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Original Article

Quantitation of Host Cell Proteins by Capillary LC/IMS/MS/MS in Combination with Rapid Digestion on Immobilized Trypsin Column Under Native Conditions

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Host cell protein (HCP) impurities are considered a critical quality attribute of biopharmaceuticals because of their potential to compromise safety and efficacy, and LC/MS-based analytical methods have been developed to identify and quantify individual proteins instead of employing enzyme-linked immunosorbent assay to assess total HCP levels. Native digestion enables highly sensitive detection of HCPs but requires overnight incubation to generate peptides, limiting the throughput of sample preparation. In this study, we developed an approach employing native digestion on a trypsin-immobilized column to improve the sensitivity and throughput. We examined suitable databases for the identification of HCPs derived from Chinese hamster ovary (CHO) cells and selected RefSeq's Chinese Hamster as the optimal database. Then, we investigated methods to identify HCPs with greater efficiency than that of denatured in-solution digestion. Native in-column digestion not only reduced the digestion time from overnight to 10 min but also increased the number of quantified HCPs from 154 to 226. In addition to this rapid digestion methodology, we developed high-throughput LC/MS/MS with a monolithic silica column and parallel reaction monitoring-parallel accumulation-serial fragmentation. The optimized system was validated with synthetic peptides derived from high-risk HCPs, confirming excellent linearity, precision, accuracy, and low limit of detection (LOD) and limit of quantification (LOQ) (1–3 ppm). The optimized digestion and analysis method enabled high-throughput quantification of HCPs, and is expected to be useful for quality control and characterization of HCPs in antibody drugs.

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1. INTRODUCTION

Host cell proteins (HCPs) are impurities derived from the host cell line, such as Chinese hamster ovary (CHO) cells, during the production of biopharmaceuticals.[1,2] HCPs must be controlled because they affect the efficacy and safety of biopharmaceuticals.[3,4] Current guidelines do not provide guidance on specific acceptable levels of HCPs in final products, but an informal target level for HCPs is around 100 ppm, based on empirical evidence.[5,6] Enzyme-linked immunosorbent assay (ELISA) is commonly used for control of HCPs.[7,8] However, ELISA is usually unable to identify specific HCPs without preparing anti-HCP antibodies for individual HCPs, making it difficult to assess the risk associated with specific HCPs.[9] Furthermore, the coverage of antibodies used in ELISA systems is not always perfect, and consequently, unexpected HCPs may not be detected.[7,10] In fact, it has been reported that even trace amounts of HCPs (much lower levels than 100 ppm) can have undesirable effects, including breakdown of additives, immune reactions, toxicity, and antibody degradation.[11–15] For example, trace amounts of phospholipase can degrade polysorbate 80, an excipient commonly used in biopharmaceutical formulations, and cause aggregation of antibody drugs.[12] In addition, HCPs such as clusterin and serine protease HTRA1 have been reported as high-risk HCPs that are difficult to remove

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with a protein A column.[16–20] Regulatory authorities are aware of the limitations of ELISA systems, and the industry is now turning to LC/MS/MS as an orthogonal method that can identify specific HCPs.[21,22] LC/MS-based protein identification requires an amino acid sequence database that combines well-curated quality with a sufficient number of entries, and UniProt/Swiss-Prot is most commonly used for this purpose. However, Swiss-Prot has only 246 Chinese hamster protein entries, and further investigation is needed to select the optimal database.

Because the percentage of HCPs in antibody drugs is very low, sample preparation by conventional digestion methods is a limiting factor in the detection of HCPs due to ion suppression caused by abundant antibodies, which prevents the identification of trace HCPs by LC/MS/MS. Therefore, native digestion was developed for the purpose of identifying trace amounts of HCPs in antibody drugs.[23–26] In native digestion, proteins are digested without denaturation, and as a result, relatively robust antibodies are less likely to be digested, while HCPs are preferentially digested. Using native digestion, Huang *et al.* extended the dynamic range of HCPs detection by one to two orders of magnitude. They successfully identified as many as 60 HCPs, twice as many as previously reported.[23] However, digestion for bottom-up proteomics is generally an overnight process, so that sample preparation is time-consuming. For faster enzymatic digestion, a method using trypsin-immobilized columns has been reported.[27,28] Masuda *et al.* performed membrane-based proteomics using trypsin-immobilized spin columns with a short digestion time of 15 min.[28] However, to our knowledge, there is no report dealing with native digestion using trypsin-immobilized columns for HCPs detection.

