digestion, incubated in the refrigerated autosampler, and analyzed at 0, 3, 9, 21, 27, and 48 h. Both stability assessments were conducted in triplicate. Any candidate that degraded more than 20% within either evaluation period was eliminated. For each remaining candidate, the S/N was evaluated under the optimal SRM conditions using pooled human liver cytosol spiked with 1 μg/mL of each CBR. The two most sensitive candidates for each enzyme were selected as the SP.

Method validation

The blank matrix used for calibration curves was pooled rat liver cytosol that we confirmed to be free of the four human-derived SP. The liver tissue was obtained as described previously [13] and processed as described for human liver samples. Prior to digestion, two ^{13}C-labeled I.S. for each CBR protein were spiked into all samples: for CBR1, DVCTELLP[L $^{13}C_6$]IK and LFSGDVVL[L $^{13}C_6$]TAR at 50 fmol on-column (assuming 100% efficiency for the entire procedure), and for CBR3, VVNISSLQCL[L $^{13}C_6$]R and VAL[L $^{13}C_6$]VTGANR, at 5 fmol on-column. The purity of all ^{13}C-labeled I.S. exceeded 98%. The calibration curves for CBR1 and CBR3 were prepared for 2–600 and 0.2–60 fmol on-column, respectively. Details are specified in the Supplemental Material.

In order to investigate matrix effects upon the quantitative results, we also spiked both enzymes at two levels (20 and 200 fmol on-column for CBR1, and 4 and 30 fmol for CBR3) into pooled human liver cytosol for which the levels of endogenous CBR1 and CBR3 had been determined previously. Analytical recovery and precision was assessed twice on two successive days (n=4). The validation data were calculated in two ways: 1) based on each SP, and ii) based on the average of the two SPs for each enzyme.

Comparison of calibration curves for CBR1 consisting of protein standards vs. synthesized peptides

To prepare calibration samples, 6 aliquots of rat liver cytosol containing 100 μg total protein were precipitated, and the two synthesized SP for CBR1 (*T1-2* and *T1-5*) were spiked into the digestion buffer for each sample at 2.2, 4.4, 22, 44, 220, and 660 fmol (0.2–60 fmol on-column). The samples were then reduced, alkylated, digested, and analyzed by nano-LC/SRM-MS as described above. Linearity of the calibration curves was evaluated. For method validation, purified CBR1 protein was spiked into aliquots of rat liver cytosol at 5, 50 and 250 fmol on-column, and analyzed using the calibration curves constructed with synthesized peptides. The analysis was performed twice on two successive days (n=4). To permit comparison of the validation data for two calibration methods, the same set of spiked samples was analyzed in parallel using a calibration curves constructed using a CBR1 protein standard.

RESULTS AND DISCUSSION

High-confidence identification of CBR1 and CBR3 tryptic peptides by nano-LC/LTQ/Orbitrap

Although the OAO strategy does not require a standard protein/peptide for optimization, the results suggest that highly pure protein standards are essential for accurate absolute target protein quantification in clinical samples. Recombinant CBR1 and CBR3 were expressed and purified from bacterial cultures, and their purity was measured by quantitative amino acid analysis. They were subjected to a sequencing experiment using nano-LC and an LTQ/Orbitrap to screen for tryptic peptides detectable with high confidence. Under a highly stringent set of criteria (Experimental section), 70.04% and 80.14% of amino acid residues were observed for CBR1 and CBR3, respectively (SI Fig. 1). Representative spectra are shown in SI Fig. 2.

The OAO strategy does not require pure target proteins for optimization, yet the results of this study suggest that highly pure protein standards are essential for accurate quantification. Therefore, the recombinant CBR1 and CBR3 were subjected to a sequencing experiment using nano-LC/LTQ/Orbitrap, to determine the maximum set of potentially observable peptides. Under a highly stringent set of criteria (*Experimental* section), 70.04% and 80.14% of amino acid residues were observed for CBR1 and CBR3, respectively (SI Fig. 1). Representative LTQ/Orbitrap spectra are shown in SI Fig. 2.

The tryptic peptides identified with high confidence using CID comprised the potential candidates for quantification. They were subjected to a preliminarily screen according to the following criteria: (i) peptides contained missed-cleavages or φ residues were eliminated; (ii) peptides containing labile amino acids such as methionine (M) and tryptophan (W) were avoided; cysteine (C) is considered a labile residue [16], but peptides containing one C were retained for investigations described below; (iii) peptides were unique to either CBR1 or CBR3 (as determined by a BLAST search against the human protein database); and (iv) peptides known to have sequence variants at specific residues were avoided. For example, single nucleotide polymorphisms result in amino acid variations at two positions in CBR1 and seven in CBR3 40. Peptides that passed the above screen are listed in SI Table 1, and were subjected to the OAO procedure.

