



Mass balance analysis for therapeutic peptides: Case studies, applications, and perspectives

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ABSTRACT

The concept of mass balance is discussed as it pertains to the pharmaceutical development of therapeutic peptides. Case studies are presented demonstrating how to perform a mass balance assessment on solid drug substance and solution drug product, and the role of mass balance in the context of the overall product control strategy is discussed. Utilizing mass balance as a specification test where the result is calculated from other critical quality attribute tests, each with their own specification, offers little value as a formalized quality acceptance criterion and may create more deviations, non-value added investigations, and potential batch failures. While useful in characterizing the performance of analytical methods and as part of a rigorous understanding of the manufacturing process and control strategy development, mass balance should not be required as a specification control and should instead be demonstrated during method development and through well-designed forced degradation experiments. Analytical method variability is discussed in relation to the analytical target profile, and the overall impact of sources of variability on the mass balance calculation is described in support of this position.

1. Introduction

Peptide- and polypeptide-based drugs represent an important class of therapeutics, with 26 new peptide and polypeptide drugs having been approved by the US FDA between 2016 and 2022 [1]. An important element of the control strategy for peptides and polypeptides includes the assessment of mass balance, which is a concept with many applications in the field of pharmaceutical development [2,3]. For example, mass balance assessments have proven useful during the development of manufacturing processes for both active pharmaceutical ingredients [4] and drug products [5,6]. Mass balance also plays an important role in the area of pharmaceutical analysis. For example, employing a mass balance approach has proven to be a robust method for establishing the defined potency of reference materials [7,8], including for synthetic peptides [9]. Additionally, mass balance is particularly useful in ensuring that analytical methods are suitable for their intended purpose [10]. In this regard, mass balance assessments facilitate addressing two fundamental questions related to the suitability of analytical methods within the context of the overall control strategy of pharmaceutical drug substances and drug products:

1. Across all methods used for release testing, is the entirety of the mass (including impurities) of drug substance detected and accounted for?
2. Do the stability-indicating purity method(s) demonstrate mass balance when comparing the decrease in assay to the increase in total impurities observed during stability studies?

For Question 1, the assessment addresses whether all components of the drug substance (e.g., active ingredient, impurities, water, solvents, ionic components, etc.) are accounted for with the suite of methods utilized to analyze the drug substance at the release stage. Mass balance for peptides and polypeptides is particularly important because the drug substance is typically isolated as a precipitated or lyophilized amorphous solid. These amorphous solids may retain impurities such as residual solvents, counterions, and peptide-related impurities. Additionally, peptide and polypeptide drug substances are generally hygroscopic. If mass balance is not demonstrated, it is possible that certain component(s) within the drug substance are unaccounted for, and additional methods, or improvements to the current methods, may be needed to ensure a robust overall control strategy. Likewise, for Question 2, the assessment addresses whether all degradation products are accurately accounted for by the stability-indicating methods. A lack

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of mass balance could indicate that degradation products are either undetected, or that if detected, the degradation products are not accurately quantitated. For both assessments, consideration must be given to the variability of the method(s) relative to the individual specification within the scope of the analysis [11].

Of note, it has been suggested that mass balance be implemented as a specification test performed at batch release for synthetic peptide and polypeptide drug substances [12] to ensure that there are no significant quantities of impurities not detected by the analytical methods. Draft regulatory guidance from the European Medicines Agency lists mass balance as an attribute to be included in the specification for drug substances [13]. However, USP <1503> Quality Attributes of Synthetic Peptide Drug Substances does not indicate that this calculated attribute should be included as a specification (routine quality control) test, but is listed as an approach to determine net peptide content (e.g., for a quantitative reference standard) [14]. To the best of our knowledge, peptides and polypeptides are the only therapeutic modality for which there is a recommendation to include a mass balance specification as a release test for each batch of drug substance. Thus, mass balance is not a common method employed for quality control testing of pharmaceutical products. Our position is that mass balance assessments should be conducted during product development as part of a rigorous understanding of the manufacturing process, control strategy, and analytical methods. Once mass balance has been demonstrated during product development, a mass balance calculation (that solely relies upon the summation of results of other critical quality attributes) is not required as a QC specification test for every batch of drug substance.

Here, case studies are presented to illustrate how mass balance can be incorporated into the pharmaceutical development process for synthetic peptides and polypeptides such that confidence in the analytical methods and overall product control strategy is established during the development phase, preventing the need for a mass balance QC specification test for routine batch release and stability studies. Likewise, case studies are presented demonstrating how mass balance can be used to demonstrate the suitability of stability-indicating purity methods for peptide and polypeptide therapeutics. The use of a mass balance assessment for understanding the UV response factor of degradation products is also discussed.

2. Materials and methods

2.1. RP-HPLC method for assay and total impurities

Assay and total impurities were determined via a gradient reversed-phase HPLC method with UV detection at 214 nm. The method employed a C18 stationary phase, 100-Å core-shell, 2.6-μm particle size, 4.6 × 250 mm reversed-phase chromatography column (Phenomenex Inc., Torrance, CA, USA) and a 10-μL injection volume. With a flow rate of 1.2 mL/min, the mobile phase gradient ramped from a mixture of 75 % mobile phase A (MPA; 0.1 % TFA in water) / 25 % mobile phase B (MPB; 0.1 % TFA in acetonitrile) to 10 % MPA / 90 % MPB over 54 minutes, followed by re-equilibration to the starting gradient conditions over 4 minutes. The column was maintained at 60°C during the run. Assay (% wt/wt) was determined by comparison of the main peak area in the samples with the corresponding peak area in an external reference standard of defined potency prepared at a single level at the target nominal sample concentration. Impurities and related substances were determined as a peak area percent (% a/a) to the total area of all peaks integrated in the chromatogram. The method was validated according to ICH Q2 guidelines [15] for accuracy, precision, specificity, limit of detection, limit of quantitation, linearity, and range. The procedure was demonstrated to be stability-indicating by its ability to resolve known impurities and degradation products from the active ingredient. For impurity integration, a reporting threshold of 0.1 % was applied, as described in Ph. Eur. 2034, Substances for Pharmaceutical Use [16].

