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A platform analytical quality by design (AQbD) approach for multiple UHPLC-UV and UHPLC–MS methods development for protein analysis

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ABSTRACT

A platform analytical quality by design approach for methods development is presented in this paper. This approach is not limited just to method development following the same logical analytical quality by design (AQbD) process, it is also exploited across a range of applications in methods development with commonality in equipment and procedures. As demonstrated by the development process of 3 methods, the systematic approach strategy offers a thorough understanding of the method scientific strength. The knowledge gained from the UHPLC-UV peptide mapping method can be easily transferred to the UHPLC–MS oxidation method and the UHPLC-UV C-terminal heterogeneity methods of the same protein. In addition, the platform AQbD method development strategy ensures method robustness is built in during development. In early phases, a good method can generate reliable data for product development allowing confident decision making. Methods generated following the AQbD approach have great potential for avoiding extensive post-approval analytical method change. While in the commercial phase, high quality data ensures timely data release, reduced regulatory risk, and lowered lab operational cost. Moreover, large, reliable database and knowledge gained during AQbD method development provide strong justifications during regulatory filling for the selection of important parameters or parameter change needs for method validation, and help to justify for removal of unnecessary tests used for product specifications.

1. Introduction

The quality by design (QbD) drug development concept has been increasingly appreciated and applied by the pharmaceutical and biopharmaceutical industry over the past 10 years following the overall guidance from International Conference on Harmonization (ICH) [1–4]. It is generally adopted by the industry as a common practice for understanding the manufacturing process control and drug product quality regardless of the regulatory submission preference. The analytical methods are critical elements in product development due to their roles in assisting with process development and product quality control. Poor analytical methods can lead to inaccurate results, resulting in misleading information that may be detrimental to the drug development program.

Quality by design application to analytical methods development has been well adopted by the pharma/biopharmaceutical analytical professionals, where one can find either extensive or partial implementation of the systematic approach presented as analytical QbD (AQbD) [5–15]. AQbD incorporates an overall consideration of scientific and regulatory knowledge, as well as quality control needs. While most of applications to-date have focused on employing design of experiments (DOEs) and statistical screening for method parameter operational spaces towards method robustness consideration [10–18], a systematic approach has also been advocated [5–8]. However, much more effort is still needed in order to perfect the AQbD procedures and propel the concept towards all methods from clinical to commercial and their lifecycle management [19,20].

1.1. Analytical QbD process

As many analytical chemists have experienced in the past, a traditional approach for method development is non-systematic and thus decisions are made based on results from sequential events of experiments. The disadvantage of this approach is that if any step is not properly designed, the method development effort often strays, leading to non-optimized methods. When the experimental results are not meaningful, additional investigations will be performed,

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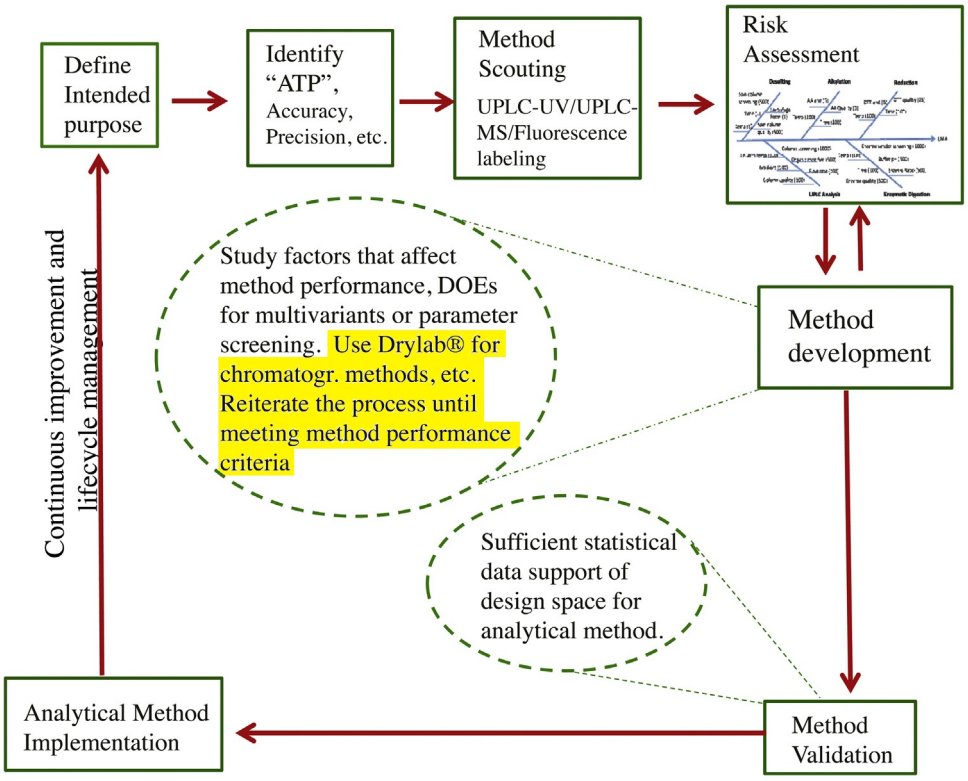

Fig. 1. A flow diagram illustrating the process of analytical quality by design (AQbD).

resulting in multiplication of lab work and lengthy development time. On the contrary, the initial time and effort that is put into the AQbD method development design is much more than the traditional manner. One has to have a significant amount of knowledge of the drug and the analytical technology in order to design the method development process correctly. Fig. 1 describes the overall AQbD process for the development of an analytical method.

The AQbD process is quite similar to the QbD process flow as described in ICH Q8 [1]. In AQbD, the starting point is to define the intended purpose of the method and analytical target profile (ATP), while in QbD, this is the step to define quality target product profile (QTPP). Each analytical method should have its intended purpose, such as whether it is to be used to support research, process and formulation development, or release and stability testing for clinical or marketed drugs, quantitative, qualitative, or limit test. The method ATP is determined combining the intended purpose and the ICH Q2 quality requirements [21], such as specificity, precision, accuracy, linearity, range, quantitation limit, and detection limit. Through method scouting experiments, the most suitable analytical technique is chosen, e.g., whether the method uses UHPLC-UV or UHPLC–MS or fluorescence labeling, and the method can meet the requirements of the intended purpose.

