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Guanidinated protein internal standard for immunoaffinity-liquid chromatography/tandem mass spectrometry quantitation of protein therapeutics

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RATIONALE: A protein internal standard (IS) is essential and superior to a peptide IS to achieve reproducible results in the quantitation of protein therapeutics using immunoaffinity-liquid chromatography/tandem mass spectrometry (LC/MS/MS). Guanidination has been used as a protein post-modification technique for more than half a century. A decade ago, the modification was applied to lysine-ending peptides to enhance their MALDI responses and peptide sequencing coverage. However, rarely has tryptic digestion of guanidinated proteins been investigated, likely due to the early conclusion that trypsin did not hydrolyze peptide bonds involving homoarginine in guanidinated proteins. In this study, the opposite was observed. Guanidinated lysine residues of proteins did not hinder the access of trypsin allowing for proteolytic digestion. Based on this observation, a new concept of internal standard, named Guanidinated Protein Internal Standard (GP-IS), was proposed for LC/MS/MS quantitation of protein therapeutics.
METHODS: The GP-IS is prepared by treating a portion of the therapeutic protein (analyte) with guanidine to convert arginine residues in the protein into homoarginine residues. After tryptic digestion, the GP-IS produces a series of homoarginine-ending peptides plus another series of arginine-ending peptides. One of the homoarginine-ending peptides, which corresponds to the analyte surrogate (lysine-ending) peptide, was chosen as a peptide internal standard (GP-PIS) for LC/MS/MS quantitation.
RESULTS: Using this GP-IS approach, a sensitive and robust immunoaffinity-LC/MS/MS assay was developed and fully validated with a linearity range from 10 to 1000 ng/mL using 200 μL of human serum for the quantitation of an Astellas protein drug in clinical development.
CONCLUSIONS: The proposed strategy allows LC/MS/MS to play an ever-increasing role in bioanalytical support for protein therapeutics development because of its capability of completely tracking all variations from the beginning to the end of sample analysis, easier preparation compared to isotope-labeled protein-IS, and greater flexibility for changing to alternate analyte surrogate peptides. Copyright © 2014 John Wiley & Sons, Ltd.

The development of protein therapeutics in the pharmaceutical industry is increasing rapidly. For example, the development of new therapeutic proteins involved more than 200 companies sharing a strong growth market of £90 billion in 2010.[1] In 2012, among 39 FDA drug approvals six were biologics.[2] According to IMS Health, it is expected that biologics will account for approximately 17% of total global spending on medicines by 2016, and seven of the top ten global medicines by spending will be biologics by mid-2017.[3] This rapid growth highlights the need for the development of quantitative bioanalytical methods with sufficient accuracy, precision and selectivity in biological matrices to support preclinical and clinical pharmacokinetic and toxicokinetic studies, as well as drug characterization at the early stage of discovery and development.

Ligand binding assays (LBAs) are currently the primary means of quantifying protein therapeutics in biological fluids. The most common format is enzyme-linked immunosorbent assay (ELISA); however, alternative technologies are also available (e.g. Luminex, Gyros, and MSD) with their own advantages and disadvantages. LBAs are popular due to their simplicity, sensitivity, and high-throughput capabilities. However, they sometimes suffer from selectivity/specificity issues or high background noise resulting from matrix interference by endogenous. Another disadvantage is lengthy and costly assay development because of the antibody production and selection process.[4]

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is the gold-standard technology for quantifying small molecule drugs in the pharmaceutical industry. Recently, it has become an increasingly popular approach to LBAs for protein quantitation. LC/MS/MS offers many inherent features such as robustness, selectivity/specificity, transferability, the ability to multiplex, relatively fast method development, and the accumulated and adaptable knowledge from small molecule analysis and proteomics. However, common approaches for LC/MS/MS

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analysis of small molecules are not readily applicable to macromolecules because of the basic differences in their chemistry and biology. Quantifying protein therapeutics using LC/MS/MS involves some unique bioanalytical challenges.[5,6] One of these challenges is the proper selection of an internal standard (IS).

Including an IS in quantitative LC/MS/MS assays is common practice for small molecule drugs, especially in a regulated environment. Typically, a stable-isotope-labeled internal standard (SIL-IS), an isotopically labeled version of the analyte, is used to better compensate for variations introduced from sample preparation and the analytical system to achieve accurate, precise and reproducible results. An IS is especially critical for protein therapeutics where sample preparation is more complicated compared to small molecule drugs.[7] Quantifying proteins by LC/MS/MS typically involves the selection and analysis of surrogate peptides generated by enzymatic digestion. In 1996, Barr[8] demonstrated the use of a chemically synthesized, stable-isotope-labeled surrogate peptide as an IS (SIL-peptide IS). It was further generalized by Gygi in 2005 as an absolute protein quantitation strategy (AQUA),[9] and is still widely used due to its simplicity.[10,11]

One drawback with using a synthesized SIL-peptide IS is that the approach involves chemical synthesis using isotope-labeled amino acids. A new synthesis would be required if the surrogate peptide is changed, which may happen frequently over the course of method development. To overcome this issue, a universal strategy was developed utilizing differential dimethyl labeling of surrogate peptides.[12] It provides a fast and low-cost approach to generate a peptide IS for method development. Similar approaches, such as isotope-coded affinity tags (ICAT),[13] enzymatic ^{18}O labeling,[14] isobaric tags for relative and absolute quantitation (iTRAQ)[15] and tandem mass tags (TMT),[16] have been developed in comparative proteomics, although they have not been used for quantitation of protein therapeutics.[17]

Another disadvantage of using a SIL-peptide IS is that it does not address variations in protein digestion. This issue could be overcome by using an enzyme-cleavable SIL-peptide IS. However, an investigation suggested that this type of IS did not show significant improvement in accuracy and precision,[18] likely because it could not completely mimic the behavior of the intact protein during enzymatic digestion.

