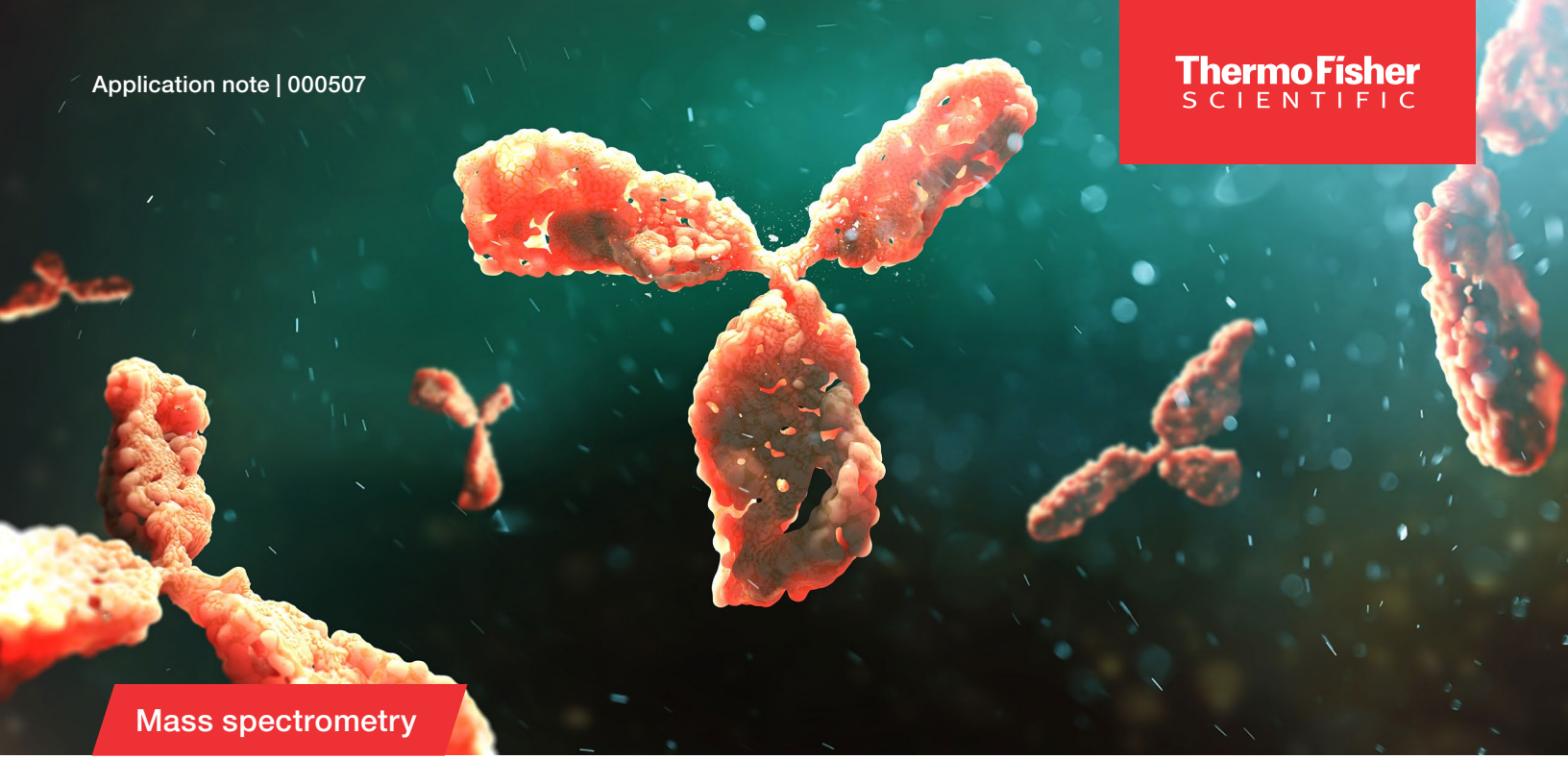

Application note | 000507

Mass spectrometry

Ultra-sensitive quantification of antibody biotherapeutics and biomarkers with a novel trapping-microLC-FAIMS/SRM-MS strategy

Authors

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Keywords

TSQ Altis Plus MS, FAIMS Pro Duo interface, biotherapeutics, differential-compensation-voltage method

Goal

To push the limit of sensitivity for the quantification of protein drugs, selective reduction of matrix interference is critical but remains a challenge. Here we took advantage of orthogonal separation and high/constant transmission efficiency provided by the Thermo Scientific™ FAIMS Pro Duo interface, as well as sensitive selected reaction monitoring (SRM)-MS on a Thermo Scientific™ TSQ Altis™ Plus triple quadrupole mass spectrometer. A novel differential-compensation-voltage (dCV) approach on a trapping-microLC-FAIMS/SRM-MS drastically improved the signal-to-noise (S/N) and limit of quantification (LOQ) for targeted quantification of monoclonal antibody (mAb) biotherapeutics and biomarkers.

