Validation of an Analytical Method to Determine Isotopic Purity by LC-MS

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Background

Labelled drug substances are used in the pharmaceutical industry to assess the pharmacokinetic profile or mode of action of a drug substance, in addition to determining release profiles and metabolism-mediated toxicity. As such, these drugs are often subject to the same specification testing requirements as their unlabelled counterparts, with additional testing to determine the extent of isotopic labelling. Whilst techniques such as HPLC with radioactivity detectors to determine the extent of labelling in radiolabelled compounds, defining the labelled extent of cold labelled compounds containing ^{13}C, ^{15}N or D (^{2}H) can prove problematic owing to a lack of radioactivity and the compounds sharing the same retention time and UV response as their unlabelled counterparts. The presented case study demonstrates the validation of a method used to assess isotopic purity using LC/QQQ-MS detection for a compound containing 5-D, manufactured in Almac Sciences. Validation tests included specificity, linearity, accuracy and precision, quantitation limit and solution stability.

Method Development Workflow

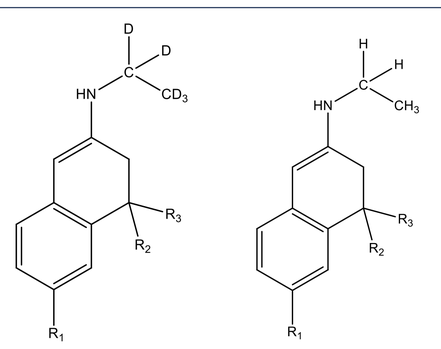

Figure 1: D5-Labelled compound of interest and Unlabelled counterpart

Method

- UHPLC-MS: Agilent 1290 Infinity II with Agilent 6460 QQQ-MS
- Column: Agilent Zorbax RRHD Eclipse Plus, C18, 50 x 2.1 mm, 1,8 µm
- Scan Type: MS2 with a range of m/z 300 – m/z 310
- The chromatography was optimised to separate the peak of interest from any interfering compounds with similar masses. Theoretical isotopic distributions of the labelled/unlabelled compound of interest were used to set the scan range of the MS and included those isotopes which gave a response exceeding the proposed LOQ. Using MassHunter Quant software, a processing method was used to extracted ion chromatograms (EICs) for the isotopes of interest from m/z 301.1 – m/z 310.1, adding each isotope as a separate quantifier ion. Ions from m/z 302.1 – m/z 306.1 were added to the method as qualifier ions. System suitability was established as adherence of the observed isotopic distribution of the unlabelled compound to the theoretical isotopic distribution.