In general, bottom-up proteomics requires not only time-consuming enzymatic digestion but also several hours per sample for LC/MS/MS measurement. This is because the measurement is performed at a low flow rate of several hundred nL/min.[29–31] HCPs characterization studies combining two-dimensional LC and Field Asymmetric Ion Mobility Spectrometry (FAIMS) with native digestion have typically required one to three hours of measurement time.[23–25] Ma and Kilby investigated high-throughput measurement of HCPs and successfully identified HCPs using a 21-minute gradient with Evosep One.[32] However, a higher-throughput method is needed for the identification and quantification of HCPs in large numbers of samples.

The data-dependent acquisition (DDA) mode of MS measurement is widely used for bottom-up proteomics, but MS/MS involves random sampling, which results in missing values. Parallel reaction monitoring (PRM) is a method that continuously acquires product ions selected as targets and does not suffer from the problem of missing values. In addition, the PRM mode quantifies peptides based on product ions, enabling quantification with higher selectivity. To further increase the selectivity, a method called prm-PASEF was recently developed using timsTOF Pro, which employs a trapped ion mobility spectrometer (TIMS).[33,34] The prm-PASEF has been reported to be more selective because it uses ion mobility separation. FAIMS is another commonly used ion mobility device, but while TIMS traps all ions, FAIMS acts as a filter that allows only ions with specific ion mobility to pass through, making it impossible to acquire multiple ions with different ion mobility simultaneously. Therefore, we considered prm-PASEF with TIMS would be suitable for rapid quantification of HCP.

In this study, we first compared databases to identify HCPs of antibody drugs produced in CHO cells. We also chose native digestion with a trypsin-immobilized column to identify HCPs more sensitively within the constraint of a short digestion time. Furthermore, we developed a high-throughput LC/IMS/MS/MS method to quantify high-risk HCPs with prm-PASEF and validated its quantitative performance.

2. EXPERIMENTAL

2.1. Materials

Dithiothreitol (DTT), iodoacetamide (IAA), urea, ammonium bicarbonate, acetonitrile (ACN), formic acid, ammonium hydroxide, and trifluoroacetic acid (TFA) were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). Formaldehyde, sodium cyanoborohydride, and triethylammonium bicarbonate (TEAB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Formaldehyde-$^{13}CD_2$ was obtained from ISOTEC Inc. (Canton, GA, USA). Modified trypsin was obtained from Promega (Madison, WI, USA). Empore disks were from 3 M (St. Paul, MN, USA). Water was purified with a Milli-Q system (Merck, Darmstadt, Germany). CHO cell-derived monoclonal antibody drugs were provided by the Japan Agency for Medical Research and Development (AMED) Manufacturing Technology Association of Biologics (MAB). Synthetic peptides of clusterin (FMDTVAEK, FM(ox)DTVAEK) and serine protease HTRA1 (TYTNLCQLR) were obtained from SynPeptide Co., Ltd. (Shanghai, China). Two types of columns of trypsin immobilized on silica monolith filters in spin column formats were supplied by Kyoto Monotech (Kyoto, Japan).

2.2. Investigation of the tryptic digestion method

For denatured digestion, 10 μg monoclonal antibody samples were denatured with 8 M urea (1 M Tris–HCl, pH 9.0). The denatured proteins were reduced with 10 mM DTT at 25°C for 30 min and alkylated with 50 mM IAA in the dark at 25°C for 30 min. The proteins were 5-fold diluted with 50 mM ammonium bicarbonate. For in-solution digestion, proteins were digested with trypsin for 16 hours (protein:enzyme = 100:1). For in-column digestion, proteins were digested on a trypsin-immobilized column. Centrifugation was performed twice at 100 *g* for 2 min.