2.2. SEC method for high molecular weight species

To quantitate high molecular weight species (HMWS), a size exclusion chromatography (SEC) method was employed. The SEC method was an isocratic HPLC method (0.5 mL/min flow rate) with UV detection at 214 nm and was designed to determine the amount of HMWS relative to the monomeric active ingredient. The method employed a 125-Å, 3.5-μm particle size, 7.8 × 300 mm size exclusion chromatography column (Waters Corp., Milford, MA, USA). The mobile phase consisted of 0.05 % TFA in 50/50 acetonitrile/water. The injection volume was 5 μL. The HMWS were determined as a peak area percent to the total area of all peaks integrated in the chromatogram. The method was validated according to ICH Q2 guidelines [15] for accuracy, precision, specificity, limit of detection, limit of quantitation, linearity, and range. The procedure was demonstrated to be stability-indicating by its ability to resolve known high molecular weight degradation products from the active ingredient.

2.3. GC method for residual solvents

Based on the manufacturing process, residual solvents tested in the drug substance were acetonitrile, isopropyl alcohol, and *tert*-butyl-methyl ether. The headspace gas chromatography method employed a 6 % polycyanopropylphenylsiloxane-94 % polydimethylsiloxane, 30 m × 0.32 mm inner diameter, 1.8-μm film thickness gas chromatography column (Agilent Technologies, Santa Clara, CA, USA) with helium as carrier gas (2.1 mL/min flow rate) and a flame ionization detector. The headspace sampler was maintained at 85°C, the injection loop (1 mL) at 95°C, and the transfer line at 130°C. A 10:1 split ratio injection was utilized. The thermal gradient started at 45°C with a 5 min hold, followed by a ramp to 175°C at 10 °C/min, and a final hold for 1 min at 175°C. Each residual solvent was determined (% wt/wt) in the drug substance by the comparison of the peak area in the sample to a calibration curve generated for each solvent with external standards. The method was validated according to ICH Q2 guidelines [15] for accuracy, precision, specificity, limit of detection, limit of quantitation, linearity, and range.

2.4. Karl Fisher method for water determination

Water content in the drug substance (% wt/wt) was determined via a volumetric Karl Fisher (KF) titration based on the USP <921> compendial method. The method was validated for accuracy, precision, linearity, and range.

2.5. RP-HPLC method for TFA and acetate

Trifluoroacetate (TFA) and acetate were determined in the drug substance by a gradient reversed-phased HPLC method with UV detection at 204 nm. The method employed a polar endcapped C18 stationary phase (Hypersil Gold AQ), 3.5-μm particle size 150 × 4.6 mm chromatography column (ThermoFisher Scientific, Waltham, MA, USA). Mobile phase A consisted of 25 mM KH₂PO₄, pH 2.2 (adjusted with phosphoric acid, 85 %) and mobile phase B was 100 % acetonitrile. With a mobile phase flow rate of 0.5 mL/min, the gradient was held at 100 % mobile phase A for 10 min, stepped to 55 % mobile phase B over 0.1 min, then held at 55 % mobile phase B for 9.9 min prior to returning to the initial gradient conditions for 0.1 min. The column was maintained at 25°C and the injection volume was 30 μL. TFA and acetate were determined (% wt/wt) in the drug substance by comparison of the peak areas in the sample to the corresponding peak area in a single-point external reference standard with a known defined potency. The method was validated for specificity, linearity, LOD, LOQ, accuracy, precision, and range.

2.6. Ion chromatography method for ammonium

Residual ammonium in the drug substance was determined by ion chromatography with conductivity detection. The method employed a CS12A cation-exchange column, 8- μ m particle size, 2 \times 250 mm (ThermoFisher Scientific). The mobile phase was 5 mM methanesulfonic acid in water. The flow rate was 0.4 mL/min and the column was maintained at 40°C during analysis. With a 10- μ L injection volume, ammonium was determined (% wt/wt) in the drug substance by comparison of the peak area in the sample to a calibration curve generated for ammonium with external standards. The method was validated for specificity, linearity, LOD, LOQ, accuracy, precision, and range.

2.7. Statistical modeling

A simulation was performed to estimate how often mass balance results would be expected to fail limits of 95 % – 105 % for varying levels of bias and key method variabilities. For simplicity, only the assay and water methods were considered since they have the highest variabilities and the largest impact on mass balance assessments from an experimental perspective. Every combination of the following conditions was evaluated:

- True assay of 95 % and water content of 5 % to give a mass balance of 100 %
- True mass balance bias: 0 %, 1 %, and 2 %
- Assay method variability (σ_A): 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3.0 %, 3.5 %, and 4.0 %
- Water method variability (σ_W): 0.25 %, 0.50 %, 0.75 %, 1.00 %, 1.25 %, and 1.50 %

Mass balance results were then calculated using simulated assay and water results where the assay and water results were assumed to be normally distributed according to Eq. 1:

$$\text{Mass Balance Result} = \text{Assay} + \text{Water} - \text{Bias} \quad (1)$$

Where,

$$\text{Assay} \sim \text{Norm}(95, \sigma_A)$$

$$\text{Water} \sim \text{Norm}(5, \sigma_W)$$

Assay was assumed to follow a normal distribution with a mean of 95 % and standard deviation σ_A . Likewise, water was assumed to follow a normal distribution with a mean of 5 % and standard deviation σ_W . For each of the above combinations of scenarios, 10,000 mass balance results were simulated and the percent of results failing to meet limits of 95 % – 105 % was determined.

2.8. Forced degradation study

To understand the stability-indicating nature of the analytical methods and assess mass balance for a synthetic peptide across a range of relevant degradation conditions, a forced degradation study was designed consistent with the principles in ICH Q1A [17]. Specifically, forced degradation studies for synthetic peptide drug substance in the solid state were performed as shown in Table 1.