Introduction

Sensitive and accurate quantification of protein biotherapeutics and related markers is critical for the discovery, development, and evaluation of these agents. In recent years, LC-MS has become a promising alternative to ligand binding assay (LBA) for the quantification of protein drugs and biomarkers in complex biological matrices.[1, 2] However, insufficient sensitivity remains a prominent challenge. For example, quantitative measurement of low ng/mL levels of monoclonal antibodies (mAbs) is often required by pharmaceutical investigations but is difficult to achieve, largely owing to high matrix interferences. As a result, immunoaffinity (IA) enrichment prior to LC-MS analysis is often necessary.[3, 4] To improve sensitivity (S/N) of LC-MS analysis, one of the most

effective means is to separate the target signal from co-eluting interferences via a separation mechanism orthogonal to that of either LC or MS.

Recently, the FAIMS Pro interface has shown great capability in improving the proteome coverage in combination with nanoflow-LC-MS, thus keeping up with the ever-increasing demands of in-depth, high-throughput proteomic analysis.[5,6] Field asymmetric ion mobility spectrometry (FAIMS) separates ions based on their differential mobility in high and low electric fields, which is a function of mass, shape, charge, center of mass, dipole moment, and the effects of clustering between ions and gas molecules (Figure 1A). Compared to conventional ion mobility spectrometry (IMS) techniques, which separate ions based on their collisional cross-sections with gas molecules at low electric fields (Figure 1B),[7] the FAIMS separation mechanism is more orthogonal to *m/z*[8,9] and can significantly improve the separation of analytes. Moreover, unlike traditional IMS, with FAIMS an appropriate compensation voltage (CV) is set to compensate for ion drift, which allows continuous sampling of the ion stream with a high ion transmission efficiency of the target species. Coupling FAIMS to the TSQ Altis Plus quadrupole mass analyzer, another continuous filter, holds considerable potential for sensitive targeted protein quantification.

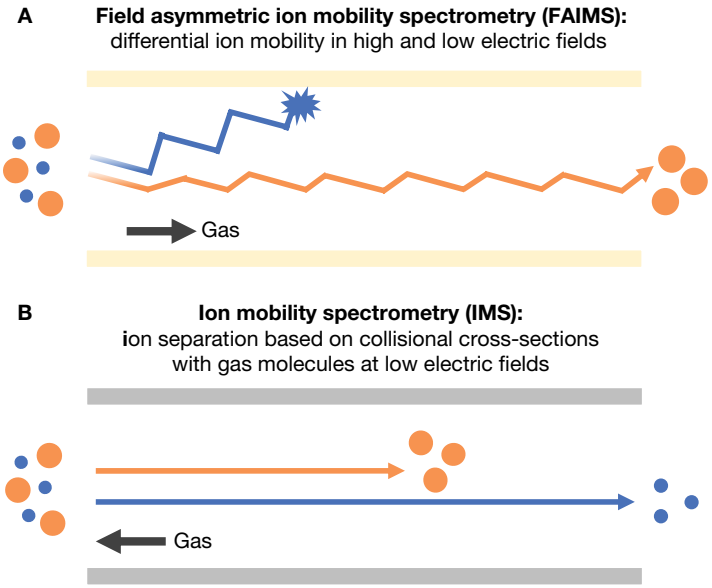

Figure 1. (A) Comparison of separation mechanism between FAIMS and (B) conventional ion mobility spectrometry (IMS). FAIMS separates ions based on their differential mobility in alternating low and high electric fields. Ions that do not transmit at the selected field are neutralized and sent to waste.

Here, we describe a sensitive LC–FAIMS/SRM-MS strategy capable of measuring low ng/mL levels of mAbs and low-abundance biomarkers in plasma, without IA enrichment. A differential-CV (dCV) approach was devised for high-throughput and accurate identification of the optimal CV that maximizes the S/N of a target peptide in complex biological matrices. The approach differentially compares the Intensity-CV profiles of the target vs. co-eluted endogenous interferences and then identifies the optimal CV achieving maximal S/N for quantification.