Accurate, high-throughput, on-the-fly OAO of SRM conditions for candidate peptides

Nano-LC/SRM-MS was chosen for quantification of the CBRs in human liver because of the high sensitivity, selectivity, and wider dynamic range it can provide [12–13, 16–18, 41–42]. It was essential to optimize SRM conditions in order to evaluate the peptide candidates for suitability as the SP, and to carry out sensitive and selective quantification. Because the optimal SRM conditions for a given precursor ion are related to numerous factors [12, 43], experimental

evaluation often is necessary [1]. However, obtaining the optimal SRM parameters for multiple candidate peptides in a complex digestion mixture is not straightforward.

In pilot studies, it was difficult to optimize SRM conditions using direct infusion of the target protein digestion mixture because of ion suppression and interference by other peptides, trypsin, and constituents of the digestion buffer. Therefore, we sought an optimization approach that employed sufficient chromatographic separation by nano-LC to avoid these effects. Given the requirement to evaluate multiple candidate peptides, and that each has several candidate transitions (*i.e*. the transitions to several abundant product ions), it is prohibitively laborious and error-prone to use trial-and-error- or one-parameter-at-a-time approaches for optimization using nano-LC/MS. Fractional factorial designs such as orthogonal arrays have emerged as an efficient means to determine how a combination of several key factors affects the outcome of an analytical experiment, and to identify the optimum for each factor [32–33]. Therefore, we employed an orthogonal array design to develop an on-the-fly method for facile, rapid, and accurate optimization of SRM conditions for all candidate peptides in a single nano-LC/MS run.

CBR1 and CBR3 were spiked into a pooled sample of human liver cytosol and processed as described above (detailed in Supplemental Materials). The resulting digest was loaded onto the same nano-LC separation system used for the identification experiment but connected to a triple-quadrupole MS. Within the 4- or 8-min window centered on the elution time of each candidate peptide, the MS was programmed to perform a repetitive series of short (20–50 ms) SRM trials. The parameters were dictated by the 3-factor/5-level orthogonal array design matrix shown in Table 1. The 3 factors evaluated were product ion, TL voltage (i.e. the de-cluster energy), and CE, which are the most important factors that affect the sensitivity of SRM [12]. Because the orthogonal array design must cover a predefined range of levels for each factor, it is important to predict strategically the regions in which optima most likely fall 32. For the product ion, the five levels for evaluation were assigned as the 5 most abundant b or y ions of the candidate peptide that were obtained from previous identification experiments. Although the m/z of precursor ions does not solely determine the optima of TL voltage and CE, as we observed previously 12, 43, it does exert a significant effect on both. Therefore, we predefined the levels for these two factors based on the m/z of the precursors (SI Table 2). Values were derived from a preliminary evaluation of >100 unique tryptic peptides derived from standard proteins. The range of levels shown in SI Table 2 was tested preliminarily for OAO of peptides derived from >20 specific proteins; for >95% of cases, the optimum for each parameter fell within the predicted range (not shown). On the few occasions in which optima were not within the predefined range, it was necessary to rerun the procedure with re-defined ranges.

Three characteristics of response were evaluated to assess the achievable sensitivity in the highly complex target matrix: peak area, height, and S/N of the XIC. The S/N appeared to be the most useful index of analytical sensitivity, and therefore was selected as the target for optimization.

The optimizations for all peptides listed in SI Table 1 were accomplished in a single nano-LC/MS run. The detailed OAO design for each is listed in SI Table 3. To enable rapid method setup, all parameters were input into pre-filled instrument method templates (*EXPERIMENTAL* section). The S/N for each trial was computed batch-wise, and optima were identified by statistical tests such as analysis of variance and backward regression. It was observed that all three parameters affected the S/N significantly. Fig 1 shows a typical set of effect charts obtained from the analysis of OAO results. For the CBR1 candidate peptide LFSGDVVLTAR (*T1-5*), the transition to the product ion of 917.5 provided the highest sensitivity by far among the 5 products (Fig 1A). TL voltage had a significant effect on sensitivity, with a maximum S/N achieved at 130 V (Fig 1B). Although the optimal TL voltage

is dependent only upon the precursor, the optimal CE for each transition depends upon both precursor and product ion. Therefore, the effect curves optimizing CE for each transition (Fig 1C–1G) were obtained by normalization against the effect of TL (see *EXPERIMENTAL* section).

Table 2 shows the optimal parameters for all candidate peptides, obtained in a single nano-LC/MS run by OAO. Also shown are the sensitivities achievable with each candidate peptide, which was evaluated after performing the OAO.

Reproducibility of the OAO results was assessed by repeating the optimization in 3 different weeks. Representative data are shown in SI Table 4. The optima determined in individual trials were highly reproducible, and agreed well with those obtained by the more time-consuming approach of direct infusion of a synthesized peptide. Two factors may contribute to this high level of reproducibility: first, for an SRM transition of a given compound, the optimal MS conditions are highly constant [43]. Second, the XIC peak, rather a single SRM spectrum, was used to evaluate the S/N for each trial. One typical XIC peak consists of 40–60 SRM data points under the LC conditions used here. This approach reduces variations arising from fluctuations in the efficiencies of ionization, ion transduction, fragmentation, etc. Given the high reproducibility achieved, it appears unnecessary to perform replicate OAO runs unless an optimum was not observed in the predicted range. Thus the OAO can be employed as a high-throughput method.

