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Study of relative response factors and mass balance in forced degradation studies with liquid chromatography/photo-diode array detector/evaporative light scattering detector/mass spectrometry system☆

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ABSTRACT

A case study was performed using photodiode array detection (PDA) in combination with evaporative light scattering (ELS) detection and mass spectrometry (MS) to assess both mass balance and the relative response factors (RRFs) in the forced degradation studies of the drug substance, glimepiride. The RRF value, which is the ratio of the response factor of the impurity to that of the API, was first determined using calibration curves of standards. This conventional technique was compared to a second, multi-detection technique, which used the PDA and ELS detectors to determine both the ultraviolet (UV) peak area and the concentration (based on measurement by ELS) in a single analysis. The resulting RRF values were then applied to forced degradation studies (acidic hydrolysis and oxidation) of glimepiride drug substance. This analysis was used to assess mass balance as well as impurity quantification. The impact of applying impurity RRF values was evaluated and found to have a significant impact on the percent of impurity quantified and on mass balance calculations at higher degradation levels. In addition, MS was used to quantify a non-chromophoric by-product and assess its impact on mass balance. Analysis of the forced degradation studies by MS detection also provided confirmation of the degradation pathway with new insights. Specifically, MS revealed differences in the stereoselectivity of the degradation processes: the acidic hydrolysis degradation was found to be stereoselective with the formation of the *trans* by-product while the oxidation degradation produced both the *cis/trans* isomers in a non-stereoselective reaction.

1. Introduction

In the pharmaceutical industry, the stability testing of drug substances and products is required to assess patient safety, quality and efficacy [1,2]. The goal is to understand the degradation process of these components; therefore, forced degradation, accelerated stability testing and real time stability testing are an integral part of the analysis. Specifically, forced degradation testing requires that drug products and substances be exposed to environmental conditions (heat, light, oxidation) more extreme than may be typically encountered [3,4]. Forced degradation provides the first assessment of understanding the degradation pathways of the active pharmaceutical ingredient (API). Furthermore, early and rough estimates of mass balance determinations of these studies can give insight to predictive issues that may arise in the longer term studies.

Mass balance is typically assessed using liquid chromatography (LC) with a photo diode array detector for most drug products and drug substances [5,6]. However, mass balance can be affected when the drug or impurity is not accurately quantified. This inaccurate quantification can result in an unexplained loss of mass or a higher than expected mass balance. In LC-PDA or UV techniques, these discrepancies in quantification can occur when there is: inadequate separation of all analytes, compounds without appreciable chromophores, or differences in response for impurities and the drug product, among additional variables [7,8]. Furthermore, the presence of limited or low level impurities in UV can also impact mass balance, as accurate quantification can be affected by limits of detection and the impact of different responses.

Regulatory agencies, such as the United States Pharmacopeia, allow impurities to be quantified based on the response of the par-

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ent compound [9]. This approach, therefore, allows the use of RRF values to quantify an impurity relative to the API, for more accurate quantification. The most common technique to measure RRF values requires the use of the slope of the calibration curves for the impurity relative to the API as measured by a UV–vis or photodiode array detector, as seen in Eq. (1) [10].

$$RRF = \frac{[Slope\ of\ Impurity]}{[Slope\ of\ API]} \quad (1)$$

This approach has been well established, and requires the preparation of a set of standards and assumes the availability of said standards. In many cases these standards may be unavailable, which lends to particular challenges when unknown impurities appear under certain stress conditions.

To address such a challenge, mass-sensitive detection techniques combined with PDA or UV detection have been proposed as a tool for determination of RRF values [8,11,12]. This approach requires the analysis by a mass based detection technique in combination with LC-UV to obtain both the response and the concentration of the analyte in an analysis. Specifically, in UV or PDA detection, the response of a compound is related to the concentration or quantity of an analyte as defined by Beer's law (Eq. (2))

$$A = \varepsilon lc \quad (2)$$

where A is the area, ε represents the molar extinction coefficient, l is the path length and c is the analyte molar concentration. Thus, combining the dual detector responses, the RRF for an impurity can be represented by the following equation:

$$RRF = \left(\frac{Peak\ Area_{Impurity}}{Conc_{impurity}}\right) / \left(\frac{Peak\ Area_{API}}{Conc_{API}}\right) \quad (3)$$

Or alternatively, as has been proposed in the literature, RRF values can be determined by using the ratio of the analyte response in a UV detector to that of a molar mass-sensitive detector, as shown:

$$RRF = \frac{UV\ Area_{Impurity}}{Conc\ Detector\ Area_{impurity}} / \frac{UV\ Area_{API}}{Conc\ Detector\ Area_{API}} \quad (4)$$

With some adjustments, this approach has been shown for estimating RRFs with both charged aerosol (CAD) [8,11], and chemiluminescent nitrogen detectors (CLND) [13].

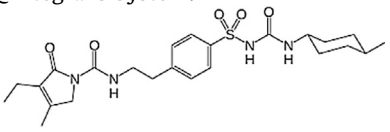
Additionally, this methodology has been postulated for evaporative light scattering (ELS) detection in combination with UV or PDA detection. Studies have indicated these alternative detection techniques may have constraints such as limited linear dynamic range and the need for volatile mobile phases [14]. Nevertheless, the challenge in obtaining relative response ratios in the absence of standards makes the orthogonal detection technique an attractive experimental approach to determine suitable RRF values.

Regardless of the method for evaluating the relative response factors of an impurity relative to the API, additional detection techniques can provide assistance in mass balance determinations. Detectors such as mass spectrometers or ELS can enable identification and quantification of non-chromophoric compounds, which are unobservable in UV. Mass spectrometry, in particular, has become more commonly used in forced degradation studies, not only for identification, but also for quantification [15]. The use of orthogonal detection techniques provide more insight into the degradation pathway as well as the ability to account for non-chromophoric compounds in mass balance calculations.