Experimental

Monoclonal antibodies 4B9 and 28H1 were provided by Roche, and AB095 was provided by AbbVie. Pure proteins of biomarkers were purchased from various commercial sources. Stable-isotope-labeled peptide internal standards with K[^{15}N,^{13}C] or R[^{15}N,^{13}C] at the C-terminus for each signature peptide were obtained from Synpeptide (Shanghai, China). To prepare calibration curves, protein standards were spiked at different concentrations in pooled mouse plasma and were proteolytically digested per a surfactant-aided precipitation/on-pellet digestion protocol[10] for analysis. To lower the LOQ of mAbs down to sub-ng/mL in plasma, Protein G (PG) enrichment was performed with Thermo Scientific™ Pierce™ Protein G magnetic beads (P/N 88848) using an automated protocol on a Thermo Scientific™ KingFisher™ Flex System. Quantification of biomarkers leptin and dipeptidyl-peptidase 4 (DPP4) was performed in pooled human plasma. A sensitive, high-throughput, and robust trapping-microflow-LC system described previously was employed[11], which contains: a Thermo Scientific™ UltiMate™ 3000 LC system consisting of a SRD-3400 degasser, NCS-3500RS pump with capillary-flow flow meter, and a WPS-3000 (RS) autosampler. A gradient separation was conducted on an in-house packed column (150 mm x 0.5 mm, 2.2 µm, 120 Å), using materials from a Thermo Scientific™ Acclaim™ C18 column (P/N 074812) in the microflow range of 25 µL/min for all targets. TSQ Altis Plus MS and FAIMS Pro Duo interface settings are listed in Table 1 and Table 2. SRM transition parameters of all analytes are listed in Table 3.

CV optimization tool to facilitate the dCV method development and to maximize S/N improvement

To facilitate ease-of-use, an on-the-fly CV optimization tool is provided in TSQ Instrument Control Software 3.4 (Figure 2A). For mAbs, a blank matrix sample spiked with isotope-labeled peptide can be used for dCV method development. Intensity-CV profiles for target and interfering signals at the elution window of the target are initially obtained within the CV range of -80 V to -20 V, at a step size of 5 V; then fine-tuned at a step size of 2 V within the peak CV±HWHM of the intensity-CV profile (Peak CV = the CV value corresponding to maximal intensity). The dCV approach is developed by differentially comparing the intensity-CV profile of the target against these of the endogenous interferences and then identifying the optimal CV with maximal S/N, shown in Figure 2B as the "Window of Opportunity".

Table 1. TSQ Altis Plus MS instrument settings

Parameter	Settings
Ion source type	Heated ESI
Positive ion spray voltage	3,500 V
Sheath gas	8 Arb
Aux gas	6 Arb
Sweep gas	0 Arb
Ion transfer tube temp.	325 °C
Vaporizer temp.	50 °C
Resolution	0.2 Da FWHM Q1 for H-SRM 0.7 Da FWHM Q1 for unit-resolution-SRM 0.7 Da FWHM for Q3 for both approaches

Table 2. FAIMS Pro Duo instrument settings

Parameter	Settings
FAIMS resolution mode	Standard resolution for all analytes except DPP4; High resolution for DPP4
Total carrier gas flow	Static
Total carrier gas flow (L/min)	3.5