The determination of potential critical method attributes (CMAs) is an analogue of critical quality attributes (CQAs) of the drug. In analytical method development, method attributes are generally defined as steps that the method must have, such as sample preparation, sample introduction, sample analysis, and data analysis, while method parameters, similar to process parameters, are each detailed step that contribute to each method attribute. Finding key method parameters (KMPs) is through iterative risk assessment and lab studies following design of experiments (DOEs), just as the process of understanding key process parameters (KPPs). Finally, the method quality control strategy is established from understanding of the criticality of each method parameter of each method attribute. The controls can then be set as procedures or acceptance criteria in the analytical method, which is an analogue to the concept of control strategy (specification) for the drug.

To determine the criticality of the method attributes and key method parameters, a fishbone diagram displaying all method attributes and parameters is an effective tool. When each analytical step is examined, risk assessment can be performed based on the step's criticality, impact, and detectability, providing the analysts good understanding of the significance of each step and effort required for consequent lab investigations. Although risk assessment appears to be straightforward based on mathematical calculation which is (criticality*impact*detectability), correct assessment of each factor relies on sound science and empirical experience. In practice, it is not unusual that the scientists have no direct or not enough experience in certain methods for proper decision making and thus, several probing experiments can be performed to facilitate the risk assessment process.

Following risk assessment, it is desirable to conduct design of experiments (DOEs) for method development to screen for the initial method conditions. The parameter setting usually covers a wide range, and it can also screen multiple parameters simultaneously, such as the Placket Burman design. Although the data points in screening experiments may not be enough, the results provide direction for the next set of DOEs. In addition, the initial DOE also offers more knowledge for risk assessment. Some risks will become low risk, while others may become more severe. The double arrows in Fig. 1 suggest the iteration between risk assessment and experimental work through DOEs. The knowledge gained from these iterative procedures allows one to establish correct KMPs, or even reduce the number of KPMs, and narrow down scope and ranges of the final experimental designs. The final DOE design defines the method acceptable operable region. It should be noted here that not all method parameters can be studied using the DOE approach. Certain method parameters are suitable to be studied in ranges, while the chromatographic separation condition development needs assistance of special software, such as Drylab®,

Table 1
Intended purpose and ATPs of 3 methods for the same protein product.

Method	Intended Purpose	Analytical Target Profile
Peptide map	Identification method	Specificity, precision, robustness, and reproducibility
Heterogeneity	Quantitation method for terminal truncation variants determination	Specificity, selectivity, precision, accuracy, linearity, range, quantitation limit, robustness, and reproducibility.
Oxidation	Quantitation for oxidation	Specificity, selectivity, precision, accuracy, linearity, range, quantitation limit, robustness, and reproducibility.

ACD/AutoChrom, or other types. The work flow described in Fig. 1 has been applied as an effective approach to developing a robust QC method with reduced time for proteins (method parameter finding in about 1 month, fine tuning to make it a QC method for another 2 months) in our laboratory.

If the method is for early phase R&D, manufacturing process development, or formulation selection, the method development may stop when the method condition is found. However, if the method is for QC use, the QC aspects of the method, such as system suitability, sample handling care details, identification of critical reagents or materials, etc., should also be quickly evaluated during method development. This way, future complication can be avoided. At a glance, it may appear to be a significant amount of work during method development. However, with such a rigor, the assurance of desired method quality being built-in during development is warranted. Significantly, with the large amount of knowledge gained during development, method validation becomes less prone to unpredictable errors. As part of the ICH Q10 quality management requirements [3], a well-documented report will capture all of the method development effort, ensures strong scientific justification for future regulatory filling needs, as well as a successful path for method lifecycle management.

1.2. AQbD applied as a platform approach for methods development

AQbD can be realized with more power when it is a platform approach. In this paper, we not only demonstrated that each method development followed the same logical AQbD process, we also exploited the platform approach to methods with commonality in equipment and conditions across a range of applications. They can be a technology-based method application to multiple products, the same type of method for different products or different methods for one product with common procedures till the diverging point when product specific procedures must be applied. For example, the platform AQbD approach applied to different proteins with the same sample preparation steps for reduction, alkylation, desalting, and digestions; different UHPLC methods with the same column and buffers; or the same CE-SDS method applications to different proteins, etc. The AQbD approach requires the development team to think broadly to consider as many methods as possible during the steps of "defining intended purpose, ATP, and method scouting". Once the final method technology is determined, one should quickly screen different products for feasibility in order to ascertain the decision.

In adopting the platform approach, one must keep the "intended purpose" in mind at all time. Using multiple methods for one product as an example, Table 1 shows the intended purpose and analytical target profiles of each method.

For risk assessment, a fishbone diagram is developed for each method, as an illustration, Fig. 2 only shows the fishbone diagram for 2 out of the 3 methods that are mentioned in this paper. The risks are ranked by scores, 1 for low, 5 for medium, and 10 for high. An arbitrarily threshold is set, and any scores combining criticality*impact*detectability with scores greater than 100 are considered significant and should be investigated. Although the peptide mapping method by UHPLC-UV and oxidation quantitation method by UHPLC–MS share a number of common steps (reduction, alkylation, desalting, enzymatic digestion, and analysis), there are still differences (non-italic portions in Fig. 2B compared to Fig. 2A) that need to be evaluated. This platform AQbD approach for methods development has simplified multiple methods development effort and enhanced the quality of the analytical methods whether they are in the clinical or commercial phases.