The results demonstrate the importance of employing experimental approaches for selection of the SP and for method optimization. It is difficult to predict the SP from the sequence of target proteins, and the sensitivity achievable for CBR1 and CBR3 depended heavily upon the peptide chosen (Table 2). Many factors exert perplexing effects on the sensitivity obtained for a peptide, including digestion efficiency, ionization, chromatographic separation, and interference from the sample matrix. Accurate prediction of these effects for candidate peptides in a biological matrix is virtually impossible.

The results also demonstrate the need to optimize SRM conditions empirically. For example, both the optimum CE and the manner in which CE affects the S/N varied significantly among the different transitions for the same precursor (Fig 1C–1G). Variation in reaction thermodynamics of bond cleavage [43] and chemical noise from the biological sample may affect individual SRM channels differently. Furthermore, the optimal SRM transition on a triple-quadrupole instrument cannot be predicted from the fragmentation data of the ion trap.

Assessment of candidate peptide stability

An additional requirement for accurate and sensitive protein quantification is that the SP must be sufficiently stable. Instability of a tryptic peptide during the digestion process would not be corrected by spiking the sample with an isotopically-labeled I.S. peptide before digestion and significant variability and quantitative errors could result [44]. Furthermore, when the number of samples to be quantified is relatively large, degradation of an unstable SP in the autosampler would diminish sensitivity significantly. Therefore, we evaluated the stability of each peptide candidate in Table 2 before selection of the SP.

Based upon the optimal parameters obtained from the OAO analysis, a nano-LC/SRM-MS method was readily developed for the simultaneous evaluation of the stability of all candidate peptides in the human liver cytosol digest. Stability was assessed under the conditions to which peptides are exposed during tryptic digestion and queuing in a cooled autosampler: (i) 24 h at 37°C in Tris buffer (pH=8.5), and (ii) 48 h at 6°C in 1% formic acid (pH=2.5). The results reveal that most candidates showed significant ($p<0.05$) instability after incubation at 37°C for 24 h, based upon a comparison of the starting and ending peptide signals (Fig. 2). However,

the extent of signal decay varied markedly (Fig. 2A and 2C). For example, the two most sensitive peptides of CBR3 (*T3-1* and *T3-7*) degraded drastically during incubation at both 37° C and 6°C (Fig 2C and 2D), leading to their disqualification as SP. Other peptides, such as *T1-1*, *T1-3*, and *T3*-5 also showed instability. This degradation occurred with or without trypsin (data not shown). Instability of most candidate peptides was significantly higher in the liver cytosol digest than in the digest solutions of the pure proteins (data not shown). Contributors to instability may include chemical modification (e.g. deamidation, oxidation), decomposition, and effects of constituents in the tissue sample matrix.

To ensure quantitative accuracy and precision, peptides showing more than 20% degradation under these conditions were eliminated. This criterion eliminated some candidates that provided the highest sensitivities (Table 2), which underscores the significant risk of selecting an SP without knowledge of its stability in the target matrix.

Sensitive, accurate quantification of CBR1 and CBR3 in human liver cytosol

(a) Development of quantitative strategy—Two unique SP from each enzyme were chosen based upon acceptable stability and sensitivity. Peptides T1-4 and T1-5 were selected for CBR1, and T3-5 and T3-7 were selected for CBR3. For each peptide, two transitions were monitored; the most sensitive transition was used for quantification, while the second was used for confirmation. Validated standards consisting of highly purified recombinant CBR1 and CBR3 (purity>97%) were used to achieve accurate quantification.

In order to minimize the concentration of endogenous non-protein compounds prior to LC/MS analysis, a highly efficient precipitation/on-pellet-digestion procedure [39] was employed for the preparation of liver cytosol samples. A high (89–92%), reproducible recovery of total protein was achieved, as well as nearly complete recovery of all SP peptides (cf. Table 3).

Because the levels of CBR3 in human liver cytosol were anticipated to be low, nano-flow LC was employed to achieve higher sensitivity [42, 45–47]. A nano-LC/nanospray configuration was employed [39], which features a low void volume, and high separation efficiency, reproducibility, and sensitivity. The RSD% of retention times during chromatographic separation was <0.15% for all candidates, which not only facilitated the OAO procedure, but also contributed significantly to reproducible quantification.

Because liver cytosol contains a highly complex mixture of proteins, a large number of tryptic peptides are produced. On the one hand, sufficient chromatographic separation is required to separate target peptides from compounds that cause chemical interference or ion suppression. On the other, shorter LC gradients improve assay throughput, which is important for clinical analysis. To balance these considerations, gradient conditions were developed that employed a shorter LC gradient (40 min) than used for identification without compromising the analysis of target peptides (*EXPERIMENTAL* Section). Typical SRM chromatograms of human liver cytosol are shown in Fig. 3.

(b) Method evaluation and validation—For quantification in biological matrices, it is important to prepare the calibration standards and perform method validation in the blank matrix (*i.e.*, the target biological sample devoid of the analytes) [48–49]. However, CBR1 and CBR3 are constitutively expressed in human liver, and a blank matrix was not readily available. Database interrogation and nano-LC analysis revealed that the rat does not express any of the four SP selected. Therefore, pooled rat liver cytosol was employed as the blank matrix, based on the reasonable assumption that the types and concentrations of most matrix components are similar in rat and human liver.