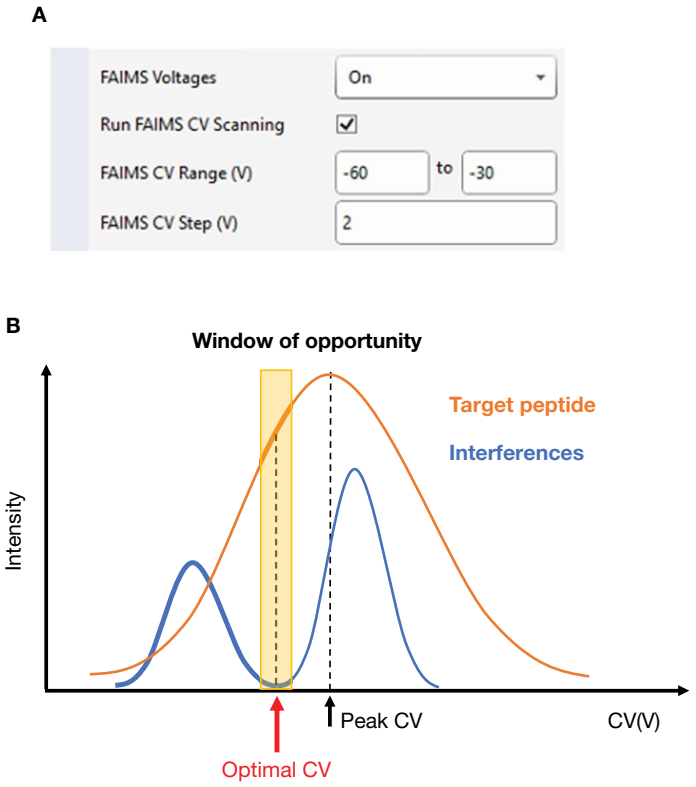

Figure 2. CV optimization tool and dCV method. A) The interface of the CV optimization tool in the SRM Properties of the instrument setup method file. B) A scheme of the rationale of dCV method.

Table 3. SRM transition parameters for quantification

Biotherapeutics or biomarkers	Signature peptide for quantification	SRM transition	Collision energy	RF lens	Optimal CV achieving the highest S/N
4B9	LLINVGSR	436.272/319.172	14	90	-50 V
28H1	LLIIGASTR	472.300/491.257	14	64	-44 V
AB095	GPSVFPLAPSSK	593.827/699.404	20	90	-48 V
Leptin	VTGLDFIPGLHPILTLSK	641.052/588.361	18	98	-59 V
DPP4	LAYVWNNDIYVK	749.391/1051.521	21	125	-36.5 V

Results and discussion

Improvement of S/N and quantitative sensitivity by the dCV method on the FAIMS Pro Duo interface

The dCV method identifies the optimal CV value that takes full advantage of the high selectivity brought by the FAIMS interface by rigorously comparing intensity-CV profiles of the target vs. co-eluting interferences. Here each signature peptide was individually optimized in spiked matrix, to achieve maximal S/N. As shown in Figure 3 and Figure 4, under the optimal CVs (values shown in Table 3), matrix interferences were drastically reduced, which substantially lowered LOQ for quantification, especially for situations where high endogenous interferences exist. For both monoclonal antibodies 4B9 and 28H1, the FAIMS-dCV method improved S/N by more than 20-fold. After Protein G (PG) enrichment, which is a quick and simple way to enrich antibodies from a biological sample,[12] mAbs can be accurately quantified with LOQs down to **0.5 ng/mL** in plasma, with a short LC separation time (Figure 3).