2. Experimental section

2.1. Materials

Tris (hydroxymethyl) aminomethane (Tris-base), tris-hydrochloride (Tris-HCl), iodoacetic acid (IAA) were purchased from Sigma. Trifluoroacetic acid (1 mL ampoules), dithiothreitol (DTT), formic acid were purchased from Thermo Scientific. Endoproteinase Asp-N, sequencing grade, 2 μg/vial and guanidine hydrochloride were purchased from EMD Millipore. HPLC grade acetonitrile and water were purchased from Burdick and Jackson and Baxter, respectively. Synthesized peptide standards were made by New England Peptide, MA. Modified Trypsin, sequencing grade, 20 μg/vial was purchased from Promega. Peptide retention standard was purchase from Thermo Scientific. The protein used in this study is from Genzyme. Acquity UPLC® BEH300C18 columns, 300 Å and 130 Å pore size, 1.7 μm, 2.1 × 100 mm or 150 mm, Acuity UHPLC column in-line filter kit, containing a holderand 0.2 μm stainless steel filters, and 300 μL polypropylene autosampler vials with snap cap and pre-slit PTFE/Silicone septa were purchased from Waters Corporation. Bio-spin® P-6 Tris columns were purchased from Bio-Rad. Protein low-binding microcentrifuge tubes (0.5 mL and 2 mL) were purchased from Eppendorf.

2.2. Instruments

The peptide mapping and C-terminal heterogeneity sequence methods were performed using Waters Acquity UPLC® H-Class system with a photodiode array or a tunable ultra-violet detector. Peptide identification was performed using a Waters H-class UPLC coupled with a Thermo Velos Pro LTQ-Orbitrap mass spectrometer. Oxidation analysis was performed on a Waters H-Class UPLC coupled with a Waters SQD mass spectrometer.

2.3. Sample preparation for reduction, alkylation, and desalting

Protein is denatured by mixing the sample with a pH 8.5 buffer containing 6 M guanidine hydrochloride. Reduction of protein is carried out in the presence of 25 mM dithiothreitol (DTT) and incubation at 60 ± 5 °C for 30 ± 5 min. The reduced protein is then alkylated with 50 mM iodoacetic acid at ambient temperature (23 ± 3 °C) for 60 ± 10 min. Prior to enzymatic digestion, the reduced and alkylated protein samples are cleaned up by a desalting step using spin columns with appropriate molecular weight cut-off.

2.4Digestion and UHPLC condition for the peptide mapping, LC–MS oxidation, and the C-terminal heterogeneity methods

Tryptic digestion for both the peptide mapping and the LC–MS oxidation methods was performed following a vendor suggested digestion conditions for this enzyme with the digestion time studied for our specific protein, which is in a pH 7.6 buffer with a substrate-to-enzyme ratio of 25:1 for 4–4.5 h at 37 °C. Separation of

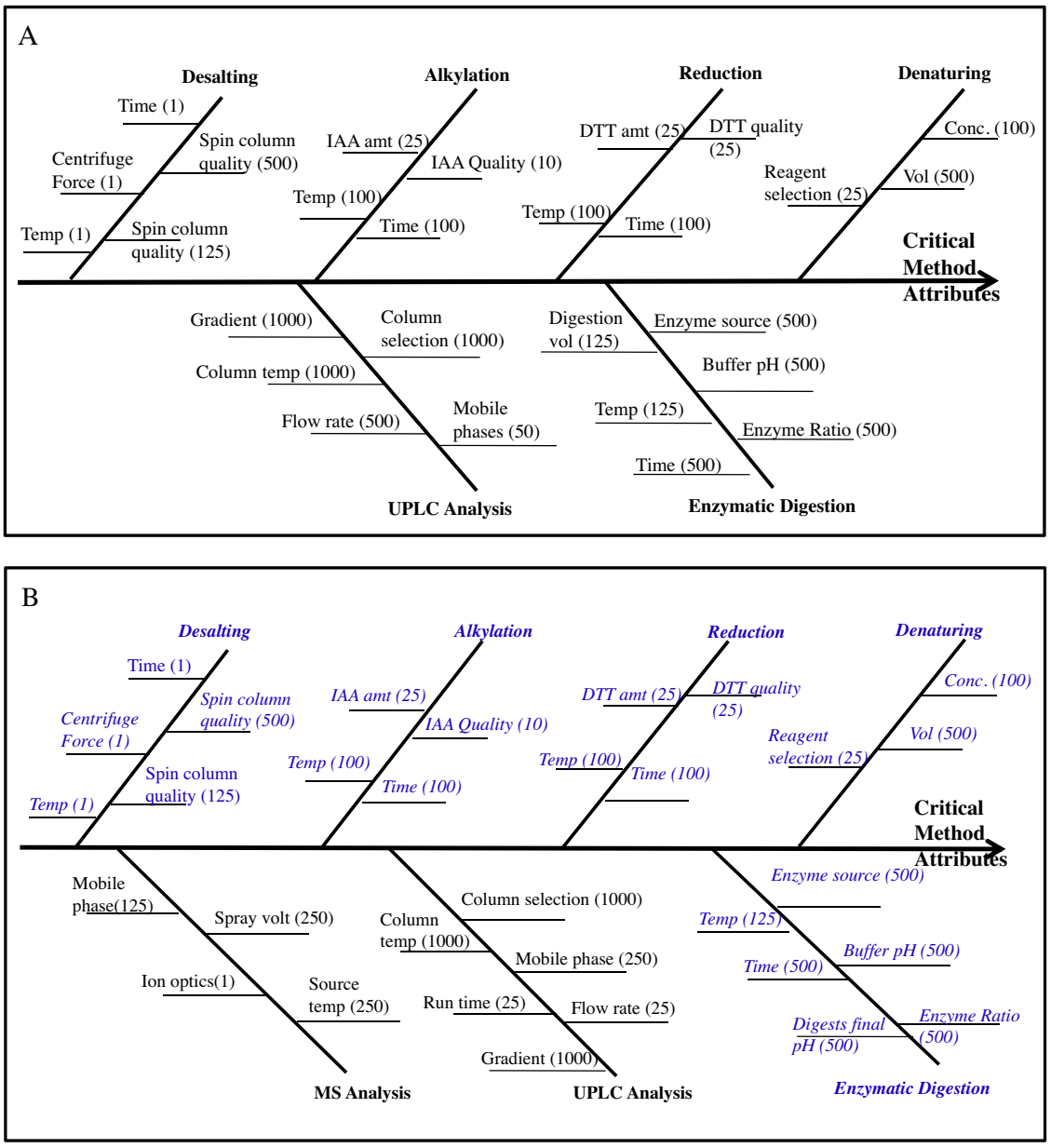

Fig. 2. Fishbone diagrams for 2 different methods, (A) peptide map by UHPLC-UV and (B) UHPLC-MS for oxidation analysis.

tryptic peptides was performed on UHPLC using a C18 UHPLC column. Water and acetonitrile containing trifluoroacetic acid were used as mobile phases for the peptide mapping method.