Furthermore, a SIL-peptide IS is incapable of compensating for variations that result from sample cleanup/enrichment prior to digestion. Common biological matrices, such as serum or plasma, contain very large amounts of endogenous proteins which are physicochemically similar to therapeutic protein drugs circulating at low concentration. Without sample cleanup or enrichment, direct digestion of protein therapeutics within the matrix is impractical. It would require a large amount of digestion enzyme and generate numerous enzymatic peptide interferences that would overwhelm both LC and MS systems, leading to ionization suppression of the analytes and ultimately compromising the entire assay performance. Therefore, some practical sample cleanup/enrichment strategies such as protein depletion[19,20] or immunoaffinity using magnetic beads[10,20,21] are often employed in order to achieve quantitation limits (ng/mL or less) necessary to support clinical or preclinical studies. Extensive sample preparation can cause sample loss resulting in irreproducible quantitation results if a proper IS is not used.[22,23]

For the reasons discussed above, a suitable protein internal standard is an ideal solution when it is introduced from the beginning of sample preparation. A full-length SIL-protein IS is the best choice for absolute protein quantitation.[24,25] It completely tracks the variations introduced by sample pretreatment, digestion, and LC/MS/MS analysis to produce accurate and reproducible results. This strategy was adopted from metabolic labeling in comparative proteomics.[26,27] However, a SIL-protein IS may not be widely adopted, especially during the early stages of drug development, because of cost, departmental resources, and slow production.

To overcome these disadvantages, we propose and demonstrate a novel internal standard for accurate LC/MS/MS quantitation of protein therapeutics named Guanidinated Protein Internal Standard (GP-IS). A GP-IS is prepared from the target protein through a simple post-translational modification, guanidination,[28] as illustrated in Fig. 1. The GP-IS still retains, or at least partially retains, the biological activity of the original protein drug allowing it to serve as an ideal internal standard throughout the entire analysis. During sample processing, tryptic digestion of the GP-IS generates two series of tryptic peptides: (A) guanidinated lysine (also called homoarginine)-ending peptides and (B) arginine-ending

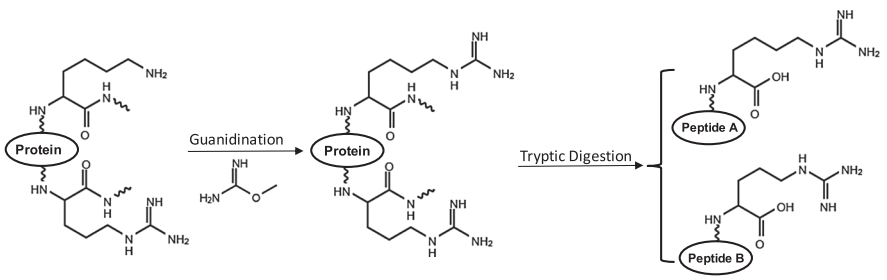

Figure 1. Scheme of the generation of guanidinated protein internal standard (GP-IS) and its surrogate peptide (GP-ISP) for absolute LC/MS/MS quantitation of proteins. Lysyl residues on proteins react with *O*-methylisourea to form homoarginyl residues. After tryptic digestion is applied, a series of peptides with a homoarginyl residue at the C-terminal are generated, one of which is selected as an internal standard (GP-ISP) for absolute LC/MS/MS quantitation of the protein therapeutic.

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peptides. From Series A, a peptide matching the analyte surrogate peptide sequence except for the terminal guanidinated lysine is selected as a GP-IS surrogate peptide (GP-ISP) for LC/MS/MS monitoring. This approach is demonstrated through a case study involving development of an immunoaffinity-LC/MS/MS assay for quantitation of Astellas protein drug ASP-P1.

EXPERIMENTAL

Materials and reagents

Model proteins including α-lactalbumin from bovine milk, papain from papaya latex, myoglobin from equine heart, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals such as dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, *O*-methylisourea hemisulfate, boric acid and sodium hydroxide were also purchased from Sigma-Aldrich. HPLC grade acetonitrile, methanol, acetic acid and formic acid were from Fisher Scientific (Hampton, NH, USA). 10 × phosphate-buffered saline (PBS) was from Teknova (Hollister, CA, USA). Sequencing grade trypsin was from Promega (Madison, WI, USA). Tosylactivated magnetic beads (Dynabeads M-280) were from Life Technologies (Carlsbad, CA, USA). Zeba spin columns were from Pierce (Rockford, IL, USA). The proprietary therapeutic protein drug, ASP-P1, and its monoclonal antibody, anti-ASP-P1, were provided by Astellas Pharma, Inc. (Tokyo, Japan). The sequence of ASP-P1 used in these studies may be found in US Patent 8,496,935 under the name D3-69-IgG2.[29]

Guanidination procedure

O-Methylisourea hemisulfate (677 mg) was dissolved in pure water (10 mL) to make *O*-methylisourea stock solution with a concentration of 0.55 mol/L. Guanidination working solution was prepared fresh before use by combining 2.2 mL of the stock solution and 0.3 mL of 1 mol/L sodium hydroxide solution.

For model proteins, a 450 µL aliquot of this fresh working solution was added to 50 µL of 10 mg/mL protein solutions prepared in water. The tube was gently rotated at room temperature for 12 h. The protein was purified using Zeba spin column against 0.1 mol/L ammonium bicarbonate buffer following the manufacturer's instructions. The purified solutions were ready for trypsin digestion.

For ASP-P1, an 80 µL aliquot of guanidination working solution was added to 20 µL of the 10 mg/mL protein solution prepared in PBS. The reaction was conducted for 4 days at room temperature, and 100 µL of guanidination working solution was added after every 24 h of incubation. The protein was purified using Zeba spin column against 0.1 mol/L ammonium bicarbonate buffer following the manufacturer's instructions.

Sample preparation for ASP-P1 quantitative method validation

Calibration standards were prepared fresh daily by diluting 10 mg/mL ASP-P1 stock solution in pooled human serum to 10, 20, 40, 100, 200, 400, 850, 1000 ng/mL. Quality control samples (QCs) were prepared from the pooled human serum at 10 ng/mL (LLOQ), 30 ng/mL (LQC), 160 ng/mL (MQC), and 800 ng/mL (HQC) and stored at –20 °C before use.