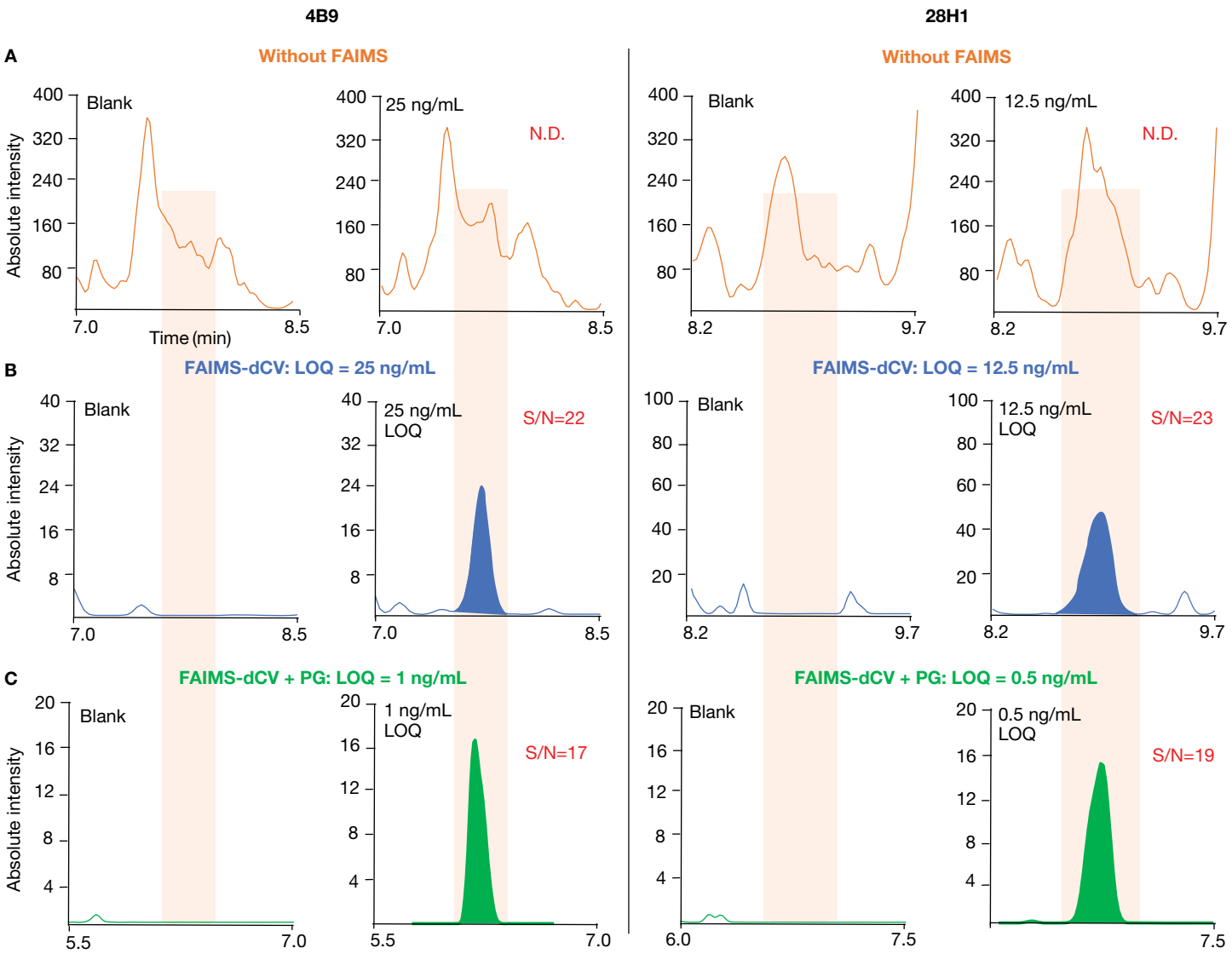

Figure 3. S/N improvement in the quantification of monoclonal antibodies from a pooled mouse plasma. With the dCV method, matrix interferences were drastically reduced, thus >20-fold S/N improvement for direct analysis of all proteins investigated (row A compared to row B). Sample preparation using Protein G capture for those mAbs further increased the sensitivity, achieving as low as 0.5 ng/mL mAb LOQ in plasma (row C).

FAIMS-dCV also provided significant S/N improvement in the quantification of endogenous circulating protein biomarkers. For example, leptin, a biomarker of obesity, was reported at low ng/mL levels in human plasma,[13,14] which typically requires antibody-based IA prior to LC-MS analysis to ensure robust quantification[15]. In this study, leptin was detected with 20-fold improvement in S/N when using FAIMS-dCV (LOQ at 2 ng/mL), permitting quantification without antibody enrichment (Figure 4).

As surveyed with the quantification of >40 target proteins, the FAIMS Pro Duo interface in standard resolution was able to markedly reduce matrix interference in most cases. In rare cases, however, the high-resolution mode was found necessary to resolve similar intensity-CV profiles between the target peptide and matrix interferent. For example, for the quantification of DPP4, an inflammation biomarker in human

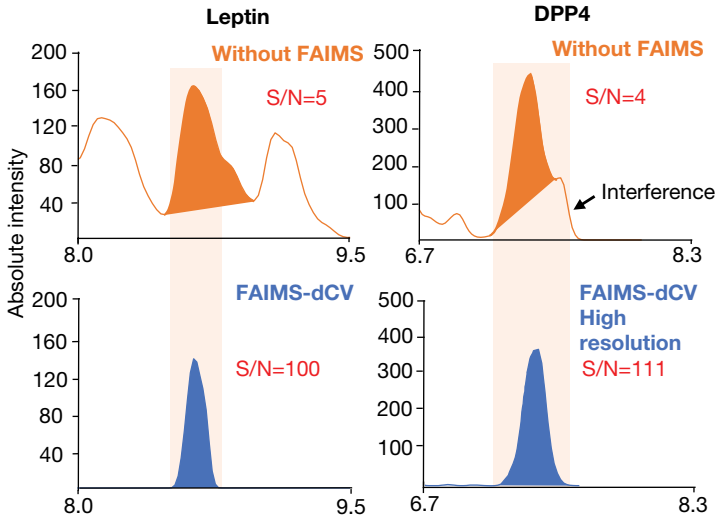

Figure 4. S/N improvement by FAIMS-dCV in the quantification of biomarkers leptin and DPP4 in pooled human plasma

plasma, intensity-CV profiles of the target and interferences were nearly identical at standard resolution, rendering it difficult to identify a "window of opportunity" to improve S/N. However, when the FAIMS Pro Duo interface was operated in high-resolution mode, it was capable of resolving the target against interference (Figure 4), providing a wide "window of opportunity" in which an optimal CV can be identified. As a result, the FAIMS-dCV at high resolution provided 28-fold S/N improvement.