Forced degradation studies for drug substance in the solution state were performed by dissolving the solid peptide drug substance at target concentrations of 5 mg/mL and 30 mg/mL in either 50/50 acetonitrile/water (v/v %) or a matrix of 140 mM sodium chloride, 5 mM phosphate buffer at pH 7.0. Table 2 shows the stress conditions the samples were exposed to. The Cu^{2+} and Fe^{3+} stress conditions shown in Table 2 were generated with cupric chloride, dihydrate and ferric chloride, anhydrous (MP Biomedicals, Solon, OH, USA), respectively. pH 9.0 and pH 2.0 stress samples were prepared by pH adjustment with 1 N NaOH and 1 N

Table 1

Forced degradation conditions for solid state samples.

Stress Type	Stress Condition	Time Point (weeks)	
		0	8
Control	N/A (−20°C)		X
Thermal	40°C	See note	X
Thermal	60°C		X
Thermal/Humidity	40°C/75 %RH		X

Note: The control sample stored at −20°C serves as the initial time point for the stress samples

Table 2

Forced degradation conditions for solution samples.

Stress Type	Condition	Time point (weeks)		
		0	2	4
Control	5°C		–	X
Thermal	40°C		X	X
Thermal	60°C		X	–
Metal-catalyzed	10 ppm Cu^{2+} , 40°C	See note	X	–
Oxidative/metal	5 ppm H_2O_2 and 25 ppm Fe^{3+} , 40°C		–	X
Basic	pH 9.0, 40°C		X	–
Acidic	pH 2.0, 40°C		X	–

Note: The control sample stored at 5°C serves as the initial time point for the stress samples.

HCl (ThermoFisher Scientific), respectively. In addition to the conditions in Table 2, a smaller proof-of concept study was conducted to generate the results in Fig. 1 and Table 6 which included stressing solution-state material at 60°C for 1, 3 and 7 days.

3. Results and discussion

3.1. Mass balance background

The assessment of mass balance at release of final drug substance across all the factors that contribute to the overall mass of the final drug substance is discussed in Section 3.2. From a total impurities standpoint, assessment at drug substance release captures the process impurities that impact the mass balance of the final drug substance. The mass balance of stability-indicating methods when degradation does occur also must be assessed and is discussed in Section 3.3.

The goal of a mass balance assessment is to determine how closely the results of all components which contribute to the mass of the final drug substance sum to 100 % on a wt/wt% basis. It is widely acknowledged that the final result will rarely equal exactly 100 % due to a variety of factors related especially to variability in sample handling and typical analytical method variability [2]. Notably, peptide and polypeptide drug substances are typically considered hygroscopic [18] and may lose or gain water depending on the relative humidity of the environment to which they are exposed. The example included in this case study demonstrates that water is the second-largest contributor to the overall mass of drug substance (after the active ingredient itself, i.e., the drug substance “assay”). Therefore, changes in water content due to sample handling can impact the deviation of the final mass balance result (MBR) from 100 %. ICH guidelines state that “due consideration of the margin of analytical error” must be considered when assessing mass balance [17], and Baertschi et al. further elaborate on the role that analytical variability plays when assessing mass balance [2]. Moreover, Swietlow et al. state that when calculating mass balance for peptides and polypeptides as described above, “the result should approach 100 %” [12]. Therefore, while the goal is to assess how closely these components sum to 100 %, it is understood that there is likely to be some variability in the result. In terms of what defines acceptable mass balance for peptide and polypeptide therapeutics, very few numerical ranges are

provided in the pharmaceutical literature. One source suggests that achieving a result of $100\% \pm 5\%$ (thus, $95\% - 105\%$) can be considered to demonstrate mass balance for peptide drug substances [18]. Further analysis of potential sources of variability in a mass balance determination specific to peptide and polypeptide drug substances is discussed in Section 3.2.2.

The stage of development at which to conduct a mass balance assessment deserves careful consideration. On one hand, it is beneficial for the drug substance manufacturing process to be representative of the final commercial process to ensure the assessment is relevant. This consideration would guide teams to conduct the assessment later in the development process to avoid, for example, early-phase manufacturing processes and methods likely to change prior to commercialization. On the other hand, waiting too late in the development process removes valuable time needed to react in case a mass balance deficit is discovered, leading to investigations and potential modifications to the control strategy (e.g., new or revised analytical methods). A reasonable development phase to conduct a mass balance assessment is beginning with the drug substance batches manufactured for pivotal clinical trials and leading up to registration stability studies. The manufacturing process for these batches should closely resemble that which is eventually commercialized, and by this time the analytical methods will have matured from early-phase methods designed to enable FHD-stage clinical trials. This stage is early enough, however, to permit time for control strategy and method modifications prior to final method validation which typically occurs prior to registration stability studies. As an added advantage, larger pivotal trials likely require more batches / manufacturing campaigns than early-stage Phase 1 and Phase 2 trials which may be supplied with a relatively smaller number of unique batches. More batches increase the “n” across which mass balance can be assessed. Mass balance should continue to be assessed as development continues, incorporating key activities such as establishment of the commercial reference standard, final ICH analytical method validation, and any post-pivotal changes to the drug substance manufacturing process.

3.2. Case study 1: Mass balance assessment at drug substance release

For this case study, the synthetic peptide batches included in the assessment were tested by validated non-compendial methods (e.g., assay and total impurities by RP-HPLC and SEC) versus the primary reference standard where appropriate, verified compendial methods (e.g., water by Karl Fisher titration), or scientifically sound characterization methods (e.g., trifluoroacetic acid/trifluoroacetate, acetate, and ammonium) for attributes contributing to the mass of the drug substance, but deemed not to require a formal specification. Specific method conditions are included in Section 2, Materials and methods. Therefore, this assessment resulted in a suitable dataset from which to demonstrate mass balance for the drug substance. The categorical components that contribute to the mass balance of the drug substance at batch release are listed in Table 3.