For the C-terminal heterogeneity method, also followed a vendor recommended digestion conditions for Asp-N with the digestion time studied for our specific protein: pH 8.0 ± 0.5, 37 ± 2 °C for 2–2.5 h. Separation of tryptic peptides was performed on a UHPLC using a C18 UHPLC column. Water and acetonitrile containing trifluoroacetic acid were used as mobile phases.

2.5. UHPLC–MS conditions for the oxidation method

The digestion method was the same as that in the peptide mapping method. The UHPLC–MS mobile phases were studied for the MS detection which contain: 0.1% formic acid (FA) and 0.009% heptafluorobutyric acid (HFBA) in water and acetonitrile for mobile phase A and B, respectively.

The mass spectrometer detection was performed using a Waters SQD instrument with the following conditions: capillary voltage: 3 KV, sources temperature: 120 °C, desolvation temperature: 250 °C, desolvation gas: 500 L/h, cone voltages at 70 V for singlet charge ions and 27 V for the doublet charged ions, respectively.

3. Results and discussion

3.1. Peptide mapping method development

Peptide mapping plays an important role for protein identification and characterization, with a leading purpose of confirming protein primary structure on a lot-to-lot comparison basis. Peptide mapping typically starts with enzymatic digestion followed by a reversed phase chromatographic separation (HPLC-UV or UHPLC-UV) [22–24].

Peptide mapping as an identification method is a qualitative method. Thus, the method ATP includes specificity, precision, reproducibility, and robustness. A peptide map procedure usually involves reduction, alkylation, desalting, enzymatic digestion, and then LC separation analysis. Each step has its critical role. For exam-

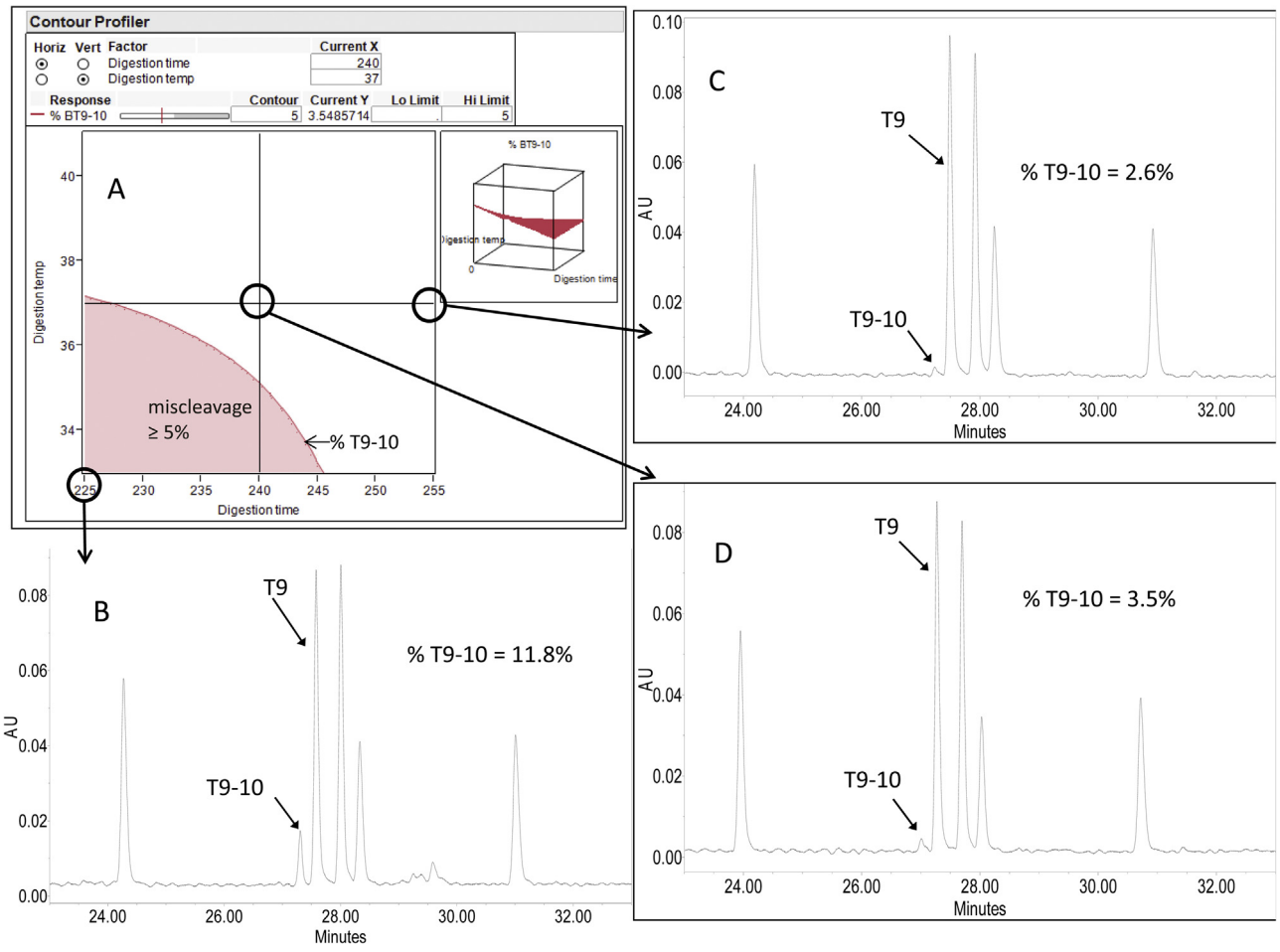

Fig. 3. (A) A contour map from the DOE study for enzymatic digestion time and temperature. The acceptable level for T9-10 is ≤ 5%. (B–D) Chromatograms at different spots on the contour map.

Table 2
Risk assessment and control strategies for 3 methods of the same protein.