The separation mechanism of FAIMS is orthogonal to *m/z*, harnessing the high-resolution-Q1 SRM (H-SRM) functionality available on the TSQ Altis Plus MS. In the case of quantification of AB095 in plasma, the combination of H-SRM and FAIMS-dCV resulted in more than 20- and 32-fold improvement of S/N and LOQ, respectively (Figure 5 and 6). We demonstrate this to be more effective than using either of the methods alone.

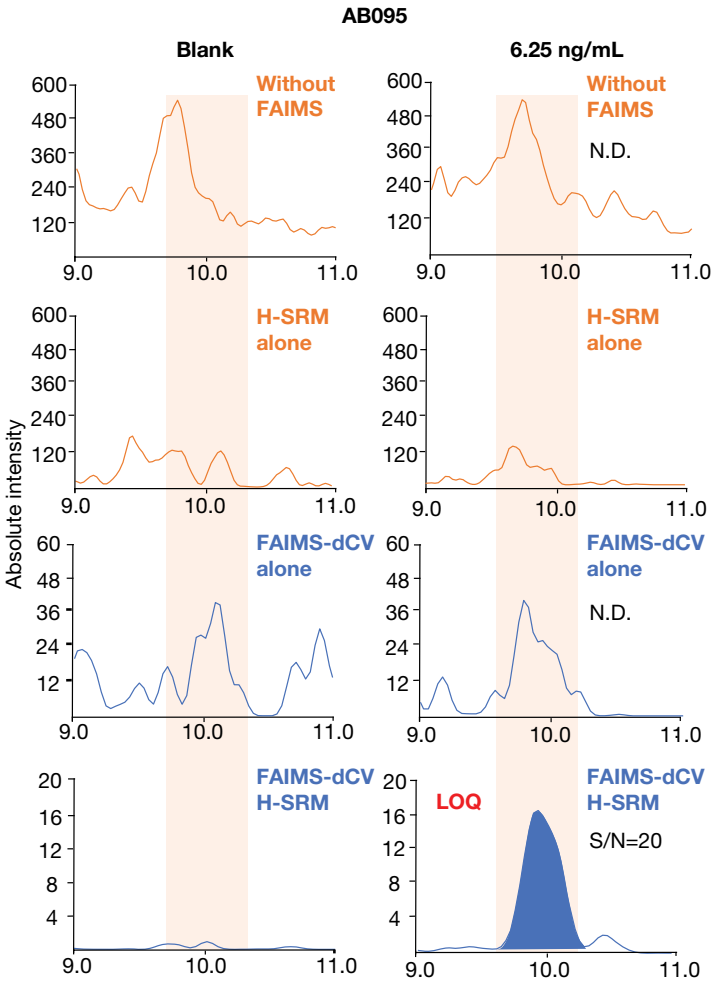

Figure 5. FAIMS separation is synergistic with high-resolution-SRM (H-SRM) for quantification of mAb AB095 in mouse plasma. The combination of H-SRM and the FAIMS-dCV method provided 20-fold S/N improvement, which was more effective at eliminating interferences than using either of the methods alone.