2. Methods and materials

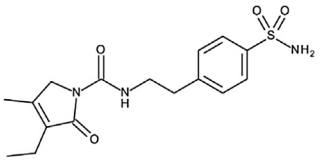
2.1. Chemicals and reagents

Glimepiride, 1-[[4-[2[(3-ethyl-4-methyl-2-oxo-3pyrroline-1-carboxamido) ethyl] phenyl]-sulphony]-3-trans-(4-methylcyclohexyl) urea], related compound B, 3-Ethyl-4-methyl-2-oxo-N-(4-sulfamoylphenethyl)-2,5-dihydro-1H-pyrrole-1-carboxamide, and related compound C, (methyl ((4-(2-(3-ethyl-4-methyl-2-oxo-2,5-dihydro-1H-pyrrole-1- carboxamido)ethyl)phenyl)sulfonyl)carbamate) were purchased from United States Pharmacopeia (Rockville, MD). Glimepiride drug substance was obtained from Alibaba.com, (Shanghai, China). Acetonitrile, 4-methylcylcohexylamine (4-MeCHA), sodium hydroxide (NaOH) and methanol were purchased from Thermo-Fisher Scientific (Pittsburg, PA). 2,2′-Azobis(2-methylpropionitrile) (AIBN), formic acid and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St Louis, MO) and purified water (18 MΩ) was provided using a EMD Millipore (Billerica, MA) Milli-Q Integral 5 System.



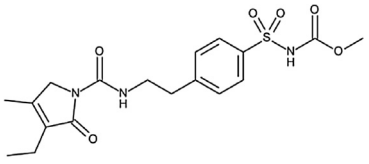
Glimepiride

MW 490.6



Glimepiride related compound B

MW 351.4



Glimepiride related compound C

MW 409.5

2.2. Instrumentation

The chromatographic system was comprised of an ACQUITY UPLC H-Class system (Waters, Milford, MA) with a quaternary solvent manager, flow through needle autosampler and a column manager. The autosampler was configured with a 50 μL extension loop. The seal wash was 90:10 water/methanol, purge solvent was methanol and the needle wash solvent was 0.1% (v/v) formic acid in 30:70 water/acetonitrile. Detection was performed with a photodiode array detector (ACQUITY UPLC PDA), an evaporative light scattering detector (ACQUITY UPLC ELSD) and a mass detector (ACQUITY QDa) (Waters, Milford, MA). The effluent flow path was plumbed from the column to a triple static tee (1:10 split flow) housed within a secondary pump (ACQUITY Isocratic Solvent Manager). The tubing from the column to the tee was 22.5″ of 0.004"ID tubing. In the first split, a portion of the flow was diverted to the PDA with 16" of 100 μm ID fused silica tubing and then, in series, to the ELS detector with 12″ of 0.004″ ID PEEK tubing. Flow from the isocratic solvent manager was introduced into the second splitter, and the final portion of flow was diverted to the mass spectrometer via the detector probe (250 mm). The chromatography data system was Empower 3 FR3 (Waters, Milford, MA).

2.3. Methods

The separation of glimepiride and related compounds B and C was achieved on a 2.1 mm x 50 mm, 1.8 μm, ACQUITY UPLC HSS T3 C_{18} column (Waters Corporation) under isocratic conditions. The mobile phase was 0.1% (v/v) formic acid in 60:40 water/acetonitrile at a flow rate of 0.8 mL/min. The column temperature was 30 °C

with mobile phase pre-heating. Injection volume was 4 μL. The PDA was set to wavelength of 228 nm and a sampling rate of 20 points per second. The ELSD was set to a gas pressure of 25 psi, the nebulizer in Cooling mode, drift tube temperature of 55 °C and the gain set to 100 with a data rate of 10 points per second. The make-up pump was used for post-column addition of 0.1% formic acid in methanol at a flow rate of 0.3 mL/min delivered to the mass detector. The mass detector utilized positive electrospray ionization (ESI+), with a cone voltage set to 5 V. The capillary voltage was 1.4 kV and the sampling rate was 5 points per second. The mass range was set to 100–600 Da. Additional single ion recording (SIR) channels were collected simultaneously for the following *m*/*z* values: 114.1, 374.3, 432.4 and 513.5. The SIR channels were measured with a cone voltage of 5 V.

2.4. Sample preparation

2.4.1. Preparation of standards for RRF determinations by LC-PDA

A solution of glimepiride and each related impurities was individually prepared at a concentration of 0.4 mg/mL in methanol. All samples were sonicated for 10 min to ensure complete dissolution. Working standards were then prepared by diluting the stock with 25:75 water/methanol. For glimepiride (API), individual calibration standards were prepared in triplicate at 0.05% (w/w), 0.1%, 0.3%, 0.4%, 0.5%, 1%, 10%, 20%, 40% and 100% of the sample concentration of 0.250 mg/mL. Related compound B and related compound C standards were prepared individually in triplicate at 0.05%, 0.1%, 0.3%, 0.4%, 0.5%, 1%, 10%, 20% and 40% of 0.250 mg/mL. The labeled purity of glimepiride was 99.6% and that of each related compound was 100%.

2.4.2. Preparation of standards for RRF determination by LC-PDA-ELS

Working standard solutions were also prepared containing all three compounds, glimepiride and related compounds (B and C). The stocks described in the previous section were used. Working standards were prepared in triplicate at 10% (w/w), 20%, 30% and 50% of 0.250 mg/mL with a final diluent composition of 25:75 water/methanol.

2.4.3. Forced degradation of drug substance studies

The glimepiride drug substance was prepared at 0.75 mg/mL stock solution in methanol and sonicated for 20 min to ensure complete dissolution. The forced degradation was performed using a water bath (Isotemp) purchased from Fisher Scientific. Acidic hydrolysis was performed at a final concentration of 0.1 M HCl, and oxidation was performed using a final concentration of AIBN at 5.7 mg/mL. All samples and controls were heated at 40 °C in a water bath. At 1, 3, 5 and 7 days, a 2 mL aliquot of the samples and controls was removed and either diluted (oxidation samples) or neutralized with NaOH (acidic hydrolysis samples). The goal of the studies was to achieve 10–20% total degradation of the API. The final sample concentration was 0.250 mg/mL of the drug substance in 25:75 water/methanol.

2.4.4. Preparation of standards of 4-Methylcylohexylamine (4-MeCHA) for quantification by mass spectrometry

A stock solution of 4-MeCHA was prepared at a concentration of 0.4 mg/mL in 25:75 water/methanol. Working standards were then prepared by diluting the stock with 25:75 water/methanol. Individual calibration standards were prepared in triplicate at 0.04% (w/w), 0.2%, 0.5%, 1%, 2% and 5%, of the sample concentration of 0.250 mg/mL. The labeled purity of 4-methylcylcohexylamine was 99.7%.