Method development (including forced degradation studies) demonstrated that impurities detected by SEC (i.e., HMWS) were not detected by the RP-HPLC method. Likewise, other process-related potential impurities such as residual solvents (e.g., acetonitrile, isopropyl

alcohol and *tert*-butyl methyl ether) and ionic species (e.g., trifluoroacetic acid, acetate, and ammonium) are not detected by either the SEC or RP-HPLC method. This understanding is important so as to not “double count” components when assessing mass balance.

To illustrate real-world challenges with demonstrating mass balance, a common scenario where all tests are not performed in the same laboratory, or even the same company, can add further challenges with regards to sample handling and should be considered. In those cases, results may be generated on physically different samples at different points in time which may contribute to inconsistencies between mass balance measurements. Additional detail is provided below where different analytical measurements were performed in different laboratories for certain tests based on instrument availability and location. For peptide drug substance batches produced by Process 1, all analytical testing was performed by Lab 1. For drug substance batches produced by Process 2, the RP-HPLC, SEC and KF testing were performed by Lab 2 while the GC testing for residual solvents and IC testing for TFA, acetate, and ammonium were performed by Lab 3. Table 4 summarizes the testing labs and methods used to test each component contributing to the mass balance for peptide drug substance, as summarized in Table 3.

3.2.1. Mass balance calculation

It is important to recognize that the results reported for the components listed in Table 3 and Table 4 are not in consistent units across all tests and this difference must be accounted for in the calculation of mass balance. For example, assay, water, residual solvents, and residual ionic species are reported on a wt/wt% basis. In contrast, total impurities and HMWS are reported on an area% basis relative to all integrated peaks in the respective UV chromatograms. The goal of a mass balance assessment is to determine how closely the results of these species in total sum to 100% on a wt/wt% basis. Thus, the total impurities and HMWS must be adjusted to a wt/wt% basis which is achieved using the equation below. Specifically, the area% results for total impurities and HMWS are accounted for by dividing the assay result by $(1 - [\text{Total Impurities}/100] - [\text{HMWS}/100])$. An assumption related to this adjustment is that the impurities detected by the RP-HPLC and SEC methods have the same response factor (i.e., molar absorptivity) as the active ingredient. While a reasonable assumption for most peptides and polypeptides, the UV detection wavelength can impact this assumption, and it should be verified for individual molecules (see Section 3.3.1). This normalization is required because only peptide-related species (i.e., total impurities and HMWS) are measured by RP-HPLC and SEC. (Note that while a RP-HPLC method is used for TFA and acetate, these species are not detected or quantitated via the RP-HPLC or SEC methods used for total impurities or HMWS, respectively). Eq. 2 is used to determine the Mass Balance Result (MBR):

$$\text{MBR} = \frac{\text{Assay}}{1 - \frac{\text{Total Impurities}}{100} - \frac{\text{HMWS}}{100}} + \text{ACN} + \text{IPA} + \text{MTBE} + \text{Water} + \text{TFA} + \text{Acetate} + \text{Ammonium} \quad (2)$$

Results for components contributing to the mass of the drug substance, and the sum of the results for these components (i.e., MBR), is presented in Table 5. The results for Total Impurities and HMWS in Table 5 are presented as area %, while other components are reported as wt/wt%. Eq. 2 is used to calculate the MBR presented in Table 5 from the individual results presented in Table 5, thus accounting for whether attributes are reported in units of area % or wt/wt%.

3.2.2. Analysis of mass balance results

As shown in Table 5, all drug substance batches met the criteria discussed in Section 3.1 for mass balance; thus, mass balance has been demonstrated for this drug substance and the holistic control strategy should be considered appropriate. In the case where mass balance has been demonstrated in this manner (i.e., on representative batches with

Table 3

Categories of components contributing to the overall mass balance of the drug substance and the analytical procedures used to quantitate those components.

Category	Analytical Procedure
API and Peptide Related Substances	RP-HPLC SEC
Moisture Content	Karl Fisher Titration
Residual Solvents	Headspace GC
Residual ionic species	RP-HPLC Ion Chromatography

Table 4

Testing lab and analytical method used for each component contributing to overall mass balance of peptide drug substance.

Category	Components	Analytical Procedure	Testing Lab	
			Synthetic Process 1	Synthetic Process 2
Assay	API	RP-HPLC		
API and Peptide Related Substances	Total Impurities			
	High Molecular Weight Species	SEC		Lab 2
Moisture Content	Water	Karl Fisher Titration		
	Acetonitrile		Lab 1	
Residual Solvents	Isopropyl alcohol	Headspace GC		
	Methyl tert-butyl ether			
	Trifluoroacetic acid	RP-HPLC		Lab 3
Residual ionic species	Acetate	RP-HPLC		
	Ammonium	Ion Chromatography		

Table 5

Mass Balance Results (MBR) for representative batches of drug substance.

Attribute	Synthetic Process 1				Synthetic Process 2					
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch 10
Assay (%)	88.4	88.0	88.2	86.2	86.6	87.2	88.6	87.3	87.2	89.0
Total Impurities (%)	2.3	2.4	3.8	2.9	2.9	2.3	2.7	2.4	2.5	2.3
HMWS (%)	0.1	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1
ACN (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IPA (%)	0.0	0.0	0.1	0.0	0.3	0.0	0.1	0.0	0.2	0.2
MTBE (%)	0.0	0.0	0.1	0.0	0.7	0.0	0.0	0.1	0.5	0.5
Water (%)	7.4	7.4	6.4	7.6	5.7	5.1	6.0	6.6	6.0	6.2
TFA (%)	0.02	0.00	0.01	0.01	0.0	0.0	0.0	0.0	0.0	0.0
Acetate (%)	0.10	0.17	0.14	0.15	0.0	0.0	0.0	0.0	0.0	0.0
Ammonium (%)	0.59	0.60	0.46	0.53	0.2	0.4	0.4	0.4	0.4	0.4
MBR (%)	99	99	99	97	96	95	98	97	97	98

Note: Results below the limit of quantitation (LOQ) established during method validation are listed as 0.0 in this table. Unrounded results were used in the calculation of MBR reported in this table.

validated methods) it should be considered unnecessary to require a formalized mass balance specification for routine batches. In other words, the control strategy has been demonstrated to be adequate. The average MBR for the 10 batches is 97.4 % with a standard deviation of 1.3 %. However, the average for Synthetic Process 1 is 98.5 % with a standard deviation of 1.0 % and the average MBR for Synthetic Process 2 is 96.8 % with a 1.2 % standard deviation. While both results are acceptable, the lower average MBR for Synthetic Process 2 may be indicative of the above-mentioned challenges of executing mass balance assessments with data generated across different laboratories.