For native digestion, 10 μg monoclonal antibody samples were digested without denaturation or chemical reaction with DTT or IAA. For in-column digestion, proteins were digested on a trypsin-immobilized column. Centrifugation was performed five times at 100 *g* for 2 min. After digestion, samples were reduced with 10 mM DTT and denatured at 90°C for 10 min. To remove undigested antibodies, the samples were centrifuged at 13,000×*g*. The supernatant was collected and alkylated with 50 mM IAA in the dark at 25°C for 30 min. All digests were desalted with reversed-phase StageTips as described previously.[35]

2.3. Stable isotope labeling

Stable isotope dimethyl labeling was carried out as previously described[36] with some modifications. Briefly, the monoclonal antibody-derived digested peptides or synthetic

peptides were dissolved in 100 mM TEAB. Then, the peptides were mixed with 4% $^{13}CD_2O$ (antibody sample) or $^{12}CH_2O$ (synthetic peptides) (final concentration 0.15%), and 600 mM sodium cyanoborohydride was added (final concentration 22 mM). The mixture was stirred for 60 min at 25°C. The reaction was stopped by adding 1% ammonium hydroxide (final concentration 0.15%), and the resulting samples were mixed with 10% TFA (final concentration 5%). The labeled peptides were desalted with reversed-phase StageTips.

2.4. Validation of high-throughput LC/IMS/MS/MS analysis of high-risk HCPs

Antibody drugs were digested in native condition with trypsin-immobilized columns (trypsin rich) in triplicate. Digested peptides were labeled with heavy isotopes, as described above. Synthetic peptides derived from serine protease HTRA1 (TYTNLCQLR) and clusterin (FMDTVAEK, FM(ox)DTVAEK) were labeled with light isotopes as described above. For 1 μg of heavy isotope-labeled antibody-derived peptide, 0.1, 0.4, 1, 4, or 10 fmol of light isotope-labeled synthetic peptide were spiked. Sample preparation was performed in triplicate, and LC/IMS/MS/MS analysis was performed for each sample.

2.5. LC/MS/MS

LC/MS/MS analyses were performed on a timsTOF Pro (Bruker, Bremen, Germany) connected to an Ultimate 3000 RSLCnano pump (Thermo Fisher Scientific, Waltham, MA, USA) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptides were separated on in-house-packed needle columns (150 mm length, 100 μm ID) of Reprosil-Pur 120 C18-AQ 1.9 μm reversed-phase material (Dr. Maisch, Ammerbuch, Germany) or C18 monolithic silica columns (200 mm length, 100 μm ID) (Kyoto Monotech, Kyoto, Japan). The injection volume was 5 μL. The flow rate was 500 or 1,500 nL/min. Measurements over 110 min were performed by applying step linear gradients of 4–8% ACN in 5 min, 8–32% ACN in 60 min, 32–80% ACN in 5 min, and 80% ACN for 10 min in 0.1% formic acid. Measurements over 40 min were performed by applying step linear gradients of 4–32% ACN in 10 min, 32–80% ACN in 1 min, and 80% ACN for 9 min in 0.1% formic acid. Measurements over 20 min were performed by applying step linear gradients of 4–32% ACN in 10 min, 32–80% ACN in 0.3 min, and 80% ACN for 3 min in 0.1% formic acid. Investigations of a suitable database for HCPs and of digestion methods were performed in the DDA mode. The TIMS section was operated with a 100 ms ramp time and a scan range of 0.7–1.4 Vs cm^{-2}. One cycle was composed of a 1-MS scan followed by 10 PASEF MS/MS scans. MS and MS/MS spectra were recorded from *m/z* 100 to 1,700. A polygon filter was applied so that singly charged ions were not selected. The isolation width was set to 2 *m/z* for precursor *m/z* <700 and 3 *m/z* for precursor *m/z* >800. Optimization of LC conditions for high-throughput measurements was done in the PRM mode. High-throughput LC/IMS/MS/MS analysis of high-risk HCPs was performed using the prm-PASEF mode.

2.6. Data analysis

Peptides and proteins were identified through automated database searching using MS Fragger[37,38] version 3.8 and Philosopher[39] version 5.0. For the identification of HCPs, RefSeq Taxonomy Chinese hamster (138,530 entries; 2023/10 release), TrEMBL Taxonomy Chinese hamster (83,287 entries; 2023/10 release), Swiss-Prot Taxonomy Rodentia (27,937 entries; 2023/10 release), Swiss-Prot Taxonomy Mouse (17,185 entries; 2023/10 release) and Swiss-Prot Taxonomy Chinese hamster (246 entries; 2023/10 release) were used as databases. For the identification of monoclonal antibodies, the AIST mAb sequence (National Institute of Advanced Industrial Science and Technology) was used as a database. Digestion mode was set Trypsin/P, allowing for up to two missed cleavages. Oxidation (M) and acetylation (protein N-term) were allowed as variable modifications. Carbamidomethylation (C) was set as a fixed modification. The false discovery rate (FDR) filter was set to 0.01 at both the peptide-spectrum match (PSM) and protein levels. Peptides and proteins were quantified by IonQuant.[40] The data acquired in the PRM or prm-PASEF[33] mode were analyzed in Skyline-daily[41] version 20.2.1.384.