3. Results and discussion

3.1. Method development and optimization

A method was developed to evaluate the forced degradation analyses of glimepiride. To satisfy the needs and ensure compatibility with UV, ELS and MS detection, an isocratic method was developed with volatile mobile phases. The isocratic method conditions ensured a similar response across the chromatogram in ELS detection while the volatile mobile phases allowed for identification of the components by MS detection. The column stationary phase was selected for greater retention of the polar impurities while still meeting the limits of quantification required for the impurities (0.05%). The k' for related compound B was 2.5 and that of related compound C was 4.5. At 0.05% impurity, the signal-to-noise was 61 for related compound B and 44 for related compound C.

3.2. Determination of RRF values by using linear calibration curves in UV detection

A relative response factor, as defined by the USP, is the response of the impurity to that of the API [9]. Therefore, to determine the RRF values by using LC-UV, the ratio of the slope of the calibration curve of the impurity to that of the API was used. In forced degradation studies, typically the goal of the analysis is to achieve 10–15% degradation with impurity reporting thresholds of 0.05% of the active pharmaceutical [9]. Therefore the range of standards were tested over the range of 0.05% to 20% for the impurities and 0.05% to 100% for the active pharmaceutical ingredient.

The calibration curves for glimepiride and related impurities met correlation coefficients (R^2) values of greater than 0.995, with 1/x weighting applied (Table 1). The signal-to-noise (S/N) for the lowest calibration point was greater than 10, thus indicating the range of standards meets the limits of quantification as defined by the USP.

The RRF factors were calculated "based on the response of the impurity relative to that of the main component" [9] using the relationship of the slope of the calibration curve for the impurity to that of the active pharmaceutical ingredient as seen in equation 1. These results produced a RRF value for related compound B of 1.32 and a RRF for related compound C of 1.15 (Table 1).

3.3. Estimation of RRF by using orthogonal UV-ELS detection

To evaluate the feasibility of the orthogonal detection approach, working standards containing glimepiride and the related compounds were analyzed on a UV-ELS system, connected in series. The calibration standards were prepared in triplicate at 10% (w/w), 20%, 30%, 40% and 50% of the assay value (0.250 mg/mL). This technique required the use of standards at higher concentrations then those analyzed by UV or PDA alone (Section 3.2) due to the sensitivity constraints of ELS detection. [8,16].

The resulting calibration curves showed a non-linear response in the ELS (as shown for related compound B in Fig. 1A). This result was expected given the response in ELS detector is based on the function[17]:

$$A = a \times m^b \tag{5}$$

where A is area, m is the mass and a and b are coefficients that are dependent on various factors, including the detector characteristics, mobile phase conditions, and the size and shape of the particles analyzed by light scattering. This function can also be represented using the log–log function:

$$\log A = b \log m + \log a \tag{6}$$

Table 1
PDA calibration curve for glimepiride and related compounds and determination of relative response factors (RRFs).

Compound	n	Fit	Weighting	y	Slope (b)	R^2	RRF
Glimepiride	10	linear	1/x	3530	16088	0.999391	1.00
Related Compound B	9	linear	1/x	−110	21421	0.999167	1.32
Related Compound C	9	linear	1/x	1550	18534	0.999315	1.15

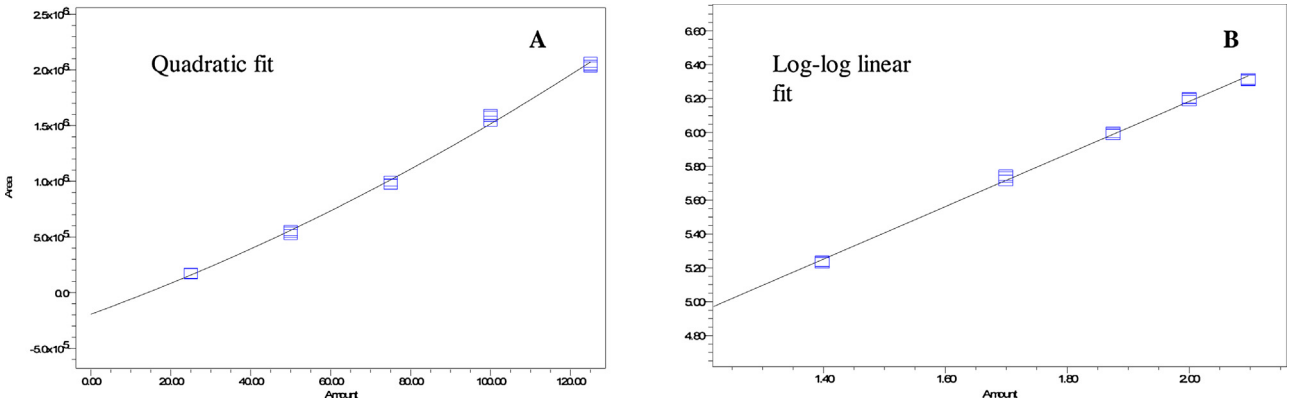


Fig. 1. ELS calibration curves for glimepiride related compound B. Detector response was found to have a quadratic fit over the range analyzed (A). Converting the response to a log–log fit resulted in a linear relationship for peak area to amount (B).

Table 2
ELS calibration curve (log–log fit) for glimepiride and related compounds.

Compound	n	Fit	Weighting	y intercept (log a)	Slope (b)	a	R^2
Glimepiride	5	Log–log linear	none	2.33	1.69	213	0.996650
Rel Cmpd B	5	Log–log linear	none	3.08	1.55	1202	0.998007
Rel Cmpd C	5	Log–log linear	none	2.90	1.57	794	0.996128

This log–log transformation of the results allows a linear calibration curve to be plotted (Fig. 1B). Thus, applying this principle, the log–log transformation produced a linear calibration curve (Table 2) for glimepiride and related impurities with correlation coefficients (R^2) values of greater than 0.995.

Going back to the original concept of using dual orthogonal detection techniques to determine RRF values, with one detector being absorptivity based and the second detector based on relative molar amounts, we can represent RRF by the following equation:

$$RRF = \frac{UV\ Area_{Impurity}}{ELS\ Area_{impurity}} \Big/ \frac{UV\ Area_{API}}{ELS\ Area_{API}} \quad (7)$$

As previously described the ELS detection typically produces a non-linear response over a wide calibration range, however, Eq. (7) assumes a linear relationship for both UV and ELS detectors. Therefore, to achieve a linear relationship between API and impurity ELS peak areas, the log–log transformation principles can be applied. Replacing the ELS peak area with the log function produces the following equation:

$$RRF = \frac{UV\ Area_{Impurity}}{ELS \log(Area_{impurity})} \Big/ \frac{UV\ Area_{API}}{ELS \log(Area_{API})} \quad (8)$$

Using this function, the RRF values were determined for both related compound B and C using standards over the concentration ranges 10–50% (w/w). The working standards contained the API, related compound B and related compound C with concentrations based on the sensitivity requirements of the ELS detector. The values obtained are shown in Table 3.