CMA	Study Type	Score	Risk Management	Control Strategies
Denaturant concentration	DOE	100	Controllable	MODR
Denaturing volume	DOE	500	Controllable	MODR
Spin column selection	Screening study	125	Controllable	Not changeable until proven equivalent
Spin column quality	Range study	500	Controllable	Visual inspection of slurry level
Reduction time and temp	DOE	100 each	Controllable	MODR
Alkylation time and temp	DOE	100 each	Controllable	MODR
Enzyme source	Studies	500	Controllable	Not changeable until proven equivalent
Enzyme ratio	Range studies	500	Controllable	Fixed
Digestion buffer pH	Range studies	500	Controllable	Fixed
Enzymatic digestion temp	DOE	125	Controllable	MODR
Enzymatic digestion time	DOE	500	Controllable	MODR
UHPLC column selection	Studies	1000	Controllable	Not changeable until proven equivalent
UHPLC mobile phase and gradient	DOE	1000	Controllable	MODR

CMA: critical method attributes, MODR: method operable design region.

ple, the reduction is to denature the proteins, alkylation is to protect the free thiols on cysteines, and desalting is to remove salt which will interfere with optimal enzyme activity. The criticality and the associated risks of each step, including the UHPLC-UV separation and analysis, were evaluated as shown in Fig. 2A. The critical steps with scores higher than 100 were chosen for further studies as listed in Table 2.

Each factor was further studied. The risk for the spin column quality and the UHPLC column were high. The reason was due to the "non-detectability" of spin column failure if any existed until completion of sample analysis. Thus, either the vendor has a strict quality control procedure, or the user needs to make an effort to monitor the spin column performance during development to ensure quality consistency, same for the UHPLC column. Other high scores are for the UHPLC mobile phase and gradient. This indicates that a significant amount of effort should be placed in UHPLC method condition finding.

For those parameters that can be studied by a low and a high value (range study), they were studied with a few experiments. Certain parameters have interactions, and they were studied together using the design of experiments (DOEs) statistical software (JMP, SAS). DOEs (as shown in Table 3, full factorial as screening experi-

Table 3
Design of experiments (DOEs) for reduction and alkylation.

Pattern	Reduction time (min)	Reduction temperature (°C)	% T9-10
−+	25	65	0.00
−−	25	55	4.93
++	35	65	0.00
+−	35	55	0.00
0	30	60	1.94
0	30	60	2.80
0	30	60	3.28
Pattern	**Alkylation time (min)**	**Alkylation temperature (°C)**	**% T9-10**
++	70	26	3.52
−+	50	26	0.00
+−	70	20	2.89
0	60	23	1.94
−−	50	20	0.00
0	60	23	2.80
0	60	23	3.28

ments) were conducted for the reduction, alkylation, and digestion steps. For the peptide mapping methods, several critical method quality attributes (reduction, alkylation, digestion, analysis, etc.) can affect the final method quality. All of these factors can be reflected by an identified output as a response factor of DOE for the peptide mapping method, which is the amount of enzymatic miscleavage (not cleaved by the trypsin) of 2 peptides T9 and T10 (T9-10).

If the response factor (amount of peptide T9-10) is less than 3.5% in the white area of the contour map for reduction and alkylation steps (Supplementary material section, Fig. S1), any condition is acceptable. Likewise for the protein digestion, the acceptable enzymatic miscleavage amount is set at <5% for T9-10 (Fig. 3). Fig. 3A is the contour map for the digestion step, the expanded chromatograms reflect the enzymatic miscleavage amount at 3 conditions out of 7 (Fig. 3B–D). In order to find the robust method operable region, another detailed optimization experiment using surface response DOE (Table 4) was performed and the results showed an excellent correlation ($r^2 > 0.98$, Fig. 4A) between the actual and predicted data for the percentage of enzymatic miscleavage of peptide T9-10, suggesting that this model can be used for setting up the method operable region. Based on the study results and model prediction, the robust method operational design region was changed for the digestion time from the original 4 h ±15 min (dash line rectangle) to 4–4.5 h, (solid line rectangle, Fig. 4B) which is the minimum time required for delivering consistent digestion results, while keeping the digestion temperature unchanged.

As for the UHPLC method separation condition development, although full factorial DOEs were conducted to assist with the method robustness determination, it was found in practice that it was much more effective when Drylab® was used to find the separation condition. This will be illustrated in the section for C-terminal variants analysis method development. In addition to method robustness evaluation, it is worth mentioning here that, all of the methods that developed following AQbD in our lab are evaluated for method reproducibility (as defined in ATP) by using different instruments and different naïve users before the methods are transferred to the validation group.

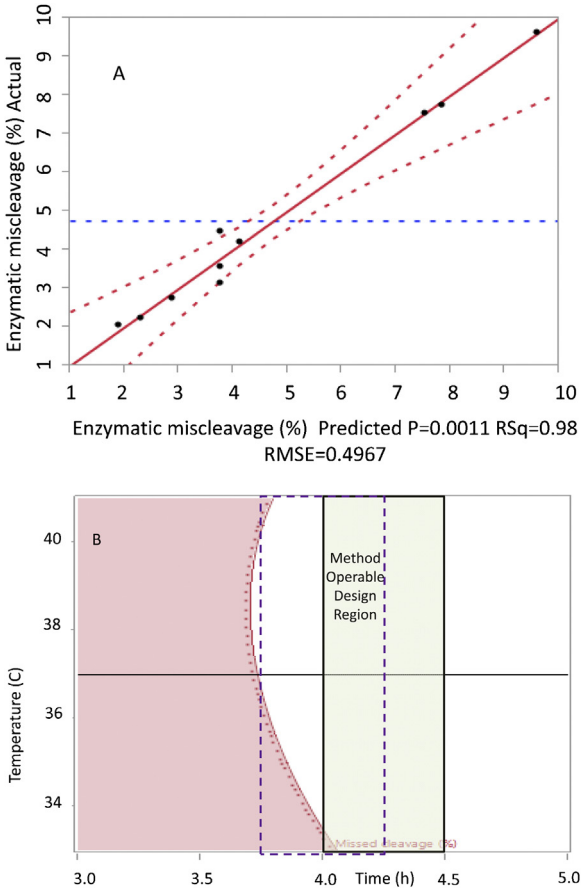

Fig. 4. Results from a surface response DOE study, (A) correlation of the predicted with the actual enzymatic miscleavage values and (B) a contour map from the DOE study for digestion time and temperature, the robust method operable design region is shifted from the dashed line rectangle to the solid line rectangle.