Immunocapture

The following procedures were used to prepare magnetic beads coated with capture antibody, anti-ASP-P1, for the capture of ASP-P1 from a 200 µL human serum sample. A 30 µL aliquot of M-280 tosylactivated beads in a Protein Lobind tube was washed twice with 0.5 mL of 0.1 mol/L borate buffer, pH 9.5 (Buffer A). The beads were separated from solution using a magnetic particle concentrator for microcentrifuge tubes (Life Technologies). After removal of the washing solution, the beads were added to 10 µL of Buffer A and 5 µL of anti-ASP-P1 at 1 mg/mL and incubated for 4 h at 37 °C with shaking at 1000 rpm for covalent binding to the beads. The beads were washed three times with 0.5 mL of PBS buffer containing 0.1% BSA (Buffer B) to remove unbound capture antibody. The beads were resuspended in 0.5 mL of Buffer B and incubated at 37 °C with shaking at 1000 rpm for 2 h to block and prevent non-specific binding. The anti-ASP-P1-coated beads were harvested and stored at 4 °C with 10 µL of Buffer B. The beads were functionally stable for at least 1 month at this condition.

Immunocapture of ASP-P1 and GP-IS was performed using the following procedures. 200 µL of serum samples were incubated at 37 °C with shaking at 1000 rpm for 1.5 h with 10 µL of the functionalized beads and 10 µL of GP-IS prepared above. After removal of the supernatant, the beads were washed three times with Buffer B and were ready for digestion after complete removal of the buffer.

Digestion

After the immunocapture step, 50 µL of 0.5 mol/L ammonium bicarbonate buffer (Buffer C) was added to the beads. Digestion was started with thermal denaturation by heating the sample plate at 95 °C for 30 min. After cooling and centrifugation, the solutions were spiked with 15 µL of 10 mmol/L DTT prepared in Buffer C and incubated at 55 °C for 60 min for disulfide bond cleavage. Alkylation was performed by adding 30 µL of 50 mmol/L IAA prepared in Buffer C and incubating for 60 min at room temperature with light protection. The digestion was conducted by incubating 10 µL of 0.1 µg/µL trypsin at 37 °C for 10 h. The reaction was stopped by adding 10 µL of 5% formic acid.

For model proteins, 50 µL of purified guanidinated solution or native protein solution was used and the same procedure as above was followed except for myoglobin, for which the cleavage and alkylation steps were omitted.

LC/MS/MS instrumentation and conditions

Qualitative and quantitative experiments were performed using a Shimadzu system comprised of LC20AD XR HPLC pumps and SIL-20AC XR autosampler coupled with an AB Sciex 5500 QTrap mass spectrometer. Separation was achieved on an Aeris Peptide XB-C18 analytical column (150 × 2.1 mm, 3.6 µm) with a UHPLC C18-Peptide column guard cartridge (Phenomenex), using the mobile phases consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN. Elution was programmed by increasing

mobile phase B from 2% at 0 min to 40% at 10 min with a flow rate of 0.35 mL/min. 10 μL of digested tryptic peptide solution, stored at 10 °C in the autosampler, was injected into the system.

The mass spectrometer was operated in positive electrospray ionization (ESI) mode. For the characterization of guanidinated model proteins, mass spectrometer parameters were optimized through the MRM/Enhanced Product Scan IDA experiment. For the quantitation of the drug, the mass spectrometer was extensively optimized using Instrument Optimization mode and Compound Optimization mode through infusing tryptic peptide solution of the standard purified by C-18 cartridges. The optimized conditions for quantitation were as follows: ionspray voltage 5500 V, source temperature 550 °C, GS1 50, GS2 60, curtain gas 10, CAD gas medium, EP 10, unit resolution for both Q1 and Q3, MRM mass transition for analyte surrogate peptide 772.4 (+2) → 969.4 (CE 33, CXP 24, DP 61, dwell time 150 ms), MRM mass transition for GP-ISP 793.4 (+2) → 1101.4 (CE 34, CXP 24, DP 61, dwell time 150 ms).

Method validation

Full validation of the LC/MS/MS method for the quantitation of ASP-P1 in human serum was performed in three batches according to the FDA guidance for bioanalytical validation.[30] The calibration standard range and linearity were evaluated using Analyst 1.5.2 software and least-squares linear regression analysis with a weighting factor of $1/x^2$.

RESULTS AND DISCUSSION

Protein guanidination and tryptic digestion

Guanidination of proteins through lysine residues became a standardized procedure in the late 1960s to study the environment and essential nature of lysine side chains in proteins.[28] A decade ago this mature technology was adapted and became popular in proteomics research. It was initially used for enhancing the signal intensities of lysine-ending tryptic peptide ions in MALDI mass spectrometry to facilitate protein sequence coverage.[31,32] Later, the applications were extended into the fields of N-terminal peptide sequencing[33,34] and quantitative proteomics.[35,36] In addition, a guanidination kit has been commercialized.[37]

However, guanidination was mostly applied to tryptic peptides post-digestion, rather than intact proteins prior to digestion. This situation may exist because of the general perception that modified lysine residues in proteins would no longer be recognized by trypsin. Indeed, it was concluded 50 years ago that trypsin did not hydrolyze peptide bonds involving homoarginine in guanidinated α-lactalbumin.[38] The conclusion was reaffirmed by experiments involving papain,[39] and later this inertness to trypsin was further generalized.[28]

Our intention to conduct more exploration was based on the fact that arginine is recognized by trypsin and it only differs from homoarginine by one CH_2 unit in its side chain. Trypsin may not have the specificity to distinguish these two residues in a complicated protein structure. The conclusion made 50 years ago could be based on the technology available at that time, such as paper chromatography and chemical analysis of amino acids. Therefore, we decided to reinvestigate tryptic digestion of guanidinated α-lactalbumin using state-of-the-art technology, LC/MS/MS.