Comparing these values to those previously described in Section 3.2, a high correlation was observed between the techniques. The mean RRF values obtained by using PDA-ELS detectors for related compound B and related compound C were 1.25 and 1.11 respectively (Table 3), while values of 1.32 for related compound B and 1.15 for related compound C were obtained using the slopes of the calibration curves in PDA detection (Table 2), indicating the utility of this approach.

Table 3
Relative response factors determined by using ratio of UV peak area to ELS log peak area function.

Standard concentration (w/w)	Sample number	RRF Rel Cmpd B	RRF Rel Cmpd C
10%	1	1.26	1.13
10%	2	1.23	1.11
10%	3	1.25	1.11
20%	4	1.26	1.11
20%	5	1.23	1.09
20%	6	1.23	1.10
30%	7	1.26	1.10
30%	8	1.25	1.11
30%	9	1.26	1.11
40%	10	1.24	1.10
40%	11	1.24	1.10
40%	12	1.24	1.11
50%	13	1.29	1.17
50%	14	1.26	1.11
50%	15	1.25	1.11
Mean		1.25	1.11
Std Dev		0.017	0.018
%RSD		1.34	1.65

The use of LC-PDA-ELS to estimate the RRF values of impurities can provide a useful tool; however, this approach has certain constraints. For example, the related impurity must be present in sufficient quantities for detection in ELS [18]. In addition, during forced degradation of a drug substance or product, it can be difficult to achieve the concentration of both the API and the impurities for quantification in the molar-mass based detector. Furthermore, non-chromophoric species in a drug substance or drug product

can impact the separation and quantification in ELS detection and should be considered when the analysis is performed on other active pharmaceutical ingredients. Lastly, the formation of very different sized particles can impact the relationship of the API and related impurities. Specifically, the size(s) and shape of the particles impact the light-scattering mechanisms (Rayleigh-Debye, Mie and refraction-reflection) [19]. Therefore, particles of very different size may not give comparable responses or relative response required for these analyses.

3.4. Degradation analysis and mass balance of drug substance under acidic hydrolysis and oxidative conditions

Reaction of glimepiride or 1-[[4-[2[(3-ethyl-4-methyl-2-oxo-3pyrroline-1-carboxamido) ethyl] phenyl]-sulphony]-3-trans-(4-methylcyclohexyl) urea], under acidic and oxidative conditions has been found to be susceptible to reaction at the carboxamide linkage and the sulfonylurea bridge. To assess this reactivity, the drug substance was exposed to 0.1N HCl for acidic stability testing and AIBN for oxidative stability testing. The goal was to achieve 10–20% degradation as quantified by UV. The samples were tested at 2 day intervals and analyzed by UV-ELS and MS detection techniques. The drug substance required exposure to acidic and oxidative conditions for 7 days to achieve the desired degradation levels. The PDA and ELS detectors were used for monitoring the analysis while the MS was used for identification and peak tracking.

Acidic hydrolysis of glimepiride (Fig. 2) produced two impurity peaks, related compound B and C, of which the confirmation was provided by MS. Under the acidic degradation conditions, sodium adducts were observed in MS for related compounds B and C, as well as glimepiride (Fig. 3). The ELS detector also showed the presence of an additional peak, eluting just prior to related compound B. This peak was also present in the control sample and can be attributed – in part – to the reagents. The degradation pathway of glimepiride has been previously described in the literature [15]. During the formation of both related compound B and related compound C, there is a loss of 4-methylcyclohexylamine (4-MeCHA), which was confirmed by MS (Fig. 3).

Under oxidative conditions, a similar chromatographic profile was observed by UV detection as observed under acidic conditions with the formation of both related compound B and C (Fig. 4). The mass spectrum profile, however, showed the presence of three additional peaks as compared to the UV chromatogram. The peak at 0.30 min was also present in the AIBN control (m/z of 177.2 Da) and could attributed to the reagent (Fig. 5). Two peaks corresponding to 4-MeCHA with a predominant m/z of 114.1 Da were also observed (Fig. 5). Based on comparison to the standard for 4-MeCHA, the two peaks were the *cis*- and *trans*- isomers.

In both of the degradation studies, mass balance and the impact of RRF values were explored. There are numerous methods to calculating and reporting mass balance. In our studies, mass balance was calculated using the peak areas as measured by UV:

$$Mass\ Balance = \frac{\sum Area_{i,x}}{\sum Area_{R,0}} \times 100 \tag{9}$$

where $\sum Area_{R,0}$ represents the sum of the peak areas or total mass of the reference sample and $\sum Area_{1,x}$ is that of the degraded sample. All peak areas with a value of greater than 0.10% total peak area were summed. The reference sample was tested at the same time as the degraded sample to reduce day-to-day variability of the method, conditions or instrument. The percent (%) impurities were calculated using peak areas.

Using this methodology, the percent degradation and the mass balance for the acidic and oxidative degradation were analyzed every two days over a 7 day time period. At the end of the 7 day period, acidic hydrolysis conditions produced API degradation of approximately 20%. Calculations of the apparent mass balance over the same time period resulted in values of 99.16–103.52% (Table 4). While these results indicate a minimal change over time, at seven days the greatest mass balance was observed with a value of 103.52%.

As described earlier differences in the response of an impurity compared to an API can impact the quantification of the impurity as well as mass balance determinations. To determine the accuracy of the mass balance and the extent of the degradation (or % impurities), calculations were also performed using the RRF values. The peak area for each impurity was corrected based on the following:

$$Corrected\ Area_i = \frac{Area_i}{RRF_i} \tag{10}$$

where RRF_i were the values experimentally determined by UV-ELS detection (Table 3, 1.25 for related compound B and 1.11 for related compound C). In this manner the impurity UV area was corrected for response differences. Since both related impurities in this study have a RRF > 1.0, corrected areas were less than the (uncorrected) peak area for each impurity.