2.7. Data availability statement

The MS raw data and analysis files have been deposited with the ProteomeXchange Consortium (https://proteomecentral.proteomexchange.org) via the jPOST partner repository (https://jpostdb.org)[42] with the dataset identifier, PXD053135.

3. RESULTS AND DISCUSSION

3.1. Investigation of suitable databases for identification of CHO-derived HCPs

First, we analyzed HCPs of CHO cell-derived antibody drugs produced by the AMED MAB by preparing samples using solution digestion under denatured conditions. LC/IMS/MS/MS measurements were performed in the DDA mode using a particle-packed column (100 μm i.d.) with a gradient time of 65 min and a total measurement time of 110 min. Since the samples were derived from CHO cells, it would be preferable to use the protein sequence database of Chinese hamsters for database searching, but the number of reviewed protein sequences of Chinese hamsters is limited (246 proteins in Swiss-Prot). Therefore, we investigated RefSeq Taxonomy Chinese hamster (138,530 entries), TrEMBL Taxonomy Chinese hamster (83,287 entries), Swiss-Prot Taxonomy Rodentia (27,937 entries), Swiss-Prot Taxonomy Mouse (17,185 entries) and Swiss-Prot Taxonomy Chinese hamster (246 entries) as candidate databases for the identification of HCPs. Searches were performed in combination with monoclonal antibody sequences. Because some sequences identical to those of monoclonal antibody-derived peptides were found in the above databases, they were excluded from the identification list of HCPs. As a result, the highest identification numbers of HCPs were obtained with the RefSeq Taxonomy Chinese hamster and the TrEMBL Taxonomy Chinese hamster databases (Table 1). The other databases were considered to lack sufficient protein coverage for the identification of HCPs derived from CHO. Since the number of identified peptides was slightly higher in the RefSeq than in the TrEMBL database, we decided to use the RefSeq database for the subsequent identification of HCPs.

Table 1. Comparison of databases for identification of HCPs derived from CHO.

Taxonomy	Database	Protein entries	Identified HCPs	Identified unique peptides (HCPs)
Chinese hamster	RefSeq	138,530	146	490
Chinese hamster	TrEMBL	83,287	146	481
Rodentia	Swiss-Prot	27,937	136	406
Mouse	Swiss-Prot	17,185	105	354
Chinese hamster	Swiss-Prot	246	14	136

Antibody drugs produced using CHO cells were analyzed using LC/MS/MS. HCPs were identified by searching with MS Fragger against the listed protein sequence databases and monoclonal antibody sequences. Some sequences identical to those of monoclonal antibody-derived peptides were found in the databases, and they were excluded from the identification list of HCPs. CHO, Chinese hamster ovary; HCPs, host cell proteins.