While within the established criteria for demonstrating mass balance, the individual and average results are consistently below 100 %. There are several reasons for observing mass balance below 100 %, including those discussed in Section 3.1 and below, which generally fall under the categories of method variability and sample handling. Notably, additional method-specific considerations (listed below by individual measurements that contribute to the MBR) explain why the MBR results are slightly below 100 %. The factors described below are within standard expectations of sample handling and method execution. Each of these factors would bias the MBR below 100 %, are additive, and when combined would cause the MBR to be less than 100 % for a given batch.

3.2.2.1. Water. As discussed in Section 3.1, peptide and polypeptide drug substances typically gain and lose water (and thus mass) as a function of the relative humidity of the environment in which they are handled. The moisture-sorption profile of the drug substance included in this case study has been thoroughly characterized and demonstrates that water content (and thus mass) changes as a function of the relative humidity at which the drug substance is equilibrated. For example, if equilibrated to 30 % RH, the water content of the drug substance could decrease by several percent (absolute) compared to the level of water targeted at isolation of the drug substance. If the same sample handled in

the same manner is used for both the RP-HPLC assay determination and KF water analysis, the RP-HPLC assay result would reflect the corresponding gain or loss in water. However, due to practical laboratory constraints, it is not always possible to use the exact same sample handled in the same manner for both KF and RP-HPLC analysis (e.g., different physical laboratories within a testing facility responsible for chromatography vs. KF measurements). Even if performed in the same laboratory, timing of the analytical tests may vary due to practical laboratory considerations such that the samples tested by RP-HPLC and KF may differ in water content.

3.2.2.2. Assay. The drug substance assay is determined by dissolving solid drug substance in diluent for analysis by the RP-HPLC method vs. external reference standard. If the drug substance was not fully in solution prior to HPLC analysis due to analyst-to-analyst sample preparation variability, the resulting assay result would be biased low.

3.2.2.3. Total impurities. The RP-HPLC impurities method applies a reporting threshold of 0.1 %, consistent with Ph. Eur. 2034, Substances for Pharmaceutical Use [16]. Individual impurities in the drug substance are not reported or included in the total impurities if below 0.1 %. Some batches may include multiple impurities below this threshold, causing the total impurities to be underrepresented in the MBR calculation of the drug substance. It is the experience of the authors that the total impurities below the Ph. Eur. reporting threshold of 0.1 % [16] could reasonably approach 0.8 % (internal unpublished data).

3.2.2.4. Residual solvents. Levels of solvents below the method LOQ would contribute to the mass of the drug substance but would not be counted in the MBR calculation since, as described above, results less than LOQ contribute a value of zero for that attribute into the MBR calculation, causing the total solvent level to be underrepresented.

3.2.2.5. Ionic species. Similar to residual solvents, levels of ionic species below the method LOQ would contribute to the mass of the drug substance but would not be counted in the MBR calculation since, as described above, results less than LOQ contribute a value of zero for that attribute into the MBR calculation, causing the total ionic species level to be underrepresented.

3.3. Case study 2: Mass balance assessment for degraded samples

Assessing mass balance at drug substance batch release demonstrates the ability of the analytical methods across the entire control strategy to capture all components contributing to the mass of the drug substance. From a RP-HPLC perspective, the assessment at drug substance batch release focuses primarily on process impurities. It is also important to assess mass balance for degraded samples, to ensure that the methods continue to demonstrate mass balance for degradation occurring on stability (i.e., when a drug substance degrades, does the increase in impurities correlate with the decrease in assay?). This type of assessment is particularly important in ensuring the suitability of the analytical methods for drug products where degradation is likely at the long-term and in-use conditions for solution-based peptide and polypeptide therapeutics. An example is presented here with a series of samples that had been stressed in drug product matrix at 60°C for up to 7 days. Samples from this study are suitable for assessing mass balance because they span the range of expected degradation observed in the drug product over the shelf-life. For example, the final total impurities (i.e., total of process and product related impurities/degradants) at the 7 day timepoint was approximately 17 % (see Table 6), which exceeds the proposed specification for total impurities in drug product at the end of shelf-life. As a result, these samples allow mass balance to be assessed over the full specification range of total impurities. Fig. 1 shows an overlay of the RP-HPLC chromatograms from the initial, 1 day, 3 day, and 7 day timepoints demonstrating the growth in individual and total impurities over the course of the study. The peaks that increase in Fig. 1 correspond to those that increase during stability testing at the long-term and accelerated conditions in the drug product. Therefore, these samples are representative of the degradation expected to occur on stability and represent a suitable data set from which to assess mass balance.

The following equation was employed to assess mass balance for degraded samples:

$$MBR = \frac{\text{Assay}_f + (\text{Impurities}_f - \text{Impurities}_i)}{\text{Assay}_i} \times 100 \quad (3)$$

Where,

MBR = mass balance result

Assay_f = assay result after stressing

Assay_i = initial assay result (prior to stressing)

Impurities_i = initial total impurities (prior to stressing)

Impurities_f = total impurities after stressing

Table 6 shows the correspondence between change in Assay (assay measured vs. external reference standard and reported as % of the label claim) and increase in total impurities, both measured by the RP-HPLC method, and an assessment of the MBR for these samples.