The corrected areas were then used in the mass balance calculations which were calculated by:

$$Corrected\ Mass\ Balance = \frac{\sum Corrected\ Area_{1,x}}{\sum Corrected\ Area_{R,0}} \tag{11}$$

where $Corrected\ Area_{1,x}$ is the total of the corrected peak areas for those compounds with a RRF value plus the peak area of the API. This can also be represented by:

$$\sum Corrected\ Area = Area_{API} + Corrected\ Area_{i,1} + Corrected\ Area_{i,2} + \ldots \tag{12}$$

With these adjustments in response, the % impurity and mass balance corrected values were determined for acidic hydrolysis (Table 4). Correction of the peak areas for the impurities (related compound B and C) under acidic hydrolysis conditions resulted in lower values for % impurity with a 3% decrease at 7 days from 19.55% to 16.69% (Table 4) as compared to using peak areas only. The corrected mass balance also decreased 3–4% at 7 days from 103.52 to 100.19% when compared to the apparent mass balance which was not adjusted using RRF values.

The same analysis was performed for the oxidative degradation studies. Under oxidative degradation conditions, less extensive degradation occurred over the same time period, with approximately 10% API degradation achieved at 7 days (Table 5). In this example, the degradation levels did not impact the apparent mass balance significantly and values between 97.60 and 100.99% mass balance were observed. As was previously described, accounting for response differences, the % impurity and mass balance corrected values were also determined (Table 5). Using RRF values, under oxidation conditions, mass balance and % impurity values showed a similar trend as observed with acidic hydrolysis: using corrected areas resulted in a decrease in both the % impurity and mass balance of 1–1.5% at 7 days (Table 5). These results show that without adjustment of RRF, both % impurities and mass balance would have been over reported. Furthermore, the greater the extent of degradation the greater the impact, with the most significant difference occurring at 7 days.

The impact of recovery with and without correcting for RRF differences is most observable at higher % degradations. Because the API peak area is unchanged, correcting impurity peak areas as shown in Eq. (11) is directly proportional to both the percent degradation and/or the deviation of the RRF's from 1. The greater the amount (%) of degradation products, the larger the change in the

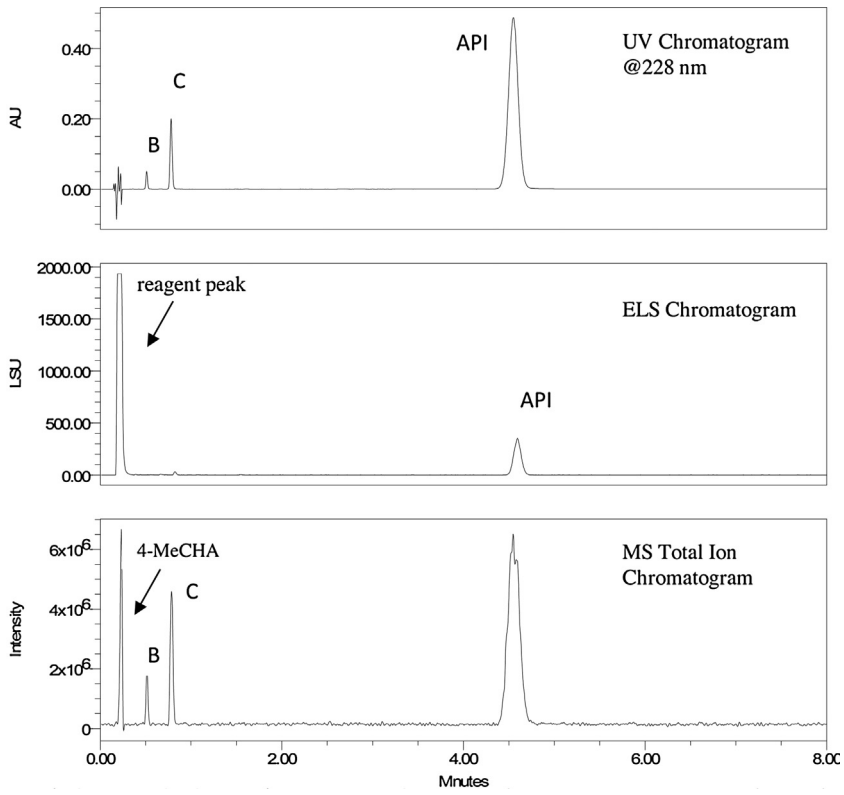


Fig. 2. Acid hydrolysis of glimepiride drug substance at 5 days. UV chromatogram (228 nm) shows the presence of related compound B (B) and related compound C (C) as well as the drug substance, glimepiride (API). ELS and MS Total Ion Chromatograms (TIC), show the presence of an additional peak eluting in the void. In MS this peak was identified as 4-MeCHA, however under ELS detection the peak is also observable in the control sample, and is likely due to the reagents. The MS TIC was processed using Component Detection Algorithm (CODA) and blank subtract in Empower 3SR3 Chromatography Data System (CDS).

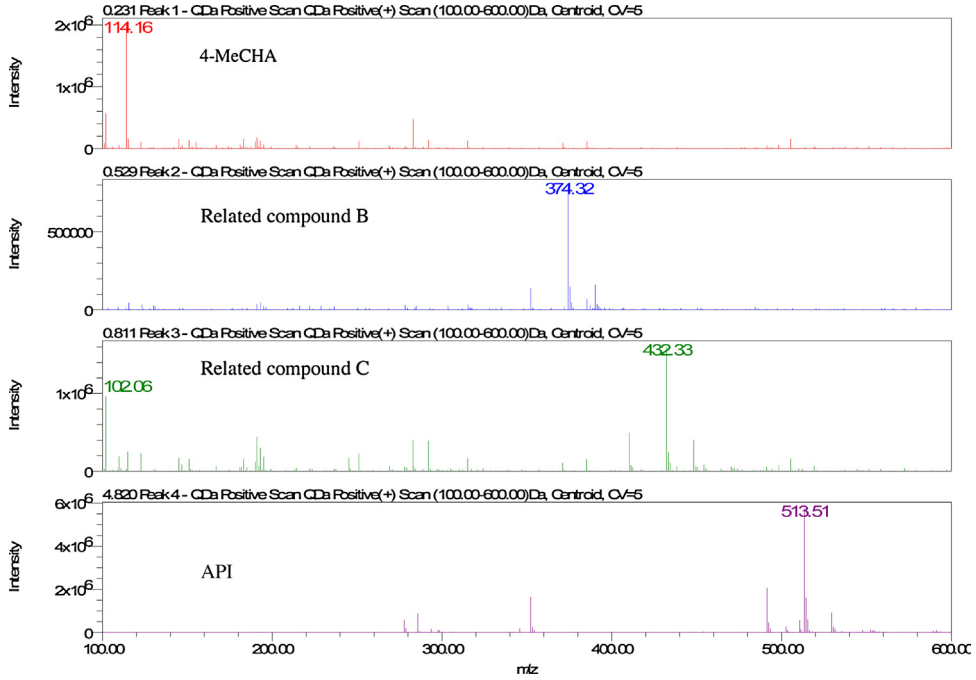


Fig. 3. Spectrum of peaks in MS, Total Ion Chromatogram (TIC) for 4-MeCHA, related compound B, related compound C and API under acid hydrolysis of glimepiride drug substance at 5 days. Impurities and the API were predominantly present as Na+ adducts under the conditions used for the analysis..

corrected mass balance. Likewise, the greater the RRF varies from 1, the greater the change in impurity quantification. The implications of this will be significant for any compounds with very different responses or higher amounts of degradations. In all examples, the % impurity and recovery were impacted similarly, however, the implications for % impurity are greater, in that impurity reporting, identification and qualification thresholds can be impacted by as little as 0.05% [20].