For CBR1, the detection limits achieved for the two SP in blank matrix were 15 amol (*T1-2*) and 80 amol (*T1-5*) on column, assuming 100% efficiency throughout the sample preparation procedure. For CBR3, the detection limits for the SP were 110 amol (*T3-2*) and 90 amol (*T3-4*) on-column. This high level of sensitivity is attributable to the accurate optimization by the OAO procedure. For each transition, the chromatographic peak was well-defined and free of interference. Moreover, no appreciable interference was observed when analyzing the blank matrix. The dynamic range of quantification was investigated for different concentration ranges of CBR1 and CBR3 (Table 3), because pilot studies showed that the endogenous levels of the two enzymes differed markedly among individuals. The standard curves for both enzymes showed good linearity (R^2=0.990 and 0.996 for the SP of CBR1, and 0.986 and 0.992 for the SP of CBR3). Quantitative accuracy was evaluated using two approaches, spiking the protein standards: (i) into blank rat liver cytosol (referred to as "*accuracy*") and (ii) into pooled human liver cytosol for which the endogenous concentrations of CBR1 and CBR3 had been determined previously ("*recovery*"). Based upon the two independent SP used for each protein, the accuracy and recovery achieved was high (Table 3). Data for the pairs of SP also agreed well, thus validating their selection. The precision of quantification also was excellent for both proteins, suggesting good reproducibility of the method.

In validation experiments, the precision and accuracy of quantification for the CBRs did not differ statistically when based on one SP vs. the average of the two SP (Table 3). Although averaging the paired values did not improve the validation data, the use of two unique peptides as SP nonetheless provides a means of quality control for quantification in complex biological samples. A valid concern is that use of a single SP could carry a significant risk of hidden bias. For example, if the target protein were truncated or modified within the lone SP, inaccurate quantification would result. The discrepancy would not be detected based on a single SP, nor would it be predicted readily, given the tremendous complexity of biological systems. Thus the use of two independent SP provides a simple gauge of whether the quantitative data are reliable (defined here as whether the difference between the concentrations determined from the SP independently were within 25% of the lower value). An example of unreliable data was observed for CBR1 in two samples (Hyt-377 and Hyt-624, SI Table 5). For cases in which a discrepancy is observed, additional investigation is necessary.

Cysteine (C)-containing peptides might be regarded as risky choices for the SP [16]. However, a number of validated methods using C-containing SP have been reported 28, 44, 50. Here we analyzed two such peptides (*T1-2* and *T3-2*) as candidates for quantification. Both were stable, provided high sensitivity (Fig. 2, Table 2), excellent accuracy, recovery, and precision (Table 3), and for the majority of clinical samples, the quantitative results derived from them agreed well with the selected SP (SI Table 5).

(c) Calibration using protein standards vs. synthesized peptide standards—

Synthesized, isotope-coded I.S. have been used for absolute quantification in numerous targeted protein quantification studies, but their non-isotope-coded cognates often are utilized for construction of the calibration curves [1, 13, 16, 24, 27–29]. Calibration based on a fragment (i.e. the SP) of the target analyte carries a risk of severe quantitative bias [51], *e.g.*, if proteolytic conversion of the target protein is not complete. A preliminary investigation was carried out to examine whether calibration curves based upon synthesized, non-isotope-coded peptides would enable accurate quantification of CBR1. For the two SP selected, the calibration curves showed excellent linearity (r^2=0.995 and 0.998 for *T1-2* and *T1-5*, respectively). Using these calibration curves, CBR1 was quantified in QC samples prepared by spiking blank matrix with known concentrations. Despite excellent quantitative precision for both SP, the CBR1 concentration was systematically and significantly underestimated (SI Table 6). Moreover, a considerable discrepancy was observed in the CBR1 concentrations determined independently from the two SP (SI Table 6). Given that the recombinant proteins and synthesized peptides

were highly purified and their concentrations were assigned by amino acid analysis, it is unlikely that these discrepancies relate to the purity of the standards. Inefficiencies in sample preparation, proteolysis, etc. more probably contributed to these quantitative errors. More extensive analysis is necessary to determine the degree to which the severe negative bias observed here represents a generalized problem in using synthesized peptides for calibration.

(d) Quantification of CBR1 and CBR3 in human liver samples—For a set of 33 human liver samples, CBR1 and CBR3 were detected with sufficient S/N (>40) in every sample. The results were normalized against the total protein concentration (w/w) of each sample, and the results are shown in SI Table 5. The average concentration of CBR1 was 93.4 ±49.7 ppm and for CBR3 was 7.69±4.38 ppm. The lower abundance of CBR3 agrees roughly with data obtained for paired mRNA samples using quantitative real-time PCR [36–38]. CBR1 levels varied approximately 9.2-fold (range: 26.2–241 ppm) among individuals in this sample set, whereas CBR3 varied 14-fold (1.26–17.9 ppm).

