DETERMINATION OF ISOTOPIC INCORPORATION IN ISOTOPICALLY LABELLED COMPOUNDS USING TIME OF FLIGHT MASS SPECTROMETRY WITH INCREASED DYNAMIC RANGE

Waters

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INTRODUCTION

Isotopically labelled compounds are very useful within pharmaceutical research. They are used to investigate metabolism of drugs and to determine their distribution between different organs. They are also used during clinical studies (e.g. bioavailability) and as internal standards for quantitation. Even though their chemical, physical and biological properties are considered more or less identical, they are easily distinguished from their non-labelled analogues using mass spectrometry.

At the present time, isotopically labelled compounds are analysed by reversed phase HPLC coupled to orthogonal acceleration time-of-flight mass spectrometry (oa-TOF-MS) at Medicinal Chemistry, Mölndal, Sweden. The degree of isotope incorporation can then be calculated from the mass spectral isotopic pattern, after subtracting the contribution from the non-labelled compound (since the degree of incorporation is not always 100% as well as from other labelled isotopes). The results have been in fair agreement with those calculated from gravimetric analyses combined with activity measurements (scintillation counter). Historically the problem with this method was the limited dynamic range of the TOF-MS detector. The TDC detector was saturated at elevated concentrations which resulted in incorrect mass information and mass intensities. To avoid saturation only scans at the slope of the peak (yielding poor ion statistics for low-concentration isotopes) could be used to calculate the isotope incorporation.

A new "Dynamic Range Enhancement" (DRE) within the oa-TOF-MS instrument extends the dynamic range for which exact mass measurements as well as ion intensity measurements can be made to up to four orders of magnitude. Merely choosing all scans across a mass spectral peak gives much improved ion statistics and more accurate mass measurements as well as ion intensities. This simplifies and renders calculations of isotope incorporations more reliable than previously possible.

Two different drug compounds from AstraZeneca have been studied: ^{2}H labelled felodipine (Calcium antagonist) and ^{2}H labelled metoprolol (β-receptor antagonist). The products were analysed using reversed phase HPLC coupled to oa-TOF-MS in non DRE and DRE mode, respectively.

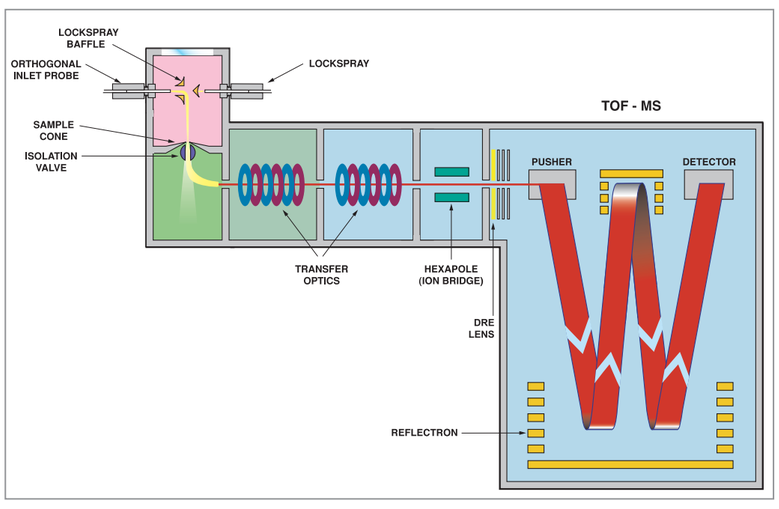

Figure 1. Oa-TOF schematic (W mode > 10000 FWHM).

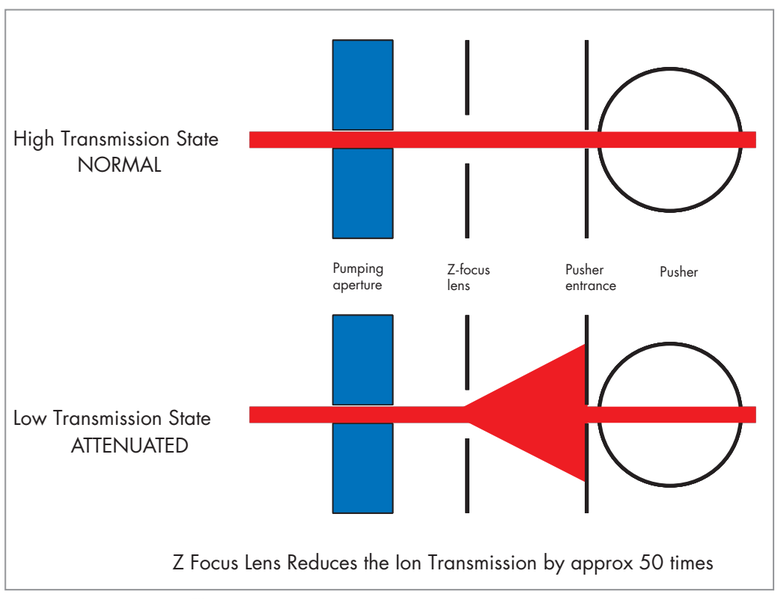

Figure 2. MarkerLynx™ Negative Ion PCA Plots.

EXPERIMENTAL

INSTRUMENTATION

Mass spectrometer:	Waters® Micromass® LCT™ and LCT Premier™
HPLC:	Waters Alliance® HT 2795
Column:	Symmetry® C_{18} (100 mm x 2.1 mm, 3.5 µm particle size)
Column temperature:	20 °C
Flow:	0.2 mL/min
Mobile phase:	A [H2O (0.1% Formic Acid)] B [MeCN]
Isocratic:	0–10 min: A (30%), B (70%)

MS

System 1:	LCT Premier
Ionization mode:	ESI+ at 3 kV
Sample cone voltage:	100 V
Desolvation temperature:	150 °C