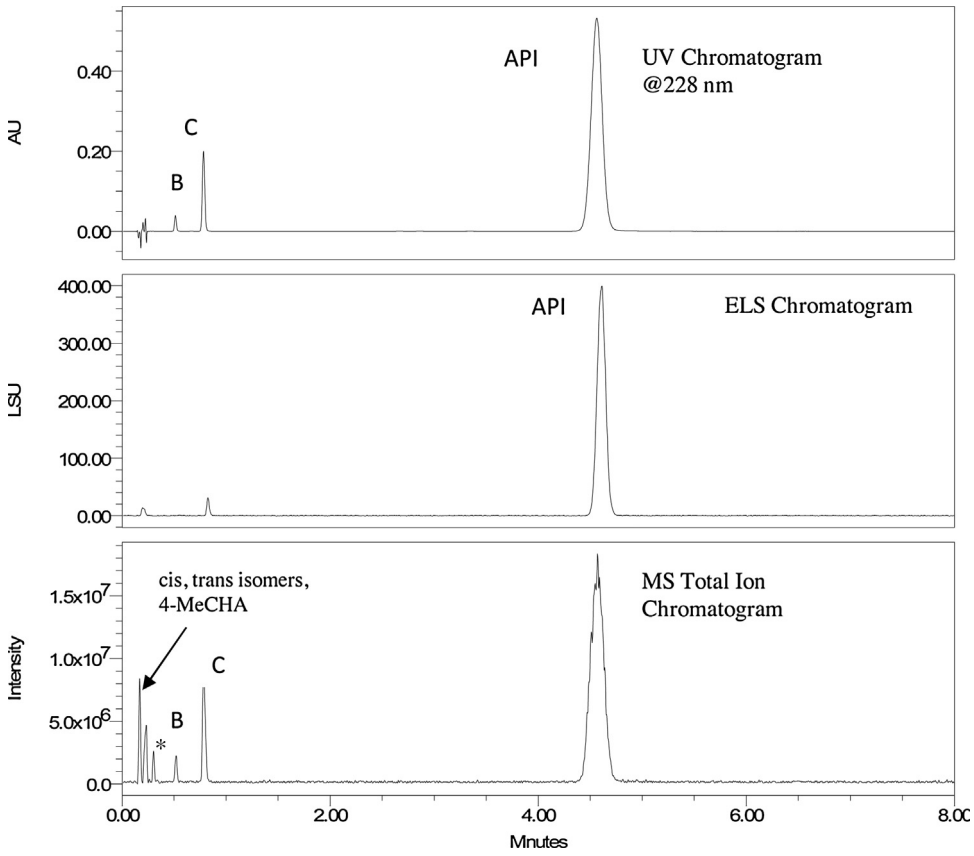


Fig. 4. Oxidation by AIBN of glimepiride drug substance at 5 days. UV chromatogram (228 nm) shows the presence of related compound B (B) and related compound C (C) as well as the drug substance, glimepiride (API). ELS chromatogram shows a small peak eluting in the void, while MS Total Ion Chromatogram (TIC), shows the presence of additional peak(s) eluting in the void. The first two partially separated peaks correspond to the cis- and trans- isomers of 4-MeCHA. The third peak is a product of the AIBN oxidation (*m*/*z* 177.2)- *- as evidenced by analysis of the control. The MS TIC was processed using CODA and blank subtract in Empower 3SR3 (CDS).

Table 4
Mass Balance of acidic hydrolyzed drug substance before and after RRF correction determined from UV/ELS method.

Time (days)	Impurities peak area	Total peak area	% Impurities	Apparent mass balance (%)	Impurities corrected peak area	Total corrected peak area	% Impurities corrected area	Corrected mass balance (%)	Total Amount (μg/mL)[b]
Reference[a]	25053	4064121	0.62	–	21606	4059339	0.53	–	250.0
1	96855	4045552	2.39	99.20	85347	4028733	2.11	98.76	246.9
3	256914	4172795	6.16	102.37	227262	4129162	5.14	101.37	253.4
5	391055	4043862	9.67	99.16	345596	3977081	8.69	97.64	244.1
7	826352	4225907	19.55	103.52	726832	4080914	16.69	100.19	250.5

[a] Reference was rerun every day prior to analysis. Values shown are for day 1.
[b] Total amount is based on reference amount multiplied by corrected mass balance (%).

Table 5
Mass balance of oxidative degraded drug substance before and after RRF correction determined from UV/ELS method.

Time (days)	Impurities total peak area	Total peak area	% Impurities	Apparent mass balance (%)	Impurities corrected peak area	Total corrected peak area	% Impurities corrected area	Corrected mass balance (%)	Total Amount (μg/mL)[b]
Reference[a]	28102	4139644	0.7	–	24414	4118924	0.6	–	250
1	73491	4184298	1.75	100.99	64589	4109561	1.57	100.7	251.8
3	203294	4210333	4.83	97.70	180076	4006617	4.49	97.00	242.5
5	320295	4192747	7.64	98.40	284040	3871958	6.28	96.92	242.3
7	425910	42503395	10.13	97.60	377805	4132242	9.14	96.10	240.3

[a] Reference was rerun every day prior to analysis. Values shown are for day 1.
[b] Total amount is based on reference amount multiplied by corrected mass balance (%).

3.5. Confirmation of degradation pathway and reconciliation of mass balance by using mass detection

The degradation pathway of glimepiride has been previously described in the literature [15]. During the formation of both related compound B and related compound C, there is a loss of 4-MeCHA. Glimepiride consists of the 4-MeCHA functional group as a *trans*-isomer, while related compound A contains the *cis*-isomer.