The protein was guanidinated as described in the Experimental section. The guanidinated protein was digested with the native protein placed side by side as a control to verify the digestion performance. *In silico* digestion using the ExPASy online program was first employed on the native protein with the setting of zero miss cleavage to predict theoretical peptides. Lysine-ending peptides with a +2 charge and *m/z* value within the mass spectrometer detection window (<1250) were selected for further evaluation. To verify the existence of a theoretical peptide, all the possible MRM mass transitions from the precursor ion (+2) to possible y-ions (+1) were monitored by LC/MS/MS. The existence of a particular peptide was confirmed if all its MRM scan peaks aligned and by analyzing the MS/MS spectrum which was acquired by MRM-Enhanced Product Ion (EPI)-IDA experiment. To search for guanidinated tryptic peptides, the same procedure was applied but MRM mass transitions were set as 'native precursor ion + 21 (+2) → native product y-ion + 42 (+1)' per guanidination modification. The results are presented in Table 1 and Figs. 2 and 3. Clearly, all the theoretical tryptic guanidinated peptides from guanidinated α-lactalbumin, corresponding to the native forms, were present. As an example, Fig. 3 shows the MS/MS spectra of Peptide 6 from native and guanidinated α-lactalbumin, which was acquired in MRM-EPI-IDA mode. The identity of the guanidinated form is confirmed because all its y-ions gained 42 *m/z* (+1) and its precursor ions gained 21 *m/z* (+2) compared to the native form resulting from guanidination of the C-terminal lysine.

Tryptic digestion of guanidinated proteins was further substantiated with papain, BSA and myoglobin. The data is included in Table 1. An extensive search also revealed a few literature references which describe this tryptic hydrolysis of guanidinated proteins, although this topic is not their main focus.[40–43] The data in Table 1 can lead to several conclusions related to guanidination of proteins and tryptic hydrolysis of peptide bonds involving homoarginine generated from guanidination, although further investigation may be needed for the confirmation. (1) Most lysine residues were completely guanidinated and completely digested resulting in >95% yield of homoarginine peptides. (2) Because of steric hindrance, not all homoarginine residues were equally accessible to trypsin. This was reflected in Table 1 by the various yields of homoarginine peptides produced by digestion. An extreme example in this case was labeled as 'Not Found, Type A', meaning all lysine residues were guanidinated to form homoarginine residues, but none were digested. (3) The various yields might also reflect that the degree of guanidination varied with the location of lysine residues in a protein. An extreme example of this case was labeled as 'Not Found, Type B', meaning no lysine residues were guanidinated. (4) Compared to the lysine-ending peptide, the corresponding homoarginine-ending peptide signal intensity was significantly reduced (Fig. 2) even for those with high conversion yield. This probably indicates sample loss during processing or protein hydrolysis during guanidination in high pH environment because changing

Table 1. LC/MS/MS data of tryptic lysine-peptides from native and guanidinated model proteins

	Native protein				Guanidinated protein				
		Mass transition monitored				Mass transition monitored			
Peptide ID No.	Sequence	Q1 ion (+2)	Q3 ion (+1)	LC RT (min)	Sequence	Q1 ion (+2)	Q3 ion (+1)	LC RT (min)	Homoarginine peptide yield (%)
α-Lactalbumin									
P1	ILDK	244.6	375.2	3.0	ILDK*	265.6	417.2	3.49	>95
P2	IWCK	303.6	493.2	3.5	IWCK*	324.6	535.2	3.82	>95
P3	EQLTK	309.6	361.2	2.4	EQLTK*	330.6	403.3	3.03	>99
P4	ALCSEK	354.2	523.2	2.4	ALCSEK*	375.2	565.2	2.91	>95
P5	LDQWLCEK	546.2	436.2	5.1	LDQWLCEK*	567.2	478.2	5.24	>99
P6	VGINYWLAHK	600.8	931.4	5.5	VGINYWLAHK*	621.8	973.4	5.66	>99
P7	FLDDDLTDDIMCVK	850.4	981.4	6.9	FLDDDLTDDIMCVK*	871.4	1023.4	7.03	>99
P8	DDQNPHSSNICNISCDK	1002.4	509.2	7.2	DDQNPHSSNICNISCDK*	1023.4	551.2	7.23	>95
Papain									
P1	ITGYK	291.2	468.2	3.1	ITGYK*	312.2	510.2	3.41	>99
P2	GAVTPVK	336.2	444.5	3.3	GAVTPVK*	357.2	486.5	3.63	>99
P3	HSYGCK		Not Found		HSYGCK*		Not Found, Type C		N/A
P4	NYIIIK	382.2	486.4	5.7	NYIIIK*	403.2	528.4	6.14	>99
P5	ATDKPGPK	407.2	641.5	1.4	ATDKPGPK*		Not Found, Type A		N/A
P6	VYPYQAK	434.7	606.3	4.1	VYPYQAK*		Not Found, Type A		N/A
P7	SSYYPFK	446.2	717.4	5.6	SSYYPFK*	467.2	759.4	5.77	>99
P8	SGVFDGPCGTK	562.7	881.3	4.8	SGVFDGPCGTK*	583.7	923.3	5.03	>99
P9	NSWGPNWGEK	587.8	787.3	5.9	NSWGPNWGEK*	608.8	829.3	6.01	>99
P10	QSGNSQGTCGVYK	693.3	784.3	3.4	QSGNSQGTCGVYK*	714.3	826.3	3.92	>99
P11	LDHAVTAVGYGTSDGK	795.5	954.2	4.6	LDHAVTAVGYGTSDGK*		Not Found, Type D		N/A
P12	IVTGNLLELSEQELVDCDK		Not Found		IVTGNLLELSEQELVDCDK*		Not Found, Type C		N/A
P13	GGYQTTSLQYVANNGVHTSK		Not found		GGYQTTSLQYVANNGVHTSK*		Not Found, Type C		
BSA									
P1	DTHK	250.6	385.2	0.8	DTHK*		Not Found, Type D		N/A
P2	HKPK	255.2	372.2	0.8	HKPK*		Not Found, Type D		N/A
P3	ADLAK	259.1	446.3	2.1	ADLAK*	280.1	488.3	3.1	>99
P4	FWGK	269.2	390.2	4.6	FWGK*	290.2	432.2	5.0	>99
P5	AFDEK	305.2	391.1	3.0	AFDEK*	326.2	433.1	3.4	95
P6	TPVSEK	330.0	462.2	2.1	TPVSEK*		Not Found, Type D		N/A
P7	NYQEAK	376.7	475.3	1.8	NYQEAK*	397.7	517.3	2.8	>99
P8	LVTDLTK	395.2	577.3	4.9	LVTDLTK*	416.2	619.3	5.1	95
P9	ATEEQLK	409.7	646.3	3.1	ATEEQLK*	430.7	688.3	3.4	>99
P10	DDSPDLPK		Not Found		DDSPDLPK*		Not Found, Type C		N/A
P11	AEFVEVTK	461.7	722.4	5.0	AEFVEVTK*	482.7	764.4	5.3	85
P12	DLGEEHFK	487.7	746.3	4.3	DLGEEHFK*	508.7	788.3	4.8	95