3.2. LC–MS oxidation quantitation method development

Protein oxidation is one of the major chemical degradation pathways for proteins, and the process involves covalent modifications of the amino acids with reactive oxygen species. The source of the reactive oxygen species can be from e.g., peroxide or light, and the side chains of cysteine (Cys), methionine (Met), tryptophan (Trp), histidine (His), and tyrosine (Tyr) residues are sensitive to oxidation [25–29]. However, Met residues are often considered as the most susceptible residue to oxidation forming sulfoxide Met[O]. Methionine sulfoxide has been readily detected in numerous pharmaceutical proteins, whether they are caused by H_2O_2, light, t-butyl hydroperoxide (BHP), and peroxodisulfate. Under extreme condition, Met oxidation forms Met sulfone [26].

For the same protein mentioned in the peptide map section, the amount of methionine oxidation needs to be quantitatively determined, and this can be done by either LC-UV or LC–MS, depending on the intended purpose of the method. Features such as method

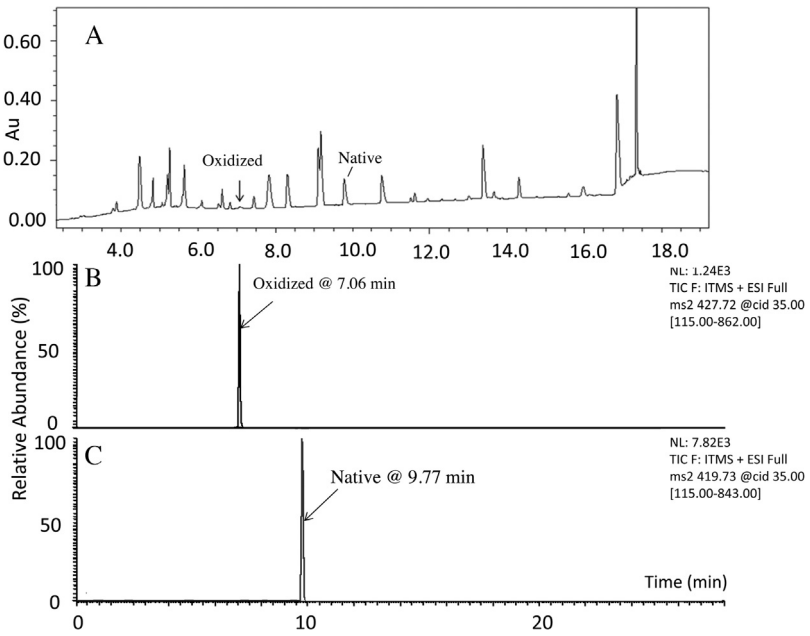

Fig. 5. A. A UV chromatogram of a protein digestion. B-C. LC–MS selected ion monitoring chromatograms of an oxidized peptide and its native peptide, respectively.

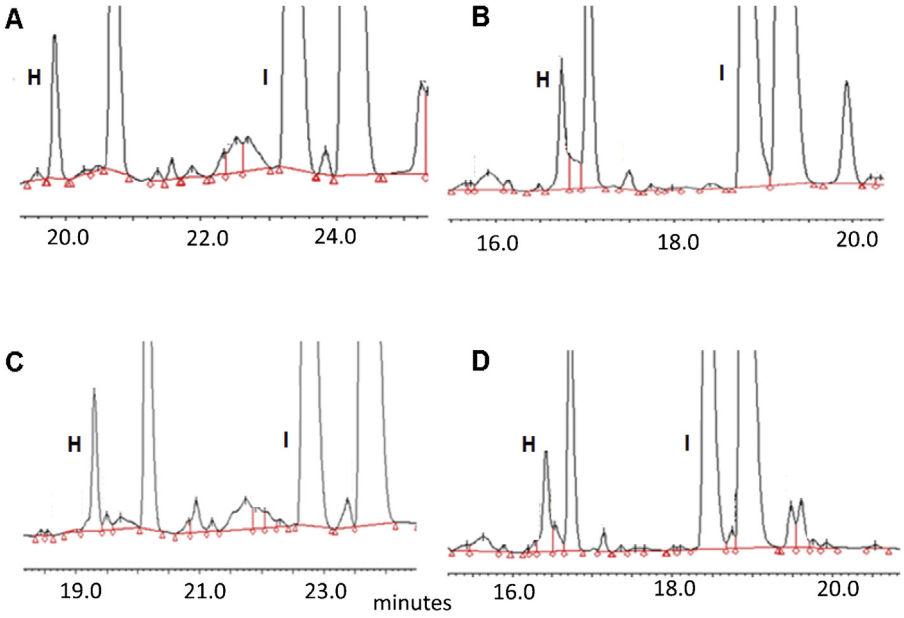

Fig. 6. Expanded chromatograms with peaks H and I from the C-terminal heterogeneity assay. All 4HPLC conditions contain 0.09%TFA in mobile phases A (water) and B (acetonitrile). (A) Column temp: 38 °C, ending mobile phase B = 23.7%, (B) column temp: 38 °C, ending mobile phase B = 26.3%, (C) column temp: 42 °C, ending mobile phase B = 23.7% and (D) column temp: 42 °C, ending mobile phase B = 26.3%.

Table 4
A surface response DOE for peptide mapping digestion time and temperature.

Pattern	Temp (°C)	Time (h)	Response Factor (% miscleavage)
–	33	3	10.33
a0	33	4	17.91
–+	33	5	2.42
0a	37	3	8.33
0	37	4	3.40
0	37	4	4.82
0	37	4	3.84
0A	37	5	2.21
+–	41	3	8.10
A0	41	4	4.54
++	41	5	2.98

sensitivity, robustness, ease of operation, and cost were considered. It was found that the quantitation limit (QL) of the UHPLC-UV method was ≥2% (relative amount to the unoxidized methionine). Thus, the UV detector is not sensitive enough, given the oxidation amount in the test article is <3%. A LC–MS method using a single quadrupole mass spectrometer was selected, since it can offer up to 10 times more sensitivity for oxidation determination as well as identification of any extraneous peaks simultaneously (Fig. 5.