Using MS detection, quantification with a single ion recording channel (SIR) for 4-methylcylcohexylamine (4-MeCHA) provided

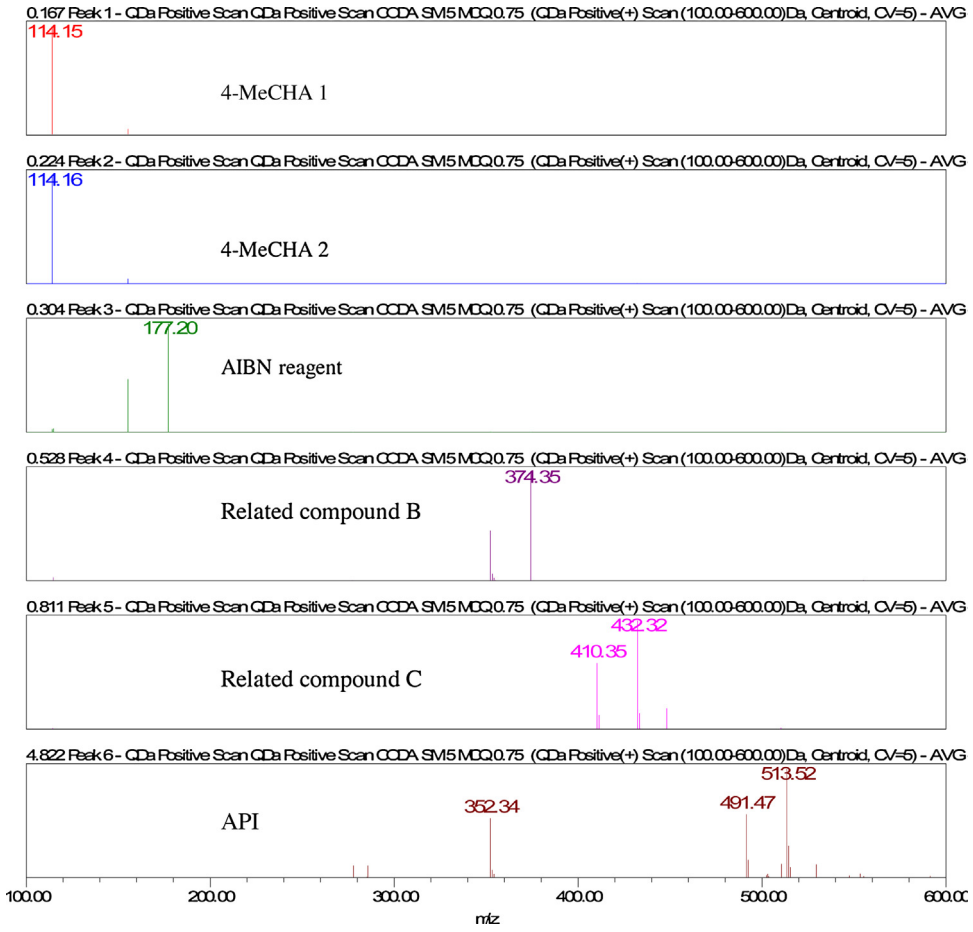


Fig. 5. Spectrum of peaks in MS Total Ion Chromatogram (TIC) for 4-MeCHA peaks 1 and 2, AIBN reagent, related compounds B, C and API under oxidative degradation of glimepiride drug substance at 5 days. Impurities and the API were predominantly present as Na+ adducts under the conditions used for the analysis.

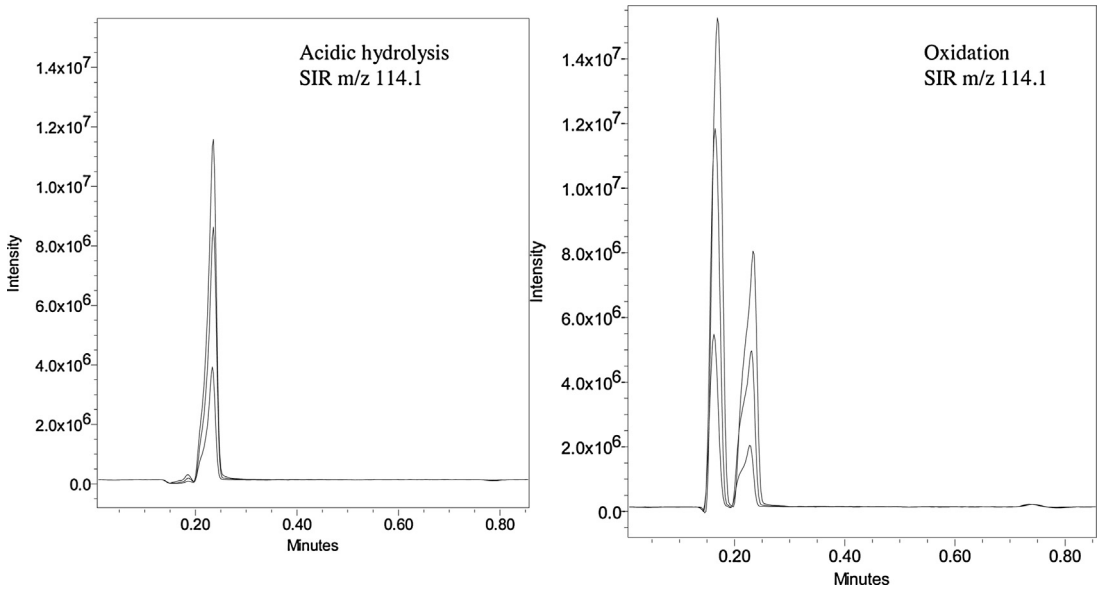


Fig. 6. Mass spectrum Single Ion Rerecording channel (SIR) of *m*/*z* corresponding to 4-MeCHA under acidic and oxidation degradation conditions at 1,3 and 5 days. Acidic hydrolysis produces a single isomer, while oxidation conditions produces two isomers, both of which increasing in area with time.

improved sensitivity and specificity as compared to a total ion channel (TIC). Analysis of the SIR confirms the presence of the by-product in the degradation reactions, with an increase observed with time for both acidic and oxidative degradation (Fig. 6). However as described earlier, under acidic degradation only a single peak was present, while under oxidative conditions two peaks were observed. This suggests a number of possibilities. For example, the drug substance may also contain the *cis*-isomer of glimepiride or

Table 6
4-Methylcylohexylamine (4-MeCHA) quantification and impact on mass balance determinations under oxidative conditions .