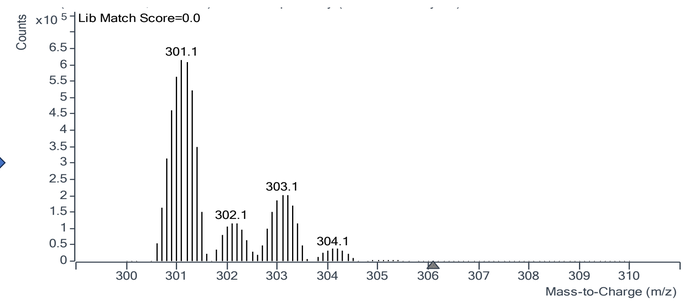

Figure 2: Spectra of Unlabelled Compound

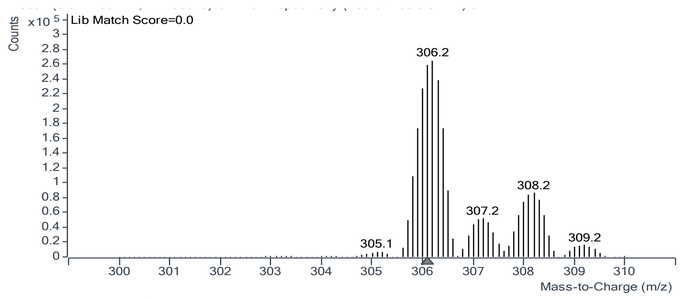

Figure 3: Spectra of D-5 Labelled Compound

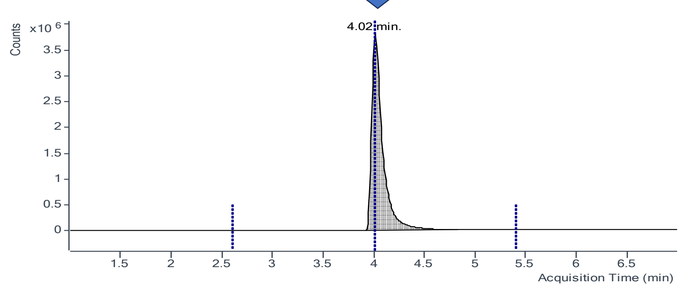

Figure 4: EIC of m/z 301.1 isotope

Figure 5: Correction for Natural Isotopic Contributions and Calculation of Isotopic Purity

Isotopomer	Response (Counts)	Blank Response (Counts)	Blank Subtract Response (Counts)	Natural Isotope Correction (Counts)	Natural Isotope Corrected (Counts)
0 x [D] 301 m/z	31228	8793	22435	0	**31228**
1 x [D] 302 m/z	9082	0	9082	6046	**3036**
2 x [D] 303 m/z	83536	0	83536	11199	**72337**
3 x [D] 304 m/z	53056	0	53056	17001	**36055**
4 x [D] 305 m/z	418798	0	418798	31934	**386863**
5 x [D] 306 m/z	16656400	0	16656400	91604	**16564796**
				Sum	**17094316**

Theoretical abundance of natural isotopes were calculated using Agilent Isotope Distribution Calculator

Theoretical Natural Isotopic Distribution for the [M+H]+ ion species of CxH12D5NyOz		Unlabelled CxH17NyOz	Mono-labelled Average Distribution	Di-labelled Average Distribution	Tri-labelled Average Distribution	Quart-labelled Average Distribution	Pent-labelled Average Distribution
Isotope	m/z	Relative Abundance (%)	Relative Abundance (%)	Relative Abundance (%)	Relative Abundance (%)	Relative Abundance (%)	Relative Abundance (%)
M	301	100.00					
M+1	302	19.36	100.00				
M+2	303	33.98	19.35	100.00			
M+3	304	6.34	33.97	19.34	100.00		
M+4	305	0.64	6.33	33.97	19.33	100.00	
M+5	306	0.05	0.64	6.33	33.97	19.32	100.00
			0.05	0.64	6.33	33.97	19.31
				0.05	0.64	6.32	33.96
					0.05	0.64	6.32
						0.05	0.64
							0.05

Isotopic Purity of Compound			
	Isotopic Enrichment Relative %		Total Labelled Atoms
0 x [D] 301 m/z	**0.18**		
1 x [D] 302 m/z	**0.02**		0.02
2 x [D] 303 m/z	**0.42**		0.85
3 x [D] 304 m/z	**0.21**		0.63
4 x [D] 305 m/z	**2.3**		9.05
5 x [D] 306 m/z	**96.9**		484.51
Sum	**100.0**	**Overall Isotopic Purity %**	**99.0**

Calculation of Isotopic Purity

Isotopic purity was calculated from the peak response for each isotope as demonstrated in Figure 5. Natural isotopic contributions were calculated from theoretical isotopic distributions and subtracted to provide the corrected isotope results. These counts were then used to calculate the relative isotopic enrichment and total isotopic purity of the sample.

Validation Results

Limit of Quantification (LOQ)

LOQ was established by injecting six replicates of the 100% unlabelled accuracy solution. The USP signal to noise ratio (S:N) for the smallest unlabelled compound isotope peak was determined in the EIC for each injection (i.e. M+5 isotope, m/z 306.1, 0.05% of theoretical abundance, >10:1).

Table 1: Limit of Quantification

Name	Isotope (m/z)	Injection Number	Signal to Noise
100% Unlabelled Accuracy Solution Prep 1 (LOQ)	306.1	1	36
		2	32
		3	33
		4	26
		5	32
		6	23

Specificity

The specificity acceptance of the method was met by demonstrating:

- There was no interference greater than or equal to the response of the smallest unlabelled compound isotope (306.1 m/z, 0.05%) in the blank.
- The mono-isotopic mass of both unlabelled (301.1 m/z) and labelled (306.1 m/z) compounds were within ±0.50 m/z of their theoretical masses.
- The isotopic distributions of both unlabelled and labelled compounds were visually concordant with their theoretical isotopic distributions.
- The retention time of the unlabelled and labelled compounds were within ±0.5mins of each other in the EIC chromatograms.