CONCLUSIONS

These studies suggest that for high-sensitivity, targeted quantification of proteins using nano-LC/SRM-MS, experimental approaches are essential for proper selection of the SP and accurate optimization of SRM conditions. Numerous factors affect the achievable sensitivity. Thus prediction of the appropriate SP, optimal transition, and SRM conditions is difficult. Furthermore, assessment of both quantitative sensitivity and stability of peptide candidates in the target matrix is essential. The experiment-based, on-the-fly OAO method developed in this study, contrasts with the more typical workflow, in which a putative SP is identified from the literature or prior proteomic analysis, synthesized, used to optimize SRM conditions, and then validated in a quantitative method [16]. In the absence of sufficient prior data supporting the chosen SP as suitable for quantification, such approaches may carry a significant risk of failure.

For quantification of multiple proteins simultaneously in biological samples, the on-the-fly OAO strategy provides the necessary throughput to evaluate numerous SP candidates. In a single nano-LC/MS run, the effects of product ions, de-clustering energy, and CE on the S/N of the candidate peptides can be identified, and optimized systematically and efficiently *via* the sequence of SRM trials conducted under the orthogonal array design. This strategy is superior to conventional one-factor-at-a-time optimization, in that it evaluates the collective effect of multiple factors rapidly and accurately.

A highly sensitive, accurate, and reproducible method for the quantification of CBR1 and CBR3 in human liver was developed as an evaluation platform for this strategy. The process yielded several valuable observations. First, although 12 peptides derived from the two enzymes in high-confidence identification experiments passed the criteria for candidate peptides, 5 were highly unstable in the biological matrix under the conditions used for digestion or queuing in an autosampler, underscoring the necessity of assessing candidate peptide stability. Second, one C residue may be acceptable in the SP, provided careful validation and evaluation are performed. Third, employment of two SP may enhance the reliability of quantification. Finally, reliance upon synthesized peptides rather than authentic protein standards for calibration can result in unacceptable quantitative bias.

The validated method revealed that the mean concentration of CBR1 in human liver was 12-fold higher than that of CBR3, and inter-individual variability in the abundance of these key drug-metabolizing enzymes is significant. To our knowledge, this is the first observation of CBR3 in human liver at the protein level, and the first absolute simultaneous quantification of both.

Although the throughput of the empirical OAO strategy is not as high as the *in silico* methods[19], it provides more accurate SRM conditions by employing experimental approach for optimization. After automation of method setup and data analysis through simple scripting, we estimate that up to 100 peptides can be optimized in a single nano-LC/MS run. By extension, the optimization necessary for multiplexed quantification of a large number of target proteins would be feasible by performing a handful of runs. Thus the OAO approach could provide a practical means for the validation of novel biomarker candidates identified by proteomic profiling, and would provide a high-throughput bridging step between biomarker discovery and the deployment of a quantitative method for clinical assessment of validated biomarkers [2, 27, 52]. Additional applications of this approach are ongoing in our lab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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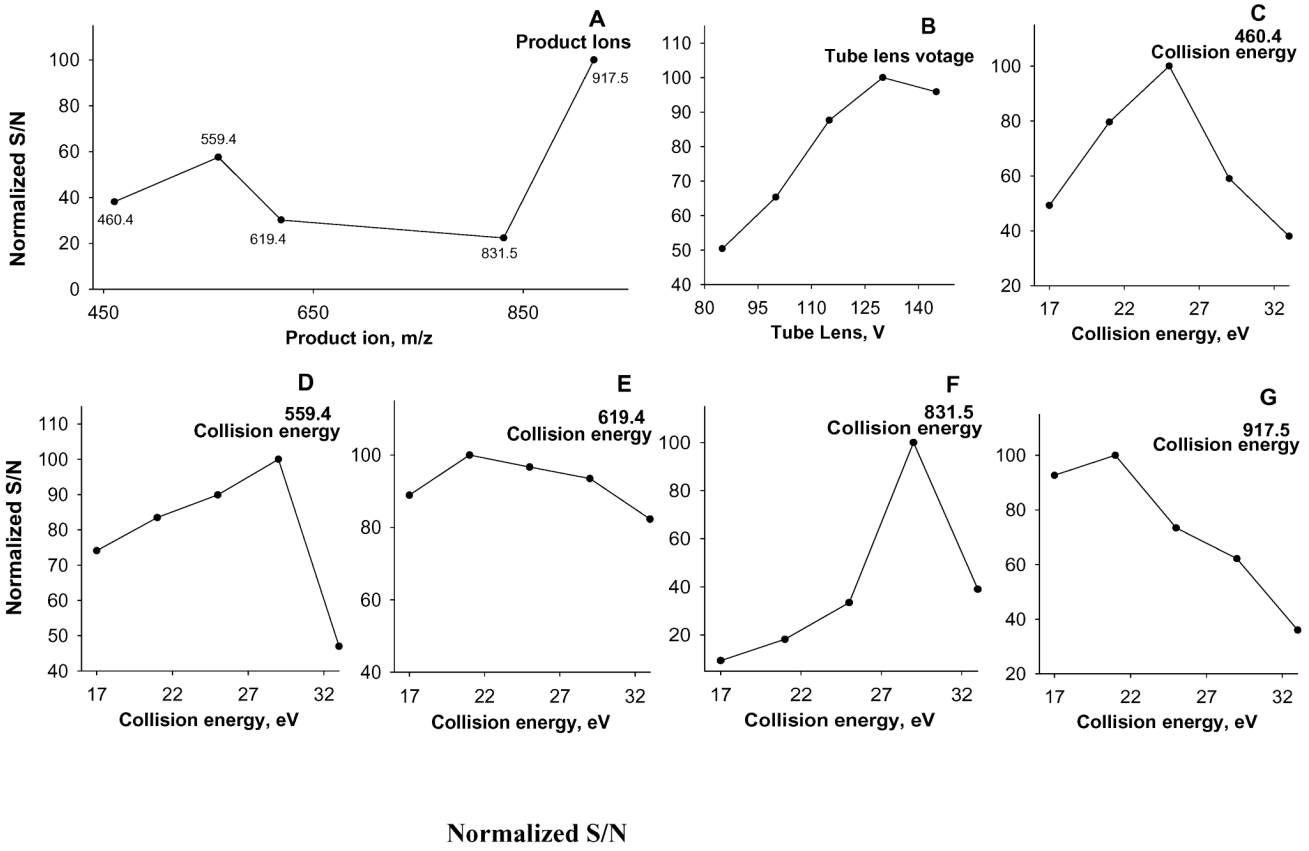