Time (days)	4-MeCHA (μg/mL) (%)	Total Amount (μg/mL)[a]	Amount + 4-MeCHA (μg/m)	Reference amount (μg/mL)[b]	Corrected mass balance (%) (not including 4-MeCHA)[c]	Final mass balance (%) (including 4-MeCHA)[d]
1	1.48 (0.59%)	251.8	253.3	250.0	100.7	101.3
3	4.02 (1.61%)	242.5	246.5	250.0	97.00	98.60
5	6.70 (2.68.%)	242.3	249.0	250.0	96.92	99.60
7	13.69 (5.48%)	240.3	254.0	250.0	97.21	101.6

[a] Amount from Table 5.
[b] Reference was rerun every day prior to analysis. Values shown are for day 1.
[c] Corrected Mass Balance from Table 5.
[d] Final Mass Balance (%) = (Amount + 4-MeCHA)/Total Amount Ref) × 100.

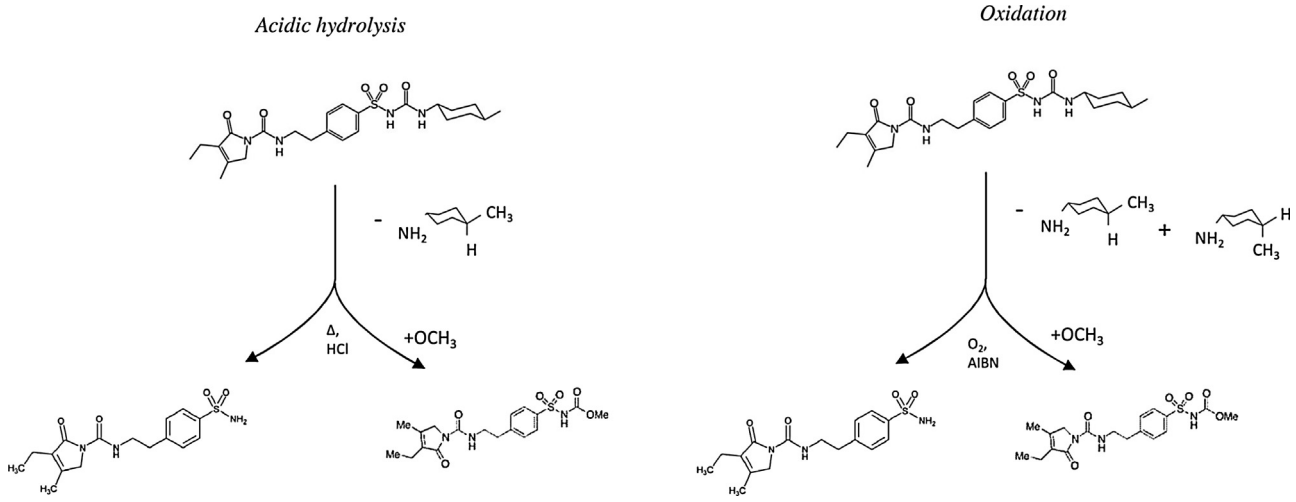


Fig. 7. Degradation pathway of glimepiride under acid hydrolysis and oxidation conditions.

related compound A[21], which only degrades under oxidative conditions. Alternatively, during oxidation by the radical generator AIBN, there may be a conversion of 4-MeCHA from the *trans*- to the *cis*-isomer. Regardless, the acidic hydrolysis reaction appears to be stereoselective while oxidation by radical generator is not (Fig. 7).

To quantify 4-MeCHA in the degradation, the calibration curve was determined using commercially available reagents. Working standards (n=5) were prepared to cover the range from 0.04 to 10% (0.1–25 μg/mL). The working standard was present as a mixture of both the *cis*- and *trans*-geometric isomers. These isomers were partially separated chromatographically, therefore the sum of the isomers was used for the calibration curve. The calibration curve had a quadratic fit with no weighting. The curve was converted to a linear relationship using a log–log fit over the quantification range with a slope of 0.835, a y-intercept of 6.77 and a correlation coefficients (R^2) of 0.997563. The signal-to-noise (S/N) for the lowest calibration point was greater than 10.

For the oxidative degradation reactions over the period of 7 days, quantification of 4-MeCHA showed an increase in the level of the by-product with impurity formation. As shown in Table 6, the amount of 4-MeCHA formed under oxidative conditions increased over the time period. At 1 day, 4-MeCHA was quantified at 1.48 μg/mL or 0.59% of the drug substance. The value increased to 13.69 μg/mL or 5.48% at 7 days. Since 4-MeCHA is formed as a by-product from related compound B and related compound C formation, the area is directly related to the extent of degradation. In addition, for every gram of related compound B and related compound C formed, only a fraction of a gram of the by-product is formed. Therefore, a correlation should exist between the formation of related compounds B and C (Table 5) and the amount of 4-MeCHA. Specifically for one gram of API that degrades to related compound B or C, 0.23% of 4-MeCHA is formed. For example, at 5 days, 15.7 μg/mL (6.28%) of the API degraded, and was accompanied by 6.70 μg/mL (2.68%) of 4-MeCHA, which was within the expected values.

The amount of 4-MeCHA in μg/mL was then added to the corrected mass balance values from Table 5. This was to assess the impact of the by-product quantification on mass balance. The total values can be represented in μg/mL or as a percent when compared to the reference standard (250 μg/mL). Comparison of the mass balance at day 7 with and without 4-MeCHA showed an increased from 97.21% (Corrected Mass Balance) to 101.6% (Final Mass Balance) as shown in Table 6. In general at higher levels of degradation the loss of the 4-MeCHA will be more impactful and will provide a more accurate mass balance.

4. Conclusions

The use of multi-detection including PDA or UV, ELS, and MS detectors allows for additional techniques for both RRF determinations as well as mass balance calculations. While UV detection alone is commonly used for these analyses, the determination of RRF using a UV detector in combination with a mass sensitive detector, such as ELS, allows the RRF value to be estimated in a single analysis. The correlation with conventional techniques is highest when compounds with similar response in ELS are present in equal amounts, due to the limited sensitivity of the ELS detector.

The application of a multi-detection technique for the study of forced degradation of pharmaceuticals provides the ability to monitor the degradation pathway and quantify all products, including non-chromophoric compounds. Specifically in this example, MS was used to quantify the non-chromophoric by-product for a more comprehensive understanding of the mass balance in acidic hydrol-

ysis and oxidative degradations. Differences in the stereospecificity of acidic and oxidation degradation reactions were also illustrated by MS. Furthermore, the addition of non-chromophoric components provides a more comprehensive mass balance evaluation, particularly in cases where extensive degradation may occur.

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