As shown in Table 6, the degradation was detected by the RP-HPLC

method by way of both the decrease in assay as measured vs. an external reference standard and the corresponding increase in total impurities (area%). Here the sample is in the solution state and % Label Claim is calculated by dividing the measured concentration in mg/mL by the target concentration, also in units of mg/mL. Thus, conversion to wt/wt %, as was performed for solid-state drug substance samples above, is not necessary in this case. The entirety of the change in assay from Initial (%) of the active ingredient is reflected by the detected change in total impurities from Initial (%). The MBR result for all samples is in good agreement with 100 %. Note that the discussion in Sections 3.1 and 3.2.2 related to method variability applies here as well. Results for HMWS from SEC analysis are not included; however, the contribution from HMWS for these samples was less than 1 % (data not shown), demonstrating that impurities detected by RP-HPLC account for the majority of the mass balance as the concentration of active ingredient decreased with degradation. Data from other methods are not applicable since these samples were in solution and the other components listed in Tables 3 and 4 are either not applicable to solution samples (e.g., water) or not stability indicating (e.g., solvents and ionic species). This analysis demonstrates that for these representative stability samples, there is no mass balance deficit [2]. Therefore, in addition to mass balance at drug substance batch release (process related impurities), mass balance has been demonstrated for chemical degradation across relevant stability samples via the stability-indicating analytical methods. Having demonstrated mass balance during product development, a formalized specification for mass balance for routine batches is not required.

With this case study in mind, mass balance was then analyzed across all samples from a stress stability study conducted for the product. As described by ICH Q1A, Stability Testing of New Drug Substances and Products, stress testing is utilized to identify the likely degradation products and understand the intrinsic stability of the molecule [17]. Further, data from stress testing demonstrates the stability-indicating nature of the analytical methods. During stress testing, samples are typically intentionally degraded to beyond the proposed specification for impurities. For this reason, stressed degradation samples represent an excellent dataset across which to calculate mass balance. Details related to the conditions used to generate the stress samples are provided in Section 2, Materials and methods. Results for all stress samples (n=12 solid state, n=25 in solution state at a peptide concentration of 5 mg/mL, and n=25 in solution state at a concentration of 30 mg/mL) are provided in Fig. 2, where mass balance is calculated with Eq. 3. The dashed horizontal lines represent the generally accepted range for demonstrating mass balance for peptides [18]. Two sets of error bars are presented for each result: the blue error bars represent one standard deviation, while the black error bars represent three standard deviations, i.e., a typical approach for establishing specifications.

As shown in Fig. 2, there is very good agreement between the change in assay for stressed samples and the increase in total impurities, as evidenced by mass balance results near 100 %. The average mass balance result across n=12 solid-state samples was 100.0 % with a standard deviation of 3.4 %. This result demonstrates that typical mass balance expectations have been satisfied; note that this assessment includes results from samples with degradation up to 23 % total impurities. Likewise, good agreement is also observed between the change in assay and increase in total impurities for samples stressed in the solution state, as

Table 6

Mass balance results for samples stressed at 60°C for 1, 3, and 7 days.

Sample	Assay (% Label Claim)	Total Impurities (%)	Change in Assay from Initial (%)	Change in Total Impurities from Initial (%)	MBR (%)
Initial	93.90	5.30	0	0	N/A
60°C 1 d	92.98	7.01	−0.92	+1.71	100.8
60°C 3 d	90.05	10.54	−3.85	+5.24	101.5
60°C 7 d	83.39	17.34	−10.51	+12.04	101.6

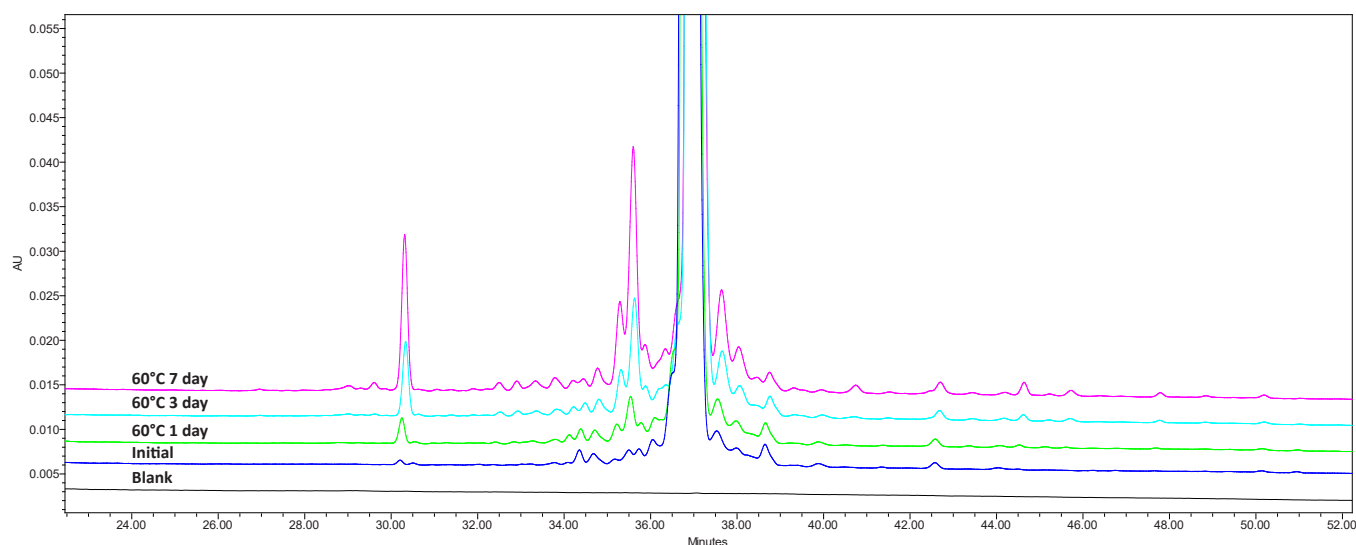


Fig. 1. Overlay of RP-HPLC chromatograms of peptide drug product solution stressed at 60°C for 1, 3, and 7 days.