(Continues)

Table 1. (Continued)

	Native protein				Guanidinated protein				
		Mass transition monitored				Mass transition monitored			
Peptide ID No.	Sequence	Q1 ion (+2)	Q3 ion (+1)	LC RT (min)	Sequence	Q1 ion (+2)	Q3 ion (+1)	LC RT (min)	Homoarginine peptide yield (%)
P13	QTALVELLK	507.8	601.2	7.1	QTALVELLK*	528.8	643.2	7.3	95
P14	LVNELTEFAK	582.3	951.5	6.6	LVNELTEFAK*	603.3	993.5	6.7	98
P15	HLVDEPQNLIK	653.4	1055.5	5.5	HLVDEPQNLIK*	674.4	1097.5	5.6	>99
P16	TVMENFVAFVDK	700.4	939.5	8.2	TVMENFVAFVDK*	721.4	981.5	8.3	95
P17	HPYFYAPELLYYANK	945.0	1110.6	7.6	HPYFYAPELLYYANK*	Not Found, Type A			N/A
P18	DAIPENLPPLTADFAEDK	978.4	1203.6	7.9	DAIPENLPPLTADFAEDK*	Not Found, Type A			N/A
Myoglobin									
P1	NDIAAK	316.2	402.2	2.5	NDIAAK*	337.2	444.2	3.0	>99
P2	ASEDLK	331.6	260.2	2.7	ASEDLK*	352.6	546.2	3.0	Low recovery
P3	TEAEMK	354.1	478.2	2.5	TEAEMK*	375.1	520.2	3.5	Low recovery
P4	LFTGHPETLEK	636.3	716.4	4.3	LFTGHPETLEK*	657.3	758.4	4.5	90
P5	HGTVVLTALGGILK	689.9	984.6	6.5	HGTVVLTALGGILK*	710.9	1026.6	6.6	>99
P6	HPGDFGADAQGAMTK	751.8	706.4	4.1	HPGDFGADAQGAMTK*	772.8	748.4	4.3	Low recovery
P7	YLEFISDAIIHVLHSK	803.9	814.6	4.9	YLEFISDAIIHVLHSK*	Not Found, Type B			0
P8	GHHEAELKPLAQSHATK	908.5	603.3	7.4	GHHEAELKPLAQSHATK*	929.5	645.3	7.5	>99
P9	GLSDGEWQQVLNVWGK	943.0	1132.6	7.8	GLSDGEWQQVLNVWGK*	Not Found, Type A			N/A

Notes: **Not Found:** No peak was found with every possible MRM transition from native proteins; **Not Found, Type A:** Neither guanidinated nor non-guanidinated peak was found in guanidinated proteins with every possible MRM transition, but peptide peak from native was good. This observation indicated the lysine residues at this location were guanidinated to form homoarginine residues, but none were digested, and further possibly implied not all homoarginine residues were equally accessible to trypsin; **Not Found, Type B:** No guanidinated was found in guanidinated proteins with every possible MRM transition, but both non-guanidinated peak in guanidinated sample and peptide peak from native proteins were good. This indicated that no lysine residue was guanidinated and further possibly implied that the degree of guanidination varied with the location of lysine residues in a protein. **Not Found, Type C**: Neither guanidinated nor non-guanidinated peak was found in guanidinated proteins with every possible MRM transition, and no peptide peak found from native either; **Not Found, Type D:** Neither guanidinated nor non-guanidinated peak was found in guanidinated proteins with every possible MRM transition, and peptide peak from native was low.

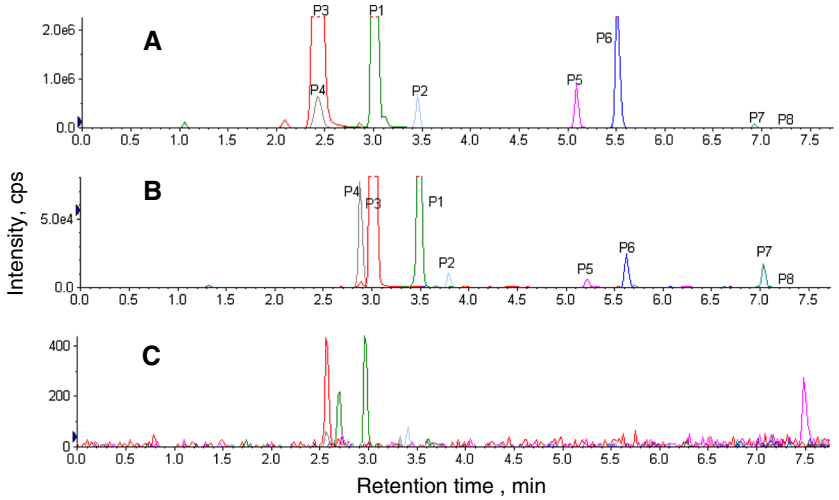

Figure 2. MRM chromatograms of (A) tryptic lysine-peptides from native α-lactalbumin, (B) tryptic homoarginine-peptides from guanidinated α-lactalbumin, and (C) remaining tryptic lysine-peptides from guanidinated α-lactalbumin. The peaks are labeled per ID numbers from Table 1. MRM mass transitions for each peptide are given in Table 1. Other conditions were as described in the Experimental section.