Figure 1.

Effect curves obtained by the on-the-fly OAO of a candidate peptide (LFSGDWLTAR) derived from CBR1. Effect of (A) product ion, and (B) tube lens voltages and (C–G) collision energies for the product ions upon the S/N. The experiment was performed with a nano-LC coupled to a triple-quadrupole MS.

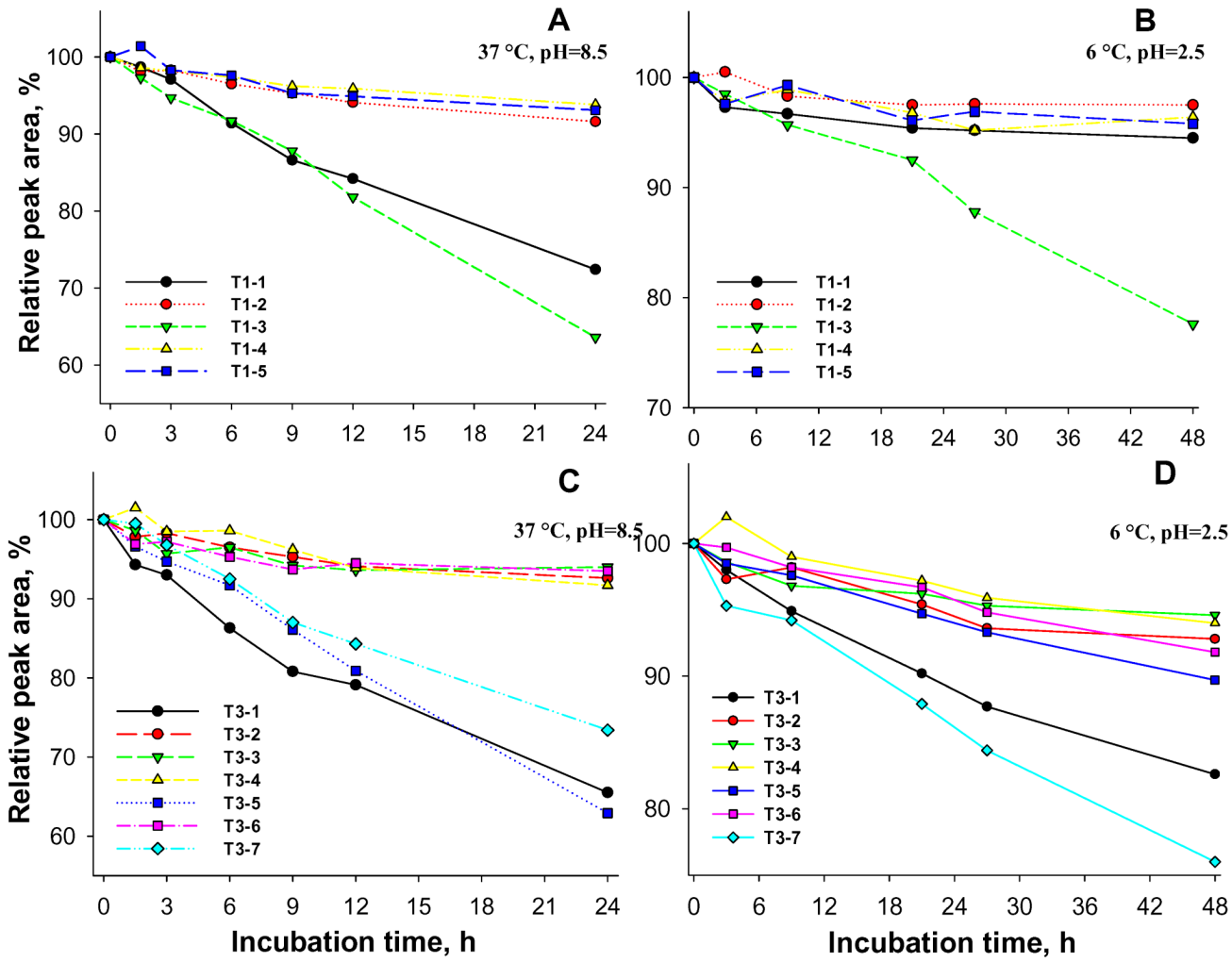

Figure 2.