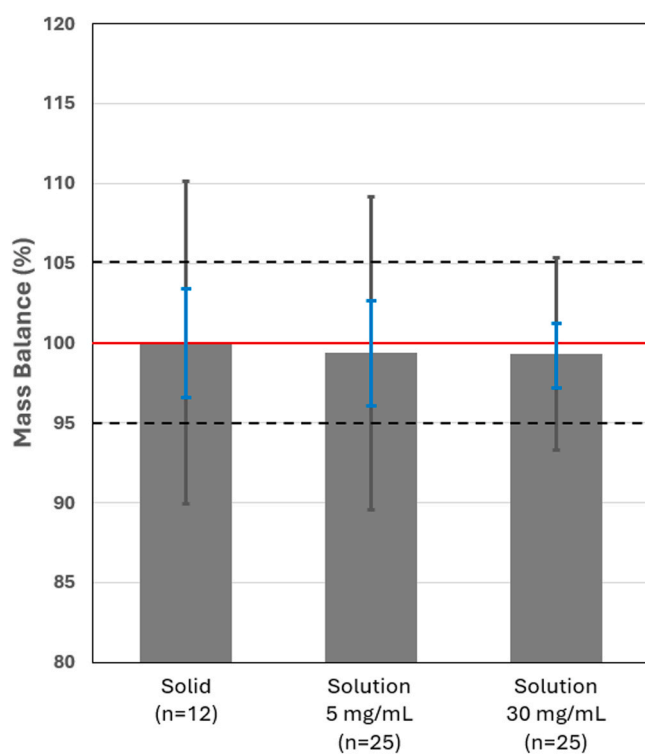


Fig. 2. Mass balance results for stress degradation samples for peptide in the solid state, solution state at 5 mg/mL, and solution state at 30 mg/mL. Error bars represent ± 1 (blue) and ± 3 (black) standard deviations.

evidenced by mass balance results that are also very close to 100 %. The average mass balance result across $n=50$ samples at both low and high concentration was 99.2 % with a standard deviation of 2.8 %. This result also demonstrates excellent mass balance and includes results from samples with degradation up to approximately 50 % total impurities representing a variety of potential degradation mechanisms. The mass balance results across stressed solid-state drug substance samples and stressed formulated drug product samples demonstrate the stability-indicating nature of the RP-HPLC method and that the change in assay is accounted for by the increase in impurities. For the purposes of analyzing mass balance results within a forced degradation study, the

± 1 standard deviation error bars (blue in Fig. 2) are useful for concluding that mass balance has been demonstrated. For example, the ± 1 standard deviation error bars are within the accepted range for demonstrating mass balance of 95 % - 105 % [18] and are in general agreement with the intermediate precision of the RP-HPLC method established during method validation. Error bars representing ± 3 standard deviations, which are typical for specification setting processes, are also shown in Fig. 2 (black error bars) and further discussed below in Section 3.4.

3.3.1. Application of mass balance for understanding UV response factor of impurities

A mass balance assessment can be beneficial in understanding if the UV response factor of impurities or degradation products is similar to that of the active ingredient. This approach leverages the assessment of mass balance analyses for demonstrating the stability-indicating performance of the assay and impurities analytical method(s) to understand UV response factors and potentially justify that there is no need to correct for UV response factors in an impurities method. Based on the expected degradation products for peptides and polypeptides, it is unlikely that a degradation product will have a UV response factor that differs meaningfully from the active ingredient (unless degraded completely to amino acid starting materials that possess very little chromophore) provided that the UV detection wavelength has been selected appropriately. For degradation products, a mass balance value significantly different from 100 %, outside the combined variability of the assay and impurities method(s), may indicate that some degradation products have differences in the relative response factors compared to the active ingredient. If degradation products have higher UV response than the active, the value will exceed 100 %, while mass balance levels below 100 % would indicate that degradation products have a lower UV response than the active. As shown in Fig. 2, there is very good agreement between the change in assay for stressed samples and the increase in total impurities, as evidenced by mass balance results within 95–105 %. The mass balance results across the stressed samples demonstrate the stability-indicating nature of the RP-HPLC method and that the change in assay is accounted for by the increase in impurities, supporting the understanding that the impurities have UV response factors similar to that of the active. Forced degradation samples represent an ideal data set with which to assess mass balance given the range of total impurities generated by the various stress conditions, including to levels well beyond the proposed specification for total impurities.

3.4. Perspectives on mass balance as a required regulatory specification

While the mass balance results presented in Table 5 and Fig. 2 demonstrate that mass balance can be demonstrated on average in very well controlled experiments, it also points to the hazards of implementing this assessment as a specification. In Fig. 2, the mass balance data from forced degradation studies are presented with error bars corresponding to ± 3 standard deviations (black error bars). The ± 3 standard deviation error bars are included to show how variability in the measurements comprising the mass balance calculation may arise over time. This analysis acknowledges that, unlike demonstrating mass balance during development where, for example, all samples are analyzed in one chromatographic run, additional variability should be considered when evaluating mass balance data in relation to a potential specification. Incorporating variability at the ± 3 standard deviation level is a typical approach in justifying specifications to account for longer-term variability such as analyses across time, by different analysts, with different instruments, etc. In other words, the ± 3 standard deviation error bars illustrate the impact of variability versus potential specifications from a capability standpoint. These error bars demonstrate that setting a predetermined specification of 95 %–105 % would be challenging to satisfy in a quality control area. In all experimental designs outlined above, it would be predicted that results will be generated outside of 95 %–105 %. Based upon an analysis with ± 3 standard deviations, a specification of 90 %–110 % would be more appropriate in order to not have an unacceptable number of specification failures because of analytical variability alone. This type of quality control failure is difficult to remediate via the deviation process, and could result in rejecting high quality, and expensive, peptide or polypeptide drug substances. Thus, the patient may not receive required medication. This analysis demonstrates how batches of acceptable quality which meet all other specification tests may be rejected due to propagated measurement variability when results from those individual tests are used to calculate a mass balance result.