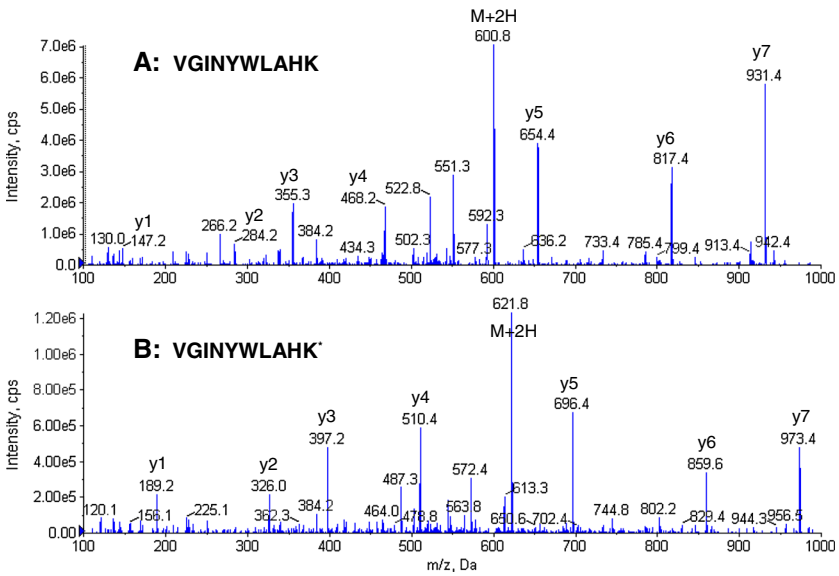

Figure 3. MS/MS spectra of Peptide 6 from native α-lactalbumin (A) and guanidinated α-lactalbumin (B). The y-ions are labeled beside the peaks in the figure. All y-ions of guanidinated α-lactalbumin gained 42 amu due to guanidination. Ions beyond y7 were outside the detection window (<1000 Da) for the MRM-Enhanced Product Ion IDA scan.

the lysine residue to a homoarginine residue should have resulted in signal enhancement, as seen in both MALDI[29,30] and electrospray ionization (ESI).[44] (5) It is evidenced in Figs. 2(A) and 2(B) that the retention times of tryptic peptides increased after guanidination. This observation was also reported in the literature.[31,44] This reflects guanidination could increase the hydrophobicity of tryptic peptides. Furthermore, the increase in retention time varied from peptide to peptide, indicating that the degree of increase in the retention time depends on the hydrophobicity of the native peptide. Typically, the impact on hydrophilic peptides (early eluted peptides) is greater than on hydrophobic peptides (late eluted peptides).

Strategic use of guanidinated protein internal standard (GP-IS) for development of immunoaffinity-LC/MS/MS assay for quantitation of ASP-P1 in human serum

ASP-P1 is a therapeutic protein being developed at Astellas. Initially, a LBA was developed to support the clinical trials, but the further implementation of this assay was hindered

by its inadequate sensitivity in a low dosing study. LC/MS/MS coupled with immunocapture sample preparation (immunoaffinity-LC/MS/MS) using capture antibody-coated magnetic beads was therefore investigated because of its proven capability in sample cleanup and enrichment to enhance assay sensitivity. Due to extensive bead washing and sample processing, it is essential to have a proper IS to mimic the analyte behavior and compensate for the variation during sample processing. GP-IS was introduced in this study to fulfill the above requirement.

In order to use GP-IS, the following three characteristics must be met. (1) The analyte surrogate peptide must end with lysine. (2) The contribution from GP-ISP to the analyte channel, due to incomplete guanidination, should be less than 20% of LLOQ, requiring a high yield of GP-ISP from guanidination and digestion. (3) Most importantly for immunocapture, the GP-IS must retain sufficient affinity to the capture antibody. Detailed discussions on these three aspects will be given in the following sections.

Selection of analyte surrogate peptide and GP-ISP

While considerations for the selection of analyte surrogate peptides have been widely discussed by many authors and good general recommendations were provided by Li,[7] some case-by-case limitations should still be taken into account. For the use of GP-IS, the analyte surrogate peptide selected must be a lysine-ending peptide. It is preferable to choose the surrogate peptide that is chromatographically well retained in order to have close elution with GP-ISP. ASP-P1 is a recombinant fusion protein with a molecular weight over 75 kDa. *In silico* digestion yields 30 tryptic peptides; 24 of which possess *m/z* (+2) within the detection window (≤1250). After a Swissprot database search, 21 peptides were excluded because they overlapped with other endogenous human proteins. For the three remaining candidates, two peptides have lysine at the C-terminal which meets the requirement for GP-IS. Finally, a peptide with the sequence ***SPGK (partially masked for proprietary reasons) was selected because it exhibited good chromatographic behavior and ESI response. This surrogate peptide contains 14 amino acids with *m/z* 772.4 (+2). Its y7 ion (*m/z* 969.4 (+1)) was a dominant ion and was selected as a fragment for MRM monitoring. The GP-ISP MRM transition is 793.4 (+2) → 1101.4 (+1). Under optimized HPLC conditions, the analyte surrogate peptide and GP-ISP eluted approximately 0.1 min apart (retention times 6.4 and 6.5 min, respectively), which enables the IS to sufficiently track the analytical system variations.

Optimization of guanidination

In the literature, optimization for guanidination of peptides has been comprehensively investigated. Using optimized conditions, which involved heating to 65°C, the complete conversion of lysines into homoarginines was accomplished in 5 min.[45] However, these optimized conditions may not directly apply to protein guanidination because milder conditions for proteins are preferred in order to retain their tertiary structure allowing for immunocapture after guanidination. Moreover, guanidination of proteins could be more difficult than for peptides because lysine residues could be buried within the 3D structure of a protein. It has been shown from the studies of the model proteins (above) that the degrees of guanidination vary between different proteins and different lysine residues. Although nearly complete guanidination was generally achieved for these model proteins in less than 12 h, conversion of the specific lysine residue located in the surrogate peptide of ASP-P1 into homoarginine was not fully completed even with thorough optimization of conditions.

After 4-day guanidination and 10-h tryptic digestion, the remaining non-guanidinated peptide was about 3% of the guanidinated peptide in terms of their MRM peak intensities. No further purification was attempted to remove this non-guanidinated portion. The spin column purified mixture was directly used as the IS spiking solution. The residual non-guanidinated peptide is essentially an impurity in the IS, contributing a response in the analyte surrogate peptide MRM channel which could interfere with quantitation. The amount of GP-IS added to each sample was reduced to a level where the interference is minimal. As shown in Figs. 4(A) and 4(B), spiking 10 µL of 1 mg/mL GP-IS into 200 µL of blank serum produces no detectable peak at the analyte surrogate peptide retention time, and the IS peak is suitable for quantitation.