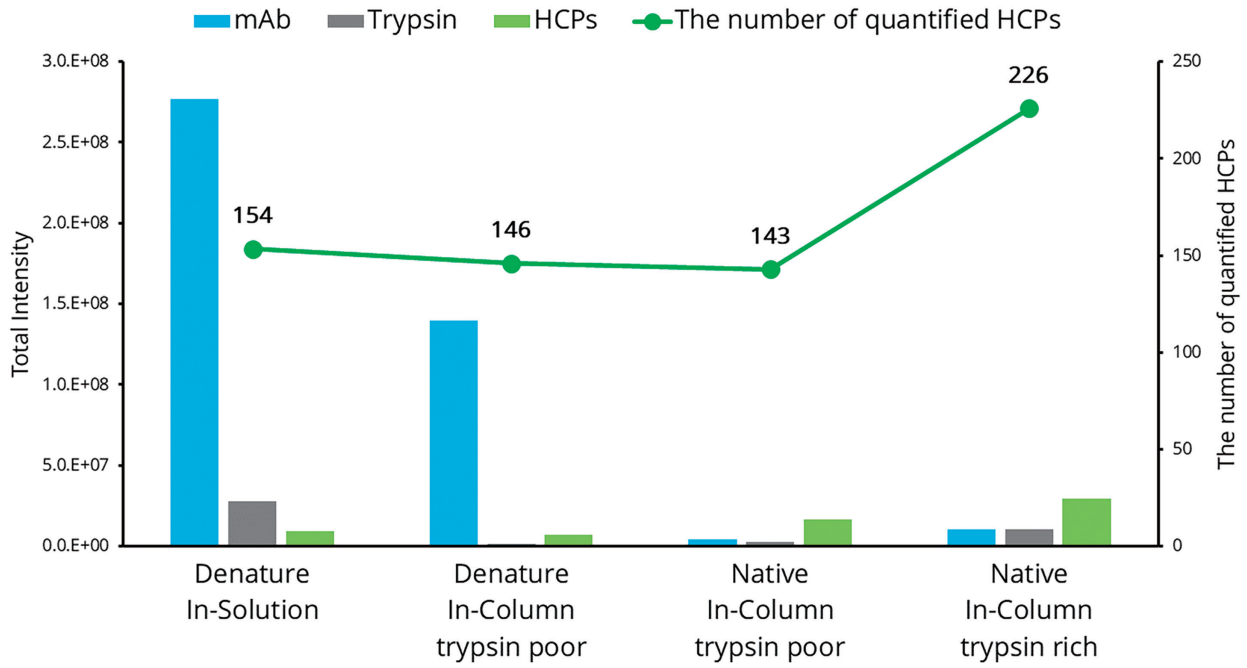

Fig. 1. Comparison of tryptic digestion methods for the quantification of HCPs. In-solution or in-column digestion was performed under denatured or native conditions. Two types of trypsin immobilization columns were prepared: one with a low level of trypsin immobilization (trypsin poor) and the other with a high level of trypsin immobilization (trypsin rich). Proteins were identified by MS Fragger and quantified by IonQuant. Proteins identified in the Chinese hamster database were determined to be HCPs. The green line shows the number of quantified HCPs for each trypsin digestion method. Bars indicate the total intensity of mAb, trypsin, or HCP-derived peptides. HCPs, host cell proteins; mAb, monoclonal antibody.

3.2. Investigation of high-throughput, high-sensitivity digestion methods

The most common digestion method in bottom-up proteomics is in-solution digestion, in which proteins are digested by adding enzymes to the sample solution. However, in-solution digestion is generally performed overnight, resulting in poor sample throughput. Therefore, in-column digestion with trypsin-immobilized spin columns has developed as an alternative to solution digestion. The advantages of trypsin-immobilized spin columns are rapidity and simplicity since digestion can be performed in a short time of only about 10 min due to the high density of trypsin immobilized on the column. Furthermore, no thermostatic bath or shaker is required. We compared in-solution digestion and trypsin-immobilized monolithic spin column digestion for the quantification of HCPs in CHO cell-derived antibody drugs under denatured conditions. The identification and quantification of HCPs were performed by MS Fragger using the RefSeq taxonomy Chinese hamster database. Reducing the digestion time from overnight (in-solution) to 4 min (in-column) slightly decreased the number of quantified HCPs (Fig. 1). To quantify HCPs more efficiently, we set out to optimize native digestion. Under native digestion conditions, antibodies are more stable, and so are less efficiently digested by trypsin compared to HCPs, thus enabling HCPs-selective digestion. However, column digestion under native conditions did not improve the number of quantified HCPs compared with denatured conditions, though the intensity of mAb showed a marked decrease (Fig. 1). This result was considered to be due to an insufficient amount of immobilized trypsin (trypsin poor) since the cleavage efficiency of trypsin is expected to be lower in the case of native digestion. Therefore, we prepared a column with a larger amount of immobilized trypsin (trypsin rich). Native digestion with this column increased the intensity of HCPs and increased the number of quantified HCPs to 226. Thus, the amount of trypsin immobilized on the column is an important consideration for native in-column digestion. We identified two HCPs that have been reported to be particularly high risk in biopharmaceutical manufacturing.[17,18] These were

Table 2. LC conditions and changes in the synthetic peptide peak.