Accuracy

Accuracy was assessed at 80%, 100% and 120% of the nominal concentration for the unlabelled compound by comparing the observed relative abundance of each isotope to the theoretical relative abundance.

The relative abundance of each compound isotope (m/z 301.1 - 306.1) were within 90% to 110% of the theoretical where the relative responses to the base isotope were ≥1.0%; within 0.2% absolute for isotopes with a relative response <1.0% and ≥0.10%; and within 0.02% absolute for isotopes with a relative response <0.1% for each level.

Precision

The precision of the method was assessed by analysing solutions at 80%, 100% and 120% of the nominal concentration of the labelled compound. The percentage isotopic enrichment for each isotope was calculated as per Figure 5.

The precision (% RSD) for the isotopic enrichment for each isotope at each level (n=3 and n=6) were <10% for isotopes with a percentage enrichment ≥1%; and <15% for isotopes with a percentage enrichment <1% but ≥0.1% which gave a peak response greater than the 0.05% relative abundance isotope in the unlabelled compound reference solution.

For intermediate precision, fresh 100% precision working standards were prepared and analysed by a second analyst on a different day using different reagents. The precision (% RSD) for the isotopic enrichment for each isotope for the second analyst (n=6) and both analysts at the 100% level (n=12) met the above criteria.

Linearity

Linearity of the method was inferred from the assessment on the 80%, 100% and 120% accuracy samples.

Solution Stability

Solution stability was demonstrated in both unlabelled and labelled compound solutions at 2–8°C and ambient conditions over a period of ~24 hours.

For the unlabelled compound, the absolute difference in percentage relative abundances of each isotope between T_0 and T_1 (m/z 301.1 – m/z 306.1) was <10% where the relative responses to the base isotope was ≥1.0%; within 0.2% absolute for isotopes with a relative response <1.0% and ≥0.10%; and within 0.02% absolute for isotopes with a relative response <0.1%.

For the labelled compound, the absolute difference in percentage isotopic enrichments between T_0 and T_1 (m/z 301.1 – m/z 306.1) for any isotopic enrichment >1.0% did not change by more than 10%. For any percentage isotopic enrichment between ≥0.1 and 1.0% the absolute change was <0.1%.

Examples of the solution stability at ambient conditions for both the unlabelled compound and labelled compound are displayed below in Table 2 and Table 3.

Table 2: Unlabelled Compound Stability at Ambient Temperature

Name	Isotope (m/z)	T_0 Response (Counts)	T_0 % Relative Abundance	T_1 Response (Counts)	T_1 % Relative Abundance	% Difference /*Absolute Difference
100% Unlabelled Compound Stability Solution	301.1	21843752	100.00	20229160	100.00	<0.1%
	302.1	3987661	18.26	3709954	18.34	-0.5
	303.1	7087751	32.45	6610606	32.68	-0.7
	304.1	1278745	5.85	1194404	5.90	-0.9
	305.1	123044	0.56	114606	0.57	<0.2%*
	306.1	8249	0.04	7195	0.04	<0.02%*

Table 3: Labelled Compound Stability at Ambient Temperature

Name	Isotope (m/z)	T_0 % Isotopic Enrichment	T_1 % Isotopic Enrichment	%Difference /*Absolute Difference
100% D-5 Labelled Compound Stability Solution	301.1	0.19	0.19	<0.1%*
	302.1	0.02	0.02	N/A
	303.1	0.43	0.43	<0.1%*
	304.1	0.21	0.21	<0.1%*
	305.1	2.26	2.30	-1.7
	306.1	96.89	96.86	<0.1%

Conclusion

The assessment of isotopic purity by LC-MS has been practiced for a number of years, however there is little evidence for GMP validated methods which have been demonstrated to meet the requirements of pre-assigned specifications. In the current study, Almac has integrated a more scrutinous GMP compliant approach which demonstrates that it is possible to accurately monitor isotopic purity down to levels as low as 0.05% with a robust and repeatable method using LC-QQQ-MS.