Optimization of immunocapture

Immunocapture using capture antibody-conjugated beads has been accepted as a sample extraction/enrichment means in protein drug LC/MS/MS quantitation. The technique is typically multistep and a long process. To minimize sample preparation time and maximize enrichment efficiency, the process was extensively optimized in this study. The optimized conditions as described in the Experimental section enable the assay to be completed in one work day, compared to 2 or 3 days of sample preparation reported in the literature.[10,18–21] The assay also achieved a very low LLOQ of 10 ng/mL from a sample volume of 200 µL. It is especially worth mentioning two observations here. First, capture antibody-conjugated beads could be prepared ahead in bulk and stored at 4°C for future use. We have validated that these beads are stable at 4°C for at least 1 month. Second, it is commonly believed that the captured drug should be dissociated from the beads prior to digestion. However, in this study we found that digestion on the beads was feasible.

The recovery of the immunocapture process for guanidinated ASP-P1 (GP-IS) was determined to be roughly 20%, compared to greater than 72% for ASP-P1. This suggests that there may be reduced affinity of the GP-IS to the capture antibody due to guanidination modification of lysine residues. Consequently, the spiking amount of the GP-IS has to be optimized as discussed above to ensure the assay has sufficient IS signal for reliable quantitation without significant contribution (less than 20% of LLOQ) to the drug MRM channel. Other than this, the guanidination modification should have no significant impact on the process after the capture step.

Optimization of digestion

Optimization of the tryptic digestion time was performed on samples comprised of both ASP-P1and GP-IS in serum matrix after the immunocapture step. As shown in Fig. 5, digestion for 6 h at 37 °C was sufficient to completely digest native ASP-P1. Further increases in digestion time resulted in more background noise and reduced signal/noise ratio. However,

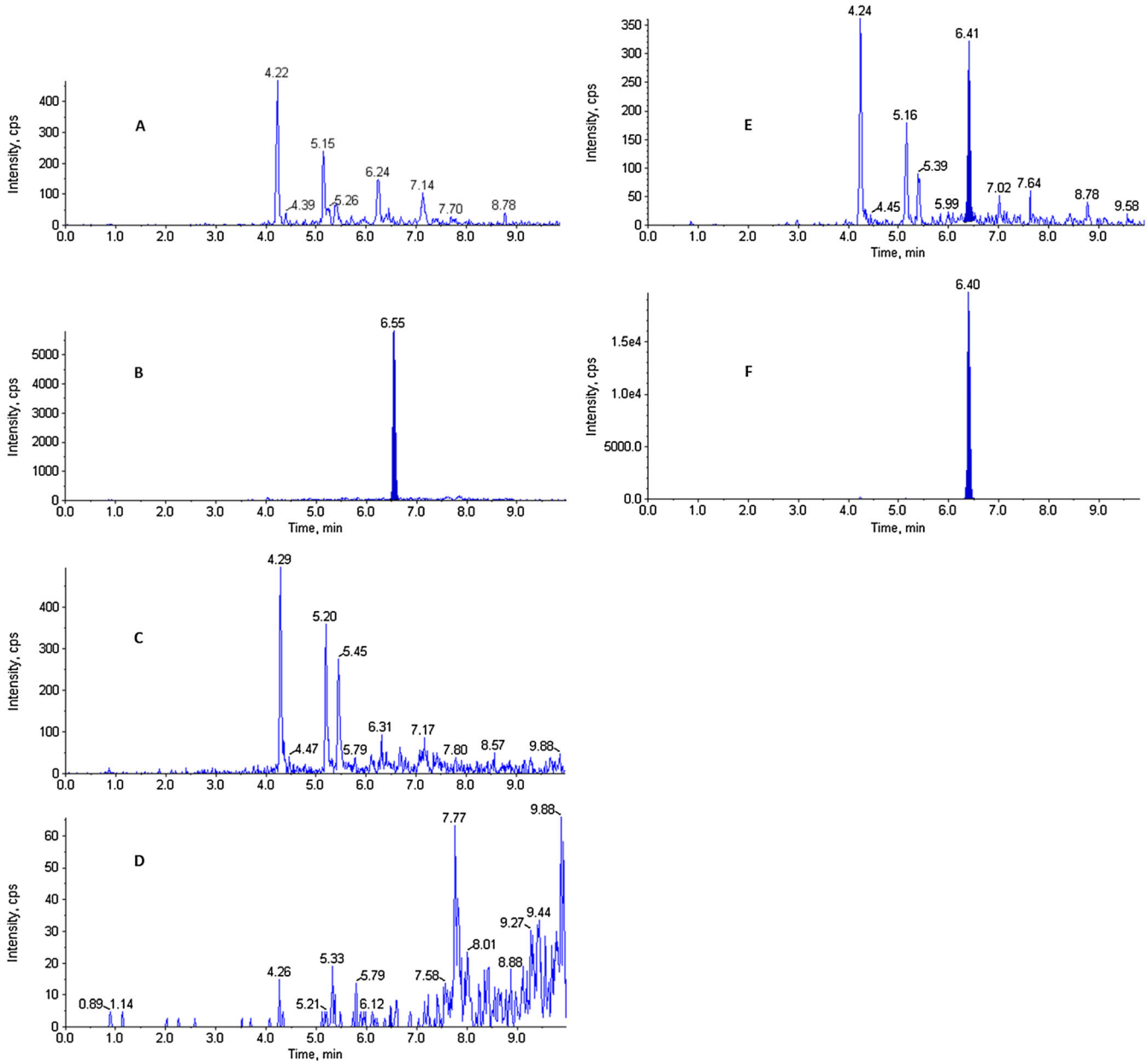

Figure 4. MRM chromatograms: (A) analyte channel and (B) IS channel of Control 0 (Blank + IS); (C) analyte channel and (D) IS channel of the pooled blank human serum; (E) analyte at LLOQ (10 ng/mL) and (F) analyte at ULOQ (1000 ng/mL). The peak highlighted in (B) at retention time of 6.55 min is the GP-ISP. No peak was observed at analyte retention time 6.4 min and IS retention time 6.5 min in blank human serum.

for GP-IS, the homoarginine peptide yield increased with digestion time up to 8 h. This indicates that the digestion efficiency of the homoarginine residue was low compared to the lysine residue. Further increases in digestion time resulted in slightly decreased yields of both guanidinated and non-guanidinated peptides. The yield ratio was highest and almost constant from 8 to 12 h; therefore, 10 h was selected as the optimal digestion time.