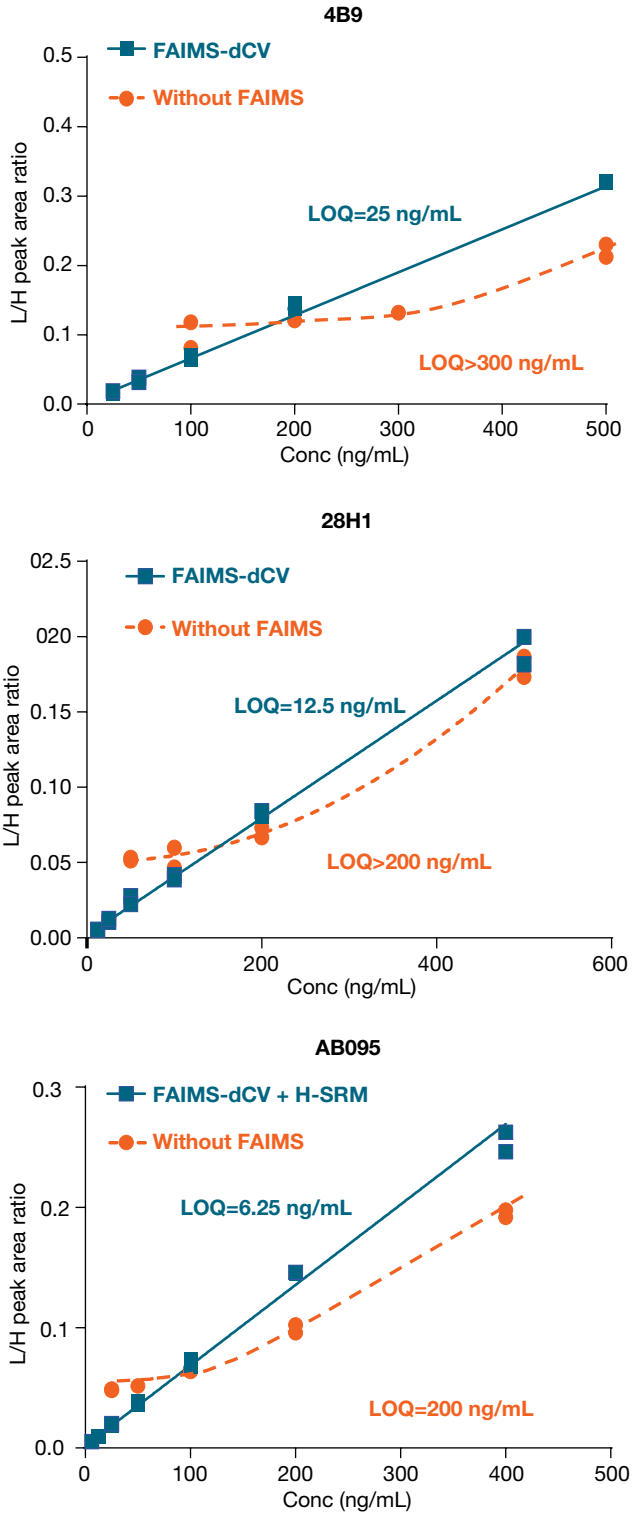

Figure 6. FAIMS-dCV method significantly extended the linearity for quantification of mAb (4B9, 28H1, and AB095) to lower concentrations (zoom-in). Calibration curves with FAIMS have good linearity compared to without FAIMS, and the LOQs are decreased significantly.

Calibration and validation results for the quantification of mAbs

Figure 5 shows the FAIMS-dCV method significantly extended the linearity for quantification of mAb to lower concentrations, and provided 12-, 16-, and 32-fold improvement of LOQ for the three model proteins 4B9, 28H1, and AB095, respectively, compared to without FAIMS (Table 4). In the case of 4B9, because of interference, the calibration curve without FAIMS was not linear at the lower levels (25 to 200 ng/mL). On the contrary, good linearity (R^2= 0.9902) was achieved after the FAIMS-dCV approach effectively lowered interference (Figure 5). In the case of AB095, H-SRM and FAIMS-dCV enabled an impressive **LOQ of 6.25 ng/mL** without any enrichment efforts. Validation results for quantification of these mAbs using the FAIMS-dCV method are shown in Table 5.

With the simple PG enrichment approach, LOQs of 0.5–1 ng/mL in plasma were achieved (Table 6) with excellent linearity across the calibration range (Figure 7). Such a high sensitivity for mAb has not been achieved by a non-antibody enrichment method. The precision and accuracy were assessed by analyzing quality control (QC) samples with four levels of each mAb. As shown in Table 6, the precision was <10% and the accuracy was <15% for plasma samples across all QC levels, indicating excellent quantitative quality.

Table 4. LOQ comparison of different quantitative methods

mAbs	Without FAIMS LOQ (ng/mL)	FAIMS-dCV LOQ (ng/mL)	FAIMS-dCV+PG LOQ (ng/mL)
4B9	>300	25	1
28H1	>200	12.5	0.5
AB095	200	6.25	1

Table 5. Validation results for quantification of anti-FAP mAbs 4B9, 28H1, and AB095 using the FAIMS-dCV method

4B9		28H1		AB095	
QC levels	Accuracy%/ Precision%	QC levels	Accuracy%/ Precision%	QC levels	Accuracy%/ Precision%
LLOQ 25 ng/mL	87.0/14.7	LLOQ 12.5 ng/mL	90.5/12.3	LLOQ 6.25 ng/mL	85.7/11.2
LQC 50 ng/mL	103.2/7.8	LQC 25 ng/mL	94.3/9.1	LQC 12.5 ng/mL	100.7/14.7
MQC 300 ng/mL	111.5/9.1	MQC 100 ng/mL	108.6/5.1	MQC 200 ng/mL	103.2/10.0
HQC 3000 ng/mL	90.2/4.9	HQC 1000 ng/mL	91.5/10.9	HQC 2000 ng/mL	105.6/7.5