	Condition 1	Condition 2	Condition 3
Gradient time (min)	65	10	10
Flow rate (nL/min)	500	500	1,500
Column	Packed column (15 cm)	Monolithic silica column (20 cm)	Monolithic silica column (20 cm)
Total run time (min)	110	40	20
Retention time (min)	21.6	18.8	6.8
FWHM (sec)	7.7	4.8	4.5
Peak height	63968	78322	72394
Peak area	589697	440523	370272

Evaluated LC conditions and characteristics of synthetic peptide peaks. A mixture of 5 fmol synthetic peptide labeled with a dimethyl light tag and 1 µg antibody sample in-column-digested under denatured conditions and labeled with a dimethyl heavy tag was measured in the PRM mode. The peaks were quantified by Skyline. LC, liquid chromatography; FWHM, full width at half maximum; PRM, parallel reaction monitoring.

clusterin and serine protease HTRA1. Therefore, we applied high-throughput enzymatic digestion and high-throughput LC/IMS/MS/MS measurements to monitor these HCPs during sample preparation.

3.3. Optimization of LC conditions for high-throughput measurements

In nanoLC/MS/MS-based bottom-up proteomics, the measurement time is generally long because the measurement is performed at a low flow rate with a long gradient. This enables the identification of many proteins, but the measurement time can be problematic when performing multiple sample measurements. Therefore, we investigated a system that can quantify several peptides in high-throughput measurements using the PRM mode, which exhibits high selectivity. The gradient time, flow rate, and analytical column were optimized to accelerate the LC/IMS/MS/MS measurement using the synthetic peptide FM(ox)DTVAEK, a tryptic peptide derived from clusterin (Table 2), as a model compound. A mixture of 5 fmol synthetic peptide labeled with a "light" dimethyl tag and 1 µg antibody sample in-column-digested under denatured conditions and labeled with a "heavy" dimethyl tag was used as the sample. In a 110 min measurement using a particle-packed column, which is a typical setup in bottom-up proteomics, the FM(ox)DTVAEK peak was detected at a retention time of 21.6 min (Fig. 2). To shorten the analytical time, we changed the analytical column from a packed column to a monolithic silica column, because packed columns have a high column pressure and are not compatible with high flow rates. Simultaneously, the gradient time was shortened to 10 min. As a result, a well-shaped peak of FM(ox)DTVAEK was observed at 18.8 min. Shortening the gradient time to 10 min decreased the full width at half maximum from 7.7 sec to 4.8 sec and increased the peak height, but the peak area was decreased. This is likely to be due to ion suppression, as the number of co-eluting peptides was increased. Even when the gradient time was shortened to 10 min, the total measurement time was still 40 min at the low flow rate of 500 L/min because 30 min was required for sample loading, column washing, and equilibration. Therefore, we increased the flow rate to 1,500 nL/min and performed the measurement for a total time of 20 min. This afforded a well-shaped peak of FM(ox)DTVAEK at 6.8 min. Although the peak height and area were decreased due to the decrease of ESI sensitivity at the high flow rate, it was still possible to detect a well-shaped peak using a 20-min total run time.