Once the method was selected, risk assessment was performed (fishbone diagram in Fig. 2B). The platform approach suggested that there was no need to change the reduction, alkylation, desalting, and digestion steps (Italic in Fig. 2A and B) for the two methods and thus, no development work was needed for the sample preparation step for the LC–MS method. However, Met is very sen-

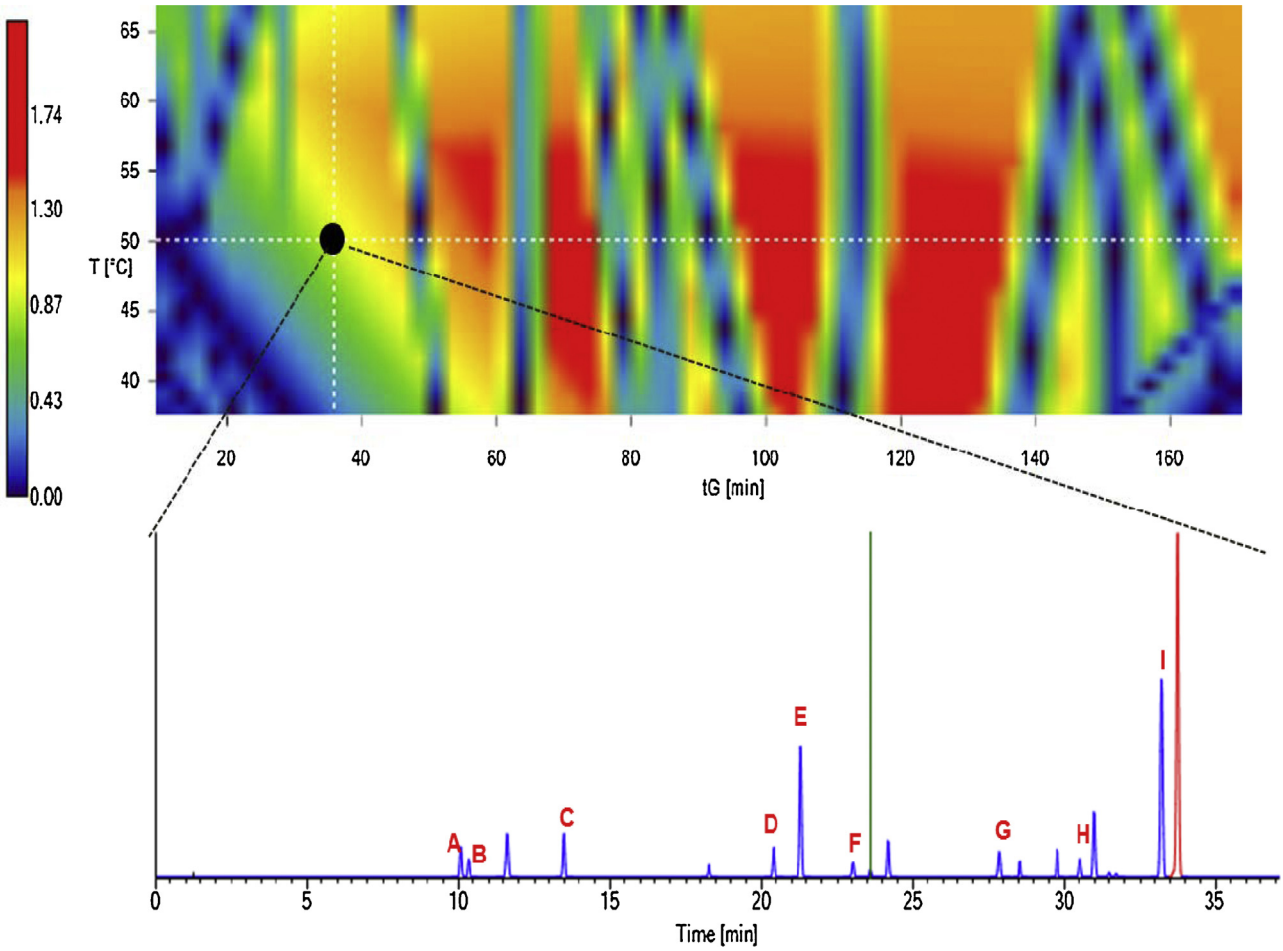

Fig. 7. A heat map generated with the Drylab® software which shows time vs resolution relationship. The bottom figure is a simulated chromatogram at a spot marked with a round dot on the top.

sitive to oxidation in general, many sources can introduce artifacts resulting in increased level of oxidation [28,29]. The LC–MS method was carefully studied and the ion source was tuned using a synthetic peptide to reduce the oxidative artifact to a non-detectable level. Some of the parameters were studied in ranges while the desolvation gas flow rate and temperature were evaluated using a full factorial study design. The final decision for MS conditions was made based on the overall consideration of instrument performance (DOE contour map) and manufacturer recommendation (data not shown).

Relatively speaking, the LC–MS method for oxidation determination was less complicated compared to the peptide mapping method, since the focus was only on 2 peptides. However, accurate quantitative analysis usually requires well resolved target peptides in order to minimize ion suppression effect during MS analysis. TFA has been commonly used as an ion pairing reagent for peptide separation in order to achieve high resolution. However, TFA causes ion suppression in MS analysis which compromises peptide signal sensitivity. HFBA is a good replacement for TFA as it is a stronger ion pairing reagent than TFA, and thus much less amount can be used. While using less HFBA creates less ion suppression, HFBA increases hydrophobic interaction leading to longer retention time of the molecule. For this LC–MS method, formic acid (FA) was also used. Thus, it was necessary to find out the optimal proportion of the 2 acids. A full factorial DOE was conducted with the goal of achieving $\geq$1.5 resolution as the response factor. The study results not only provided solution, it also showed that the separation resolution was extremely sensitive to the amount of HFBA (sorted parameter estimates section) as shown in Supplementary material section, Fig. S2. These outputs were statistically significant which means that problems related to the inaccurate addition of HFBA is likely to reoccur in future experiments. The sample preparation procedures of the method were thus carefully designed to ensure that HFBA amount can be delivered exactly using a gas tight syringe to ensure consistency over time. With the correct mobile phase and gradient, the final method meets the acceptance criteria as a quantitative method per ICH guidance. Using samples spiked with different levels of the oxidative peptide in the range of 0.8–20%, the method quantitation limit (QL) at 0.8% was achieved.