Method validation

Three validation batches were conducted on three different days using QCs and calibrators prepared from pooled human serum. As shown in Figs. 4(C) and 4(D), this matrix does not present quantifiable peaks in the analyte or IS MRM channels at their respective retention times. Assay selectivity was further validated using six individual lots of human serum. As shown in Fig. 4(E), the S/N was >5 at the 10 ng/mL LLOQ. The recovery of the assay was >72% as determined at the high, middle, and low QC levels. The matrix effect was investigated by preparing QCs at low and high QC levels in human serum from six individual donors. Compared to the nominal concentrations, the measured concentrations had acceptable accuracy (relative error <20%), indicating lot-to-lot matrix variability was acceptable. No measurable carryover, which was evaluated by placing a matrix blank after the highest calibration standard, was observed. The validation also demonstrated ASP-P1 in human serum had

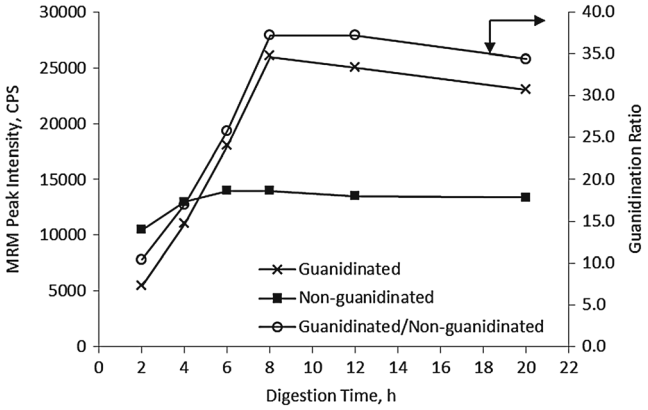

Figure 5. Effect of digestion time on the yield of tryptic peptides of guanidinated ASP-P1 and native ASP-P1. (×) Guanidinated surrogate peptide. (■), Remaining non-guanidinated surrogate peptide. (○) Ratio of guanidinated to remaining non-guanidinated surrogate peptide. Line × and Line ■ use left y-axis and Line 3 uses right y-axis. The data for Line ■ was amplified 20-fold to make the change more visible.

Table 2. Inter-day calibration curve (n = 3) statistics for ASP-P1 in human serum

Calibration standards	Nominal conc. (ng/mL)	Accuracy (%RE)	Precision (%CV)
Cal 1	10	2.5	3.7
Cal 2	20	–7.5	10.1
Cal 3	40	2.5	9.8
Cal 4	100	–8.7	9.1
Cal 5	200	–0.2	13.2
Cal 6	400	14.4	0.5
Cal 7	850	–3.0	6.8
Cal 8	1000	–2.5	15.2

Correlation coefficient (r): 0.9911–0.9940

at least 24 h of bench-top stability, six cycles of freeze/thaw stability, 27 h of reinjection stability at +10°C, and 49 h of processed sample stability at +10°C.

The statistical analysis results for the calibration curves and QCs are listed in Tables 2 and 3, respectively. Because this assay included immunocapture, we adopted the typical LBA validation acceptance criteria. Accuracy must be within ±20% (±25% at the LLOQ and ULOQ). Precision must not exceed 20% (25% at the LLOQ and ULOQ). Calibration curves must have a correlation coefficient (r) ≥0.99. All data presented in Tables 2 and 3 meet these acceptance criteria. Over three validation batches, only one out of 24 calibrators and one out of 72 QCs failed the acceptance criteria.

Additionally, a very similar assay had been developed and validated by using a SIL-peptide IS. In that validation, one out of five batches failed the acceptance criteria and was rejected. In the four successful batches, three out of 32 calibrators and 18 out of 90 QCs failed the acceptance criteria individually. The accuracy and precision data for standard curves and QCs are provided in the Supporting Information. Compared to these results, the current assay with GP-IS is more favorable in terms of assay accuracy, precision and ease of performance.

Table 3. Intra- and inter-day accuracy and precision of quality control sample data for ASP-P1 in human serum

Quality control samples	Nominal conc. (ng/mL)	Accuracy (%RE)	Precision (%CV)
Intra-day (6 replicates, 3 days)			
LLOQ	10	–13.5 to 6.3	7.0–15.0
LQC	30	–7.5 to –13.3	11.2–14.4
MQC	160	–10.5 to –1.9	4.6–12.4
HQC	800	–5.6 to 7.4	5.7–13.3
Inter-day (n = 3)			
LLOQ	10	–6.9	14.0
LQC	30	1.7	13.3
MQC	160	–6.2	9.2
HQC	800	0.1	10.8

CONCLUSIONS

As the rapid development of biotech pharmaceuticals increases, demands for capable bioanalytical assays are expected. While LBA is still dominating this area, LC/MS/MS has emerged as a promising approach because of its advantages as discussed earlier. However, application of LC/MS/MS is challenging in situations where assay sensitivity is a concern. It is difficult to have highly sensitive serum assays without comprehensive sample preparation such as immunocapture. To improve the accuracy and precision for this sample preparation approach, a proper internal standard is desired to off-set the variations from the entire sample analysis process. The GP-IS strategy proposed in this work has been proven for this purpose. Its advantages include: (1) better able to track assay variation than stable-isotope-labeled peptide-IS, or other protein analog-IS, since it can be spiked at the beginning of sample processing and has very similar functionality as the analyte; (2) easier preparation than stable-isotope-labeled protein-IS because it only involves a simple chemical reaction; (3) it provides greater flexibility for changing to alternate analyte surrogate peptides. If the selected analyte surrogate peptide has to be changed during method development, it is possible to select a different corresponding peptide as an IS (GP-ISP) from the digested GP-IS. One restriction when using the GP-IS approach is that the analyte surrogate peptide must have a lysine residue at the C-terminal, because only this type of peptide can be guanidinated to generate a GP-ISP. Overall, the strategy presented in this work allows LC/MS/MS to play an ever-increasing role in bioanalytical support for protein therapeutics.

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