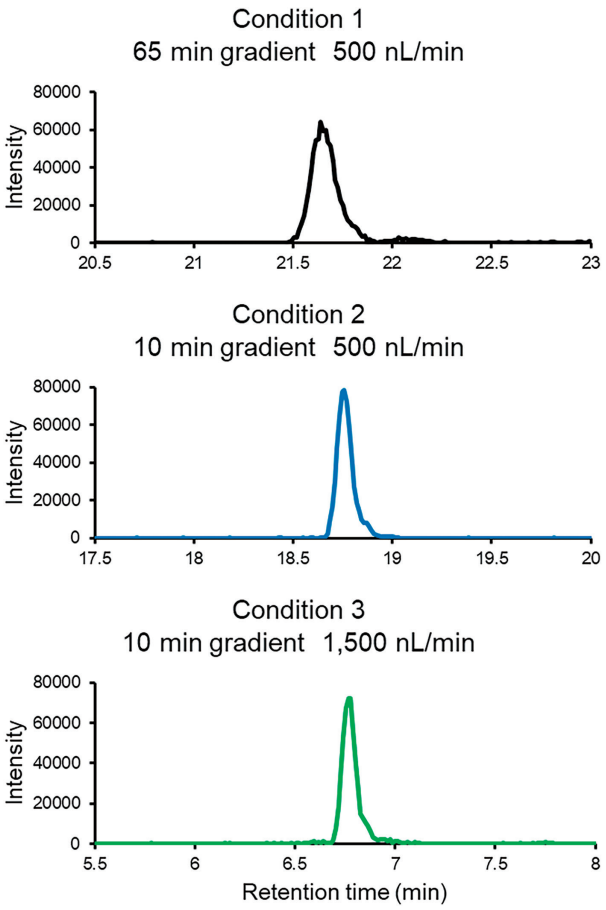

Fig. 2. Extracted ion chromatograms of FM(ox)DTVAEK under different LC conditions. A mixture of 5 fmol synthetic peptide labeled with a light dimethyl tag and 1 µg antibody sample in-column-digested under denatured conditions and labeled with a heavy dimethyl tag was measured in the PRM mode. LC, liquid chromatography; PRM, parallel reaction monitoring.

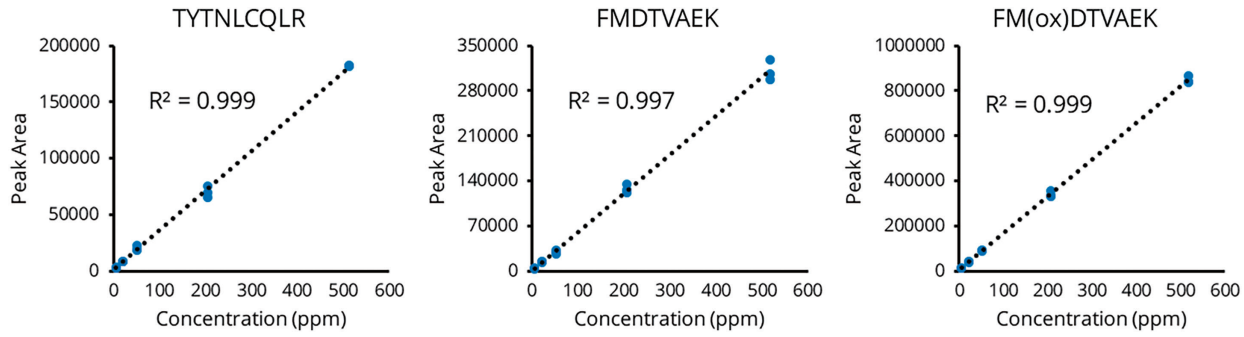

Fig. 3. Calibration curves of synthetic peptides. For synthetic peptides derived from high-risk HCPs, technical triplicates were performed for five amounts (serine protease HTRA1: 5–512 ppm, clusterin: 5–517 ppm) with a matrix of antibody drug samples. The *x*-axis displays the amounts of the light-labeled peptides, and the *y*-axis shows the peak area. Coefficients of determination (R^2) are indicated. HCPs, host cell proteins.

Table 3. Summary of validation test results.

Peptide sequence	TYTNLCQLR	Peptide sequence	FMDTVAEK	FM(ox)DTVAEK
LOD	0.67 ppm	LOD	0.97 ppm	0.37 ppm
LOQ	2.03 ppm	LOQ	2.95 ppm	1.13 ppm
5 ppm		5 ppm		
Accuracy [%]	113	Accuracy [%]	97	96
Precision [%]	2.2	Precision [%]	4	1.2
20 ppm		21 ppm		
Accuracy [%]	106	Accuracy [%]	109	105
Precision [%]	1.4	Precision [%]	3	3.5
51 ppm		52 ppm		
Accuracy [%]	107	Accuracy [%]	91	100
Precision [%]	7.7	Precision [%]	6.3	1.1
205 ppm		207 ppm		
Accuracy [%]	96	Accuracy [%]	102	99
Precision [%]	5.6	Precision [%]	4.6	2.9
512 ppm		517 ppm		
Accuracy [%]	100	Accuracy [%]	100	100
Precision [%]	0.2	Precision [%]	4.2	1.5

Validation using synthetic peptides of high-risk HCPs. The LOD and LOQ were determined based on the noise level (σ) and the slope of the calibration curve (slope) according to the 18th Japanese Pharmacopoeia using the following equations: LOD = 3.3 σ/slope, LOQ = 10 σ/slope. The accuracy was calculated from the deviation from the calibration curve. Precision was calculated from relative standard deviations. HCPs, host cell proteins; LOD, the limit of detection; LOQ, the limit of quantification.