Time-dependence of the stability of the candidate peptides derived from CBR1 at (A) 37°C and pH=8.5 for 24 h and (B) 6°C and pH=2.5 for 48h, and from CBR3 at (C) 37°C and pH=8.5 for 24 h and (D) 6°C and pH=2.5 for 48h. The evaluations were performed in triplicate; error bars are not shown in order to ensure the clarity of the plots. Individual time profiles with error bars are shown as supplemental data (SI Figure 3).

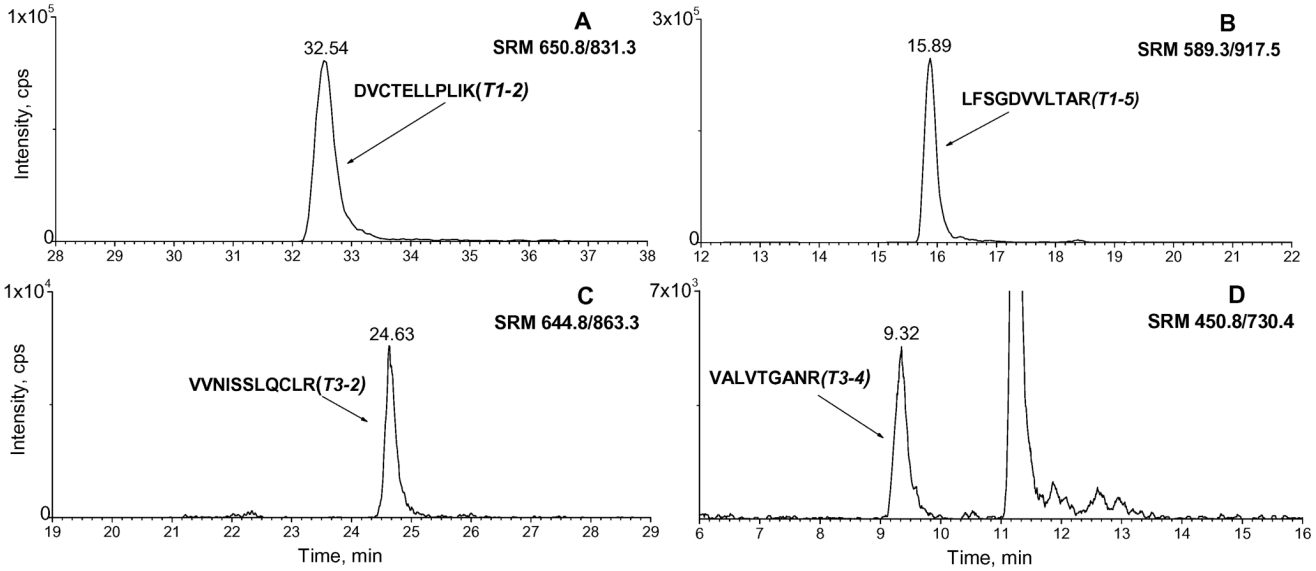

Figure 3.

Typical SRM chromatograms for the quantification of CBR1 and CBR3 in a human liver sample, using a nano-LC coupled to a triple-quadrupole MS (Sample ID is Hyt-369): (A) DVCTELLPLIK(T1-2); (B) LFSGDVVLTAR(T1-5); (C) VVNISSLQCLR(T3-2); (D) VALVTGANR(T3-4).

Table 1

[a]The orthogonal array design (L_{25}) for the on-the-fly optimization of the SRM conditions for a candidate peptide

SRM Trials	[b]Factors		
	Product ion	Tube lens (V)	Collision energy (eV)
Trial1	P1	TL1	CE1
Trial2	P1	TL2	CE2
Trial3	P1	TL3	CE3
Trial4	P1	TL4	CE4
Trial5	P1	TL5	CE5
Trial6	P2	TL1	CE2
Trial7	P2	TL2	CE3
Trial8	P2	TL3	CE4
Trial9	P2	TL4	CE5
Trial10	P2	TL5	CE1
Trial11	P3	TL1	CE3
Trial12	P3	TL2	CE4
Trial13	P3	TL3	CE5
Trial14	P3	TL4	CE1
Trial15	P3	TL5	CE2
Trial16	P4	TL1	CE4
Trial17	P4	TL2	CE5
Trial18	P4	TL3	CE1
Trial19	P4	TL4	CE2
Trial20	P4	TL5	CE3
Trial21	P5	TL1	CE5
Trial22	P5	TL2	CE1
Trial23	P5	TL3	CE2
Trial24	P5	TL4	CE3
Trial25	P5	TL5	CE4

[a]The levels for product ions were obtained from the LTQ/Orbitrap data and the other two factors were assigned according to SI Table 2. Each trial required 20–50 ms and the entire cycles were performed repetitively within the 4- or 8-min elution time window of the target peptide. The experiment was performed on a nano-LC/triple-quadrupole MS.