3.3. C-Terminal heterogeneity method development

Cleavage of residues at the terminals of the protein sequence during protein expression is one of post-translational modification events which may influence the biological activity of proteins. Using the same protein mentioned above, we also developed a C-terminal heterogeneity method which focuses on only the C-terminal of the protein primary structure. Based on the sequence, selection of a proper enzyme is critical. Trypsin would generate too many small pieces, while endoproteinase Asp-N, which specifically cleaves peptide bonds from N-terminus at both glutamic (E) and aspartic acid (D) residues, was appropriated as it gives proper length of peptides for UV detection.

Again, a fishbone diagram was used to assist with the determination of method parameter priority (data not shown). The prior steps to enzymatic digestion from the peptide mapping method were

Table 5
Comparison of resolutions of peak H and peak I before and after DryLab® optimization for the UHPLC method conditions.

	Initial Method (Empirical method development)		Improved Method (DryLab® assisted method development)	
Peak	front resolution	back resolution	front resolution	back resolution
Peptide H	0.88	3.52	2.66	3.52
Peptide I	0.72	1.89	1.24	3.45

shared, such as reduction, alkylation, and desalting. The focus of the C-terminal heterogeneity method development was immediately onto the enzymatic digestion condition which includes enzyme to substrate ratio, digestion time and temperature with reference to the vendor suggestions. However, the real challenge for this method development came from finding a robust chromatographic separation condition. The reason was that among the overall more than 40 peaks, only 9 of them were needed for quantitation, and they had to be reasonably resolved from the background peaks. The initial chromatographic method was developed based on empirical chromatography experience. Although the 9 peaks appeared to be well resolved from one another, during robustness study with statistical DOEs, it was found that 2 peaks were moving into adjacent peaks which would potentially cause method inaccuracy (peaks H and I in Fig. 6). The method was considered not robust. For such a difficult case, a chromatographic method development software Drylab® was employed.

The Drylab® software also indicated that the originally used column could not give acceptable separation. Thus, a similar column but with longer length (15 cm as opposed 10 cm) and smaller particle pore size (130 Å as opposed to 300 Å) was used. Simulation results from the initial 4 experimental runs while varying both temperature and gradient time generated a "heat map" as shown in Fig. 7, which indicated quality (good vs bad) of the chromatographic runs with respect to critical resolution, temperature, and gradient time. For final method selection, it was important to make a comprehensive decision for gradient time, column temperature, and resolution. As shown in Fig. 7, the selected gradient time was 35 min which was not too long for a complex UHPLC method. The column temperature was set at 52 °C since the solvent is not too corrosive and thus, the column life was not impacted significantly at this temperature. At the selected "set point" on the heat map, the target peak resolutions were sufficient, allowing the final method to meet acceptance criteria for accuracy (recovery of >95%), precision, linearity, quantitation limit, reproducibility, and robustness during method assessment. The UHPLC method condition predicted from the software was performed experimentally, and the results matched the theoretical prediction (data not shown). Compared with initial chromatographic method, resolution for peaks H and I in Fig. 7 were greatly improved using the DryLab® optimized method (results are shown in Table 5). When the method parameters were varied in a full factorial mode with a DOE design, such as column temp, mobile phase B ending percentage, and TFA amount in mobile phases A and B, the response factor "% each peak area from the C-terminal" had RSDs less than 3.6% which is considered low for a trace level quantitative method. Importantly, these variable parameters were changed together, which would rarely occur in real analysis, further supporting that the method was robust.

Using the Drylab® simulation, the cost saving was tremendous. It has shortened the method development time from the typical 1–3 months to about 1 week predictably. Importantly, the unknown factors in chromatography become more predictable. The direct cost saving involves labor, consumable, and instrument time. The lateral benefits are even more significant, since the productivity of scientists can be increased with multiple factors.

4. Developing analytical methods using AQbD through product life cycle management

Application of AQbD infuses rigorous science into analytical methods. As demonstrated above in this paper, a platform AQbD approach is an extremely effective way to improve method development efficiency, method quality, and reduce operation cost. Analytical methods have life cycles, going from clinical to commercial phase when process and formulation change. In addition, requirements for methods vary, and the product specifications usually become tighter. Thus, the analytical methods need to be assessed and changed accordingly in order to remain suitable for use along the continuum of drug development path [19,20]. Moreover, when the analytical methods are validated and implemented for routine use, issues that one does not foresee during method development will show up. The methods should be assessed for the frequency of out-of-specification and out-of-trend data, data accuracy and variability, failure rate, easiness of method execution, and cost of operation. These details guide the effort for the next generation method improvement. Although method development strategy as depicted in Fig. 1 applies to any size of molecules and any dosage forms of drugs, small molecules vs large proteins, solid vs parenteral dosage forms, detailed differences in scientific knowledge and specific regulatory requirements should be emphasized.

5. Conclusion

A platform AQbD approach for analytical methods development is presented in this paper. As demonstrated by the development process of 3 methods, the systematic approach strategy offers a thorough understanding of the method scientific strength, risk, and control strategy. This approach will greatly reduce the method development time and cost. The use of statistical or simulation software offers unbiased experimental designs and data output which guides correct decision making. With the robustness of the methods being built-in during method development, methods will be used with reduced error rate. Accurate data will be generated over time and this will help to build solid database for product life cycle management. The large knowledge base gained during AQbD method development supports the selection of important parameters during method validation, or future needs for different parameters to be validated, with a strong justification during regulatory filling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.03.031.

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