Taken a step further, simulations of potential mass balance results can be analyzed based on expected results for assay, water, assumed uncertainties associated with those measurements, and potential bias that could be imparted due to the impact of a method reporting threshold (as discussed above) or from inaccuracies associated with reference standard assignment. Results for these simulations are summarized in Table 7. Table 7 shows the simulated percentage of batch failures as a function of bias (determined for a Bias % of 0 %, 1.0 %, and 2.0 %), water standard deviations (ranging between 0.25 % and 2.0 %), and assay standard deviations (ranging between 1.0 % and 4.0 %).

1.50 %), and assay standard deviations (ranging between 1.0 % and 4.0 %). All of these ranges, based upon the author's experiences, are practical results that are reasonable even for well-designed methods. For each of these combinations of scenarios, 10,000 mass balance results were simulated and the percent of results not meeting limits of 95–105 % was determined as outlined in Section 2.7, Statistical modeling. The results of this simulation are shown in Table 7, which is color-coded to illustrate combinations of bias and measurement variability that result in batch failure rates of less than 1.0 % (green), 1.0 %–5.0 % (yellow), and greater than 5.0 % (red). It is important to note that these simulated “batch failures” occur as a result of combining individual measurements (assay and water) which could pass the individual criteria (i.e., specification limits for assay and moisture) but fail a proposed mass balance specification derived from these results due to combined measurement variability from the constituent tests. The data illustrated in Table 7 reveal that even with reasonable assumptions for measurement variability, the simulated batch failure rate quickly approaches unacceptable levels from the standpoint of manufacturing capability. For example, when there is no bias, an assay method variability of 2.0 %, and a water method variability ranging from 0.25 % – 1.50 %, the probability of not meeting limits of 95 % – 105 % ranges from 0.54 % – 2.64 %. Increasing the assay method variability from 2.0 % to 2.5 %, with no bias and water method variabilities ranging from 0.25 % – 1.50 % increases the probability of not meeting limits of 95 % – 105 % to 2.68 % – 6.04 %. The simulation demonstrates that even when there is no bias, the probability of not meeting limits of 95 % – 105 % is greater than 1 % for assay method variabilities of 2.0 % or greater and water method variabilities of 0.75 % or greater. The simulated failure rate quickly increases above 6 % when the assay method variability increases to 3.0 %. These rates of artificial batch failures illustrate the supply chain risk to imposing a specification that is mathematically derived from other tests which adds no additional assurance of product quality.

These simulations, along with the experimental data presented above, demonstrate the risks associated in setting a mass balance specification. These data also reinforce the opinion and recommendation that mass balance assessments are important to understand with regard to method capability and should be assessed throughout development; however, there is little value in implementing a specification where the summation of other critical quality attributes would need to be confirmed during routine batch release and stability studies.

Table 7

Probability of failing mass balance limits of 95 % – 105 % for varying levels of assay method variability, and water method variability as a function of 0 %, 1 %, and 2 % biases. Results are color-coded to illustrate simulated batch failure rates of less than 1.0 % (green), 1.0 %–5.0 % (yellow), and greater than 5.0 % (red).

Bias	Water SD	Assay SD						
		1.0	1.5	2.0	2.5	3.0	3.5	4.0
0	0.25	0	0.07	0.54	2.68	6.92	11.23	17.56
	0.50	0	0.03	0.80	3.27	7.45	12.42	16.98
	0.75	0	0.13	1.12	3.53	7.62	13.39	17.56
	1.00	0	0.17	1.34	3.97	8.18	13.20	17.53
	1.25	0.05	0.51	1.72	5.03	8.94	14.32	19.57
	1.50	0.20	0.94	2.64	6.04	9.91	15.02	19.23
1.0	0.25	0	0.15	1.50	3.92	8.20	12.64	19.18
	0.50	0	0.23	1.49	4.59	8.38	13.63	18.50
	0.75	0.01	0.52	1.79	4.64	9.15	13.66	19.24
	1.00	0.09	0.57	2.25	5.34	9.61	14.30	19.03
	1.25	0.22	1.01	3.28	6.70	10.47	15.04	20.50
	1.50	0.68	1.84	3.91	7.46	11.90	16.16	20.77
2.0	0.25	0.04	1.19	4.14	8.19	12.53	17.25	22.24
	0.50	0.06	1.55	4.58	8.27	13.21	17.33	22.33
	0.75	0.20	1.91	5.33	9.14	13.59	18.12	23.23
	1.00	0.72	2.37	6.06	10.03	14.17	18.89	22.66
	1.25	1.31	3.83	7.08	10.62	15.36	19.34	23.82
	1.50	2.62	4.86	8.41	12.02	15.86	20.08	25.38

4. Conclusions

The mass balance determination for a therapeutic peptide is presented above. In addition to batch release, mass balance has also been assessed and demonstrated for degraded stability samples. In all cases, the results meet the criteria established for demonstrating mass balance for synthetic peptides and polypeptides. Thus, mass balance for this product at batch release as well as on stability has been demonstrated, and a formalized mass balance specification for routine batches is not required. The assessment of stability samples demonstrates that the analytical methods are stability-indicating based on the mass balance correspondence between assay and total impurities results. Collectively, this analysis demonstrates that further evaluation of mass balance (including a formal mass balance specification at drug substance) is not required nor does it provide any additional assurance of quality beyond the methods and controls already in place. Furthermore, requiring a mass balance specification may result in an unacceptable number of specification failures because of analytical variability alone.

CRedit authorship contribution statement

Brian Pack: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Chad Wolfe:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Evan Hetrick:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Meng Zhao:** Writing – review & editing, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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