3.4. High-throughput LC/IMS/MS/MS analysis of high-risk HCPs

As the next step, we evaluated the quantitative performance of the combination of high-throughput native digestion on a trypsin-immobilized column with high-throughput LC/IMS/MS/MS measurement within 20 min. We validated the method for HCPs quantification with respect to linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) using synthetic peptides derived from serine protease HTRA1 (TYTNLCQLR) and clusterin (FMDTVAEK, FM(ox)DTVAEK), which are high-risk HCPs identified in CHO cell-derived antibody drugs produced by AMED MAB. As a sample matrix, 1 μg of antibody drug was native-digested using a trypsin-immobilized column. Synthetic peptides were labeled with light isotopes, while matrix peptides derived from antibody drugs were labeled with heavy isotopes. For 1 μg of heavy isotope-labeled antibody-derived peptide, 0.1, 0.4, 1, 4, or 10 fmol of light isotope-labeled synthetic peptide were spiked. Based on the injected amount of synthetic peptides, the weight ratio of each HCP to the antibody drug was calculated to be 5–512 ppm for serine protease HTRA1 and 5–517 ppm for clusterin. LC/IMS/MS/MS measurements were performed using a relatively new technique, prm-PASEF. PRM is a selective measurement method because it continuously isolates and fragments specific precursor ions, and quantifies them based on the fragment ions. Moreover, prm-PASEF can also isolate targets based on ion mobility, making it even more selective. Six fragment ions with high intensity were chosen for extracting chromatograms and quantification. The peak areas of the three synthetic peptides showed good linearity (Fig. 3). The LOD, LOQ, accuracy, and precision for each peptide are shown in Table 3. As regards accuracy, recovery rates of 91–113% were consistently obtained. As for precision, the relative standard deviation (RSD%) was in the range of 0.2–7.7%. The LOD and LOQ were determined based on the noise level (σ) and the slope of the calibration

curve (slope) according to the 18th Japanese Pharmacopoeia using the following equations: LOD = 3.3 σ/slope, LOQ = 10 σ/slope. The LOD and LOQ values were 0.37–0.97 ppm and 1.13–2.95 ppm, respectively. Thus, the high selectivity of prm-PASEF contributed to the high sensitivity and quantitative character of the system. The results for linearity, accuracy, and precision corresponding to the injection amount are shown in Fig. S1 and Table S1. The contents of HCPs in heavy-labeled antibody drugs were estimated from the peak area ratio of the synthetic peptides, and it was calculated that the antibody drugs contained 20.3 ppm of serine protease HTRA1 and 728.3 ppm of clusterin. The relative standard deviations of the contents of the three replicates were 1.5% (serine protease HTRA1) and 0.5% (clusterin), indicating that the reproducibility of this method is very high. These results demonstrate that the combination of our high-throughput digestion method (10 min) and high-throughput LC/IMS/MS/MS analysis (20 min) could sensitively and selectively quantify HCPs.

4. CONCLUSION

In this study, we developed an approach employing native digestion on a trypsin-immobilized column to improve the sensitivity and throughput of HCP quantification in antibody drugs. First, we examined suitable databases for the identification of HCPs derived from CHO cells and concluded that RefSeq's Chinese hamster database was the best choice for the highly sensitive identification of HCPs. Next, we compared in-solution and in-column denatured and native digestion strategies to determine the best method for quantifying HCPs with high sensitivity. Native digestion in-column required optimization of the amount of trypsin immobilized on the column. Compared to the in-solution digestion of denatured samples, the optimized conditions not only reduced the digestion time from overnight to 10 min but also increased the number of HCPs quantified from 154 to 226. Among the identified HCPs, we focused on clusterin and the serine protease HTRA1, which have been reported as high-risk HCPs. For rapid analysis of these HCPs, we used monolithic silica columns at high flow rates. In addition, high sensitivity and selectivity were obtained by using a prm-PASEF data acquisition method that enables ion mobility separation. The optimized system was validated with synthetic peptides, confirming excellent linearity, precision, accuracy, and low LOD and LOQ (1 to 3 ppm). The optimized digestion and analysis method enables high-throughput quantification of HCPs and is expected to be useful for quality control and characterization of antibody drugs.

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COMPETING INTEREST

The authors declare the following competing financial interest(s): R.T. is an employee of Shionogi & Co., Ltd. The remaining authors declare no competing interests.

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