Table 6. Validation results for quantification of anti-FAP mAbs 4B9, 28H1, and AB095 with PG capture and FAIMS-dCV method

4B9		28H1		AB095	
QC levels	Accuracy%/ Precision%	QC levels	Accuracy%/ Precision%	QC levels	Accuracy%/ Precision%
LLOQ 1 ng/mL	109.7/8.6	LLOQ 0.5 ng/mL	98.5/5.9	LLOQ 1 ng/mL	107.0/9.7
LQC 3 ng/mL	109.7/4.5	LQC 1 ng/mL	107.8/9.8	LQC 3 ng/mL	98.9/6.0
MQC 15 ng/mL	107.3/8.8	MQC 15 ng/mL	93.3/8.0	MQC 15 ng/mL	87.4/2.5
HQC 160 ng/mL	94.5/5.6	HQC 160 ng/mL	112.2/8.0	HQC 160 ng/mL	112.3/7.0

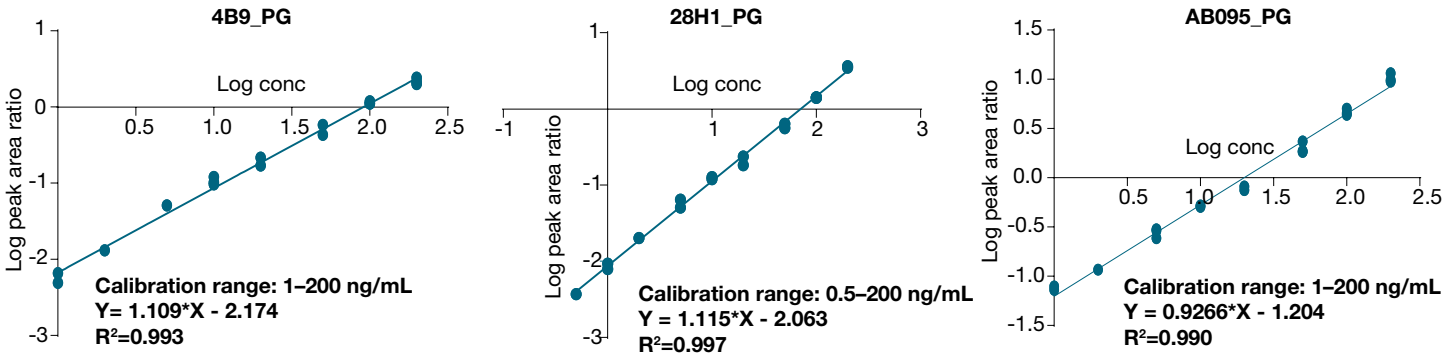

Figure 7. Calibration curves of mAbs 4B9, 28H1, and AB095 in mouse plasma with PG capture and FAIMS-dCV method

Conclusion

We present a robust, selective, and sensitive LC-FAIMS-SRM/MS approach for the quantitative measurement of low ng/mL levels of mAbs and biomarkers in the plasma samples. A novel dCV method using an on-the-fly CV optimization tool is devised to guide the method development process, quickly identifying the optimal CV that maximizes S/N the target in complicated biological samples.

This strategy increases the quantitative sensitivity of the three mAbs and two biomarkers we investigated by more than 20-fold. Moreover, in rare cases where the target and interferences show similar intensity-CV profiles, we found the high-resolution mode available on the FAIMS Pro Duo interface was highly effective at resolving the target against interference. It is also found that FAIMS separation is synergistic with H-SRM, which is useful in cases where higher selectivity is desired.

Finally, with a simple, generic protein G capture, mAbs can be reliably quantified by the LC–FAIMS-SRM/MS method down to 0.5 ng/mL in plasma samples 25-fold lower than initially observed. Such sensitivity is unprecedented for quantification of mAb without antibody enrichment and is more than sufficient for most pharmacokinetic and clinical analysis.

Acknowledgments

We would like to thank Roche for kindly providing the mAbs 4B9 and 28H1 and thank AbbVie for kindly providing the mAb AB095. We thank Prof. Weijun Qian from Pacific Northwest National Laboratory who provided SIL peptides for biomarkers leptin and DPP4.

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