[b]The numbers 1–5 in the table denote the five different levels for each factor.

Table 2

The optimal SRM conditions of candidate peptides obtained by the OAO procedure, and the S/N achievable in human liver cytosol

Candidate Peptide	Optima of SRM conditions			[c]S/N
	Optimal transition	[a]TL (V)	[b]Product ion /Optimal CE in eV	
CBR1				
[d#]TNFFGTR *(T1-1)*	421.7→627.3	120	627.3/23; 216.1/27; 480.3/19; 363.2/27; 333.3/27	3.4E3
[e*]DVCTELLPLIK*(T1-2)*	650.8→831.3	115	831.3/24; 470.4/29; 1154.6/24; 543.8/34; 718.4/29	1.6E4
[#]SCSPELQQK *(T1-3)*	538.7→829.5	100	829.5/25; 281.1/29; 742.4/21; 516.4/29; 248.1/29	6.5E2
GIGLAIVR *(T1-4)*	399.8→458.4	105	458.4/19; 628.4/19; 341.2/23; 171.0/27; 387.4/23	2.1E3
[*]LFSGDVVLTAR*(T1-5)*	589.3→917.5	130	917.5/21; 559.4/29; 460.4/25; 619.4/21; 831.5/29	6.3E4
CBR3				
[#]EYGGLNVLVNNAAVAFK *(T3-1)*	890.0→1064.5	150	1064.5/27; 715.4/32; 834.5/27; 933.5/32; 1414.6/22	7.7E4
[*]VVNISSLQCLR*(T3-2)*	644.8→863.3	115	863.3/29; 776.4/24; 1090.7/19; 312.8/24; 576.1/29	1.5E4
LGVTVLSR*(T3-3)*	422.8→575.4	90	575.4/23; 375.3/27; 674.5/19; 470.3/23; 474.5/27	2.6E4
[*]VALVTGANR*(T3-4)*	450.8→730.4	115	730.4/21; 518.3/25; 417.3/29; 617.5/21; 383.3/33	2.1E4
[#]AFENCSEDLQER*(T3-5)*	749.3→876.4	155	876.4/24; 432.1/24;660.1/29; 218.9/34; 640.5/24	3.9E2
GIGLAIAR*(T3-6)*	385.7→600.4	105	600.4/19; 430.2/19; 341.2/23; 359.3/23; 412.3/27	2.7E3
[#]QFSGDVVLTAR*(T3-7)*	596.8→917.5	130	917.5/21; 460.4/25; 559.4/29; 658.5/21; 347.3/29	3.8E4

[a]Tube lens voltage.

[b]The fragments were sorted in the order of the most abundant to least abundant; only the top two most abundant transitions were used for analysis;

[c]The S/N for the most sensitive transition, evaluated by spiking 1 μg/mL proteins into a pooled human liver cytoso preparation;

[d]"#" denotes the peptide was not stable as evaluated by the stability examination (*cf.* Fig. 5).

[e]"*" denotes that the peptide was chosen as a signature peptide (SP).

Table 3

Linearity, Detection Limits , Accuracies and Variability for the Quantification of CBR1 and CBR3 (*n*=4)

	[a]DL (fmol)	[a]Linear range (fmol)/r^2	[b]Accuracy% (RSD%)	[c]Recovery% (RSD%)	[d]Mean accuracy% (RSD%)	[d]Mean recovery% (RSD%)
CBR1						
T1-2	0.08	2-600 /0.996	88(11); 93(7); 87 (7)	92(6); 94(7)	89(7); 93(5); 92(5)	89(4); 96(9)
T1-5	0.015	2-600/0.990	91(4); 93(3); 96(5)	86(6);97(10)		
CBR3						
T3-2	0.11	0.2-60/0.992	97(9); 91(3); 94(4)	92(5); 88(4)	92(7); 93(4); 94(5)	93(6); 90(3)
T3-4	0.09	0.2-60/0.986	87(5); 96(7); 95(8)	95(11); 91(5)		

[a]Detection limits were measured with the most sensitive peptide and are expressed as the protein amounts on column (assuming 100% efficiency for sample preparation).

[b]Accuracies were measured by spiking the standard proteins at three levels (5, 50 and 250 fmol on-column for CBR1 and 1, 10 and 40 fmol for CBR3) into the blank matrix (rat liver cytosol), and then analyzing the samples twice in each of two successive days (*n*=4).

[c]Recoveries were determined by spiking the standard proteins at two levels (20 and 200 fmol on-column for CBR1 and 4 and 30 fmol for CBR3) into a pooled human liver cytosol sample, and then measuring the samples twice in each of two successive days (*n*=4);

[d]Accuracy and recovery calculated based on the averages of protein concentrations obtained independently by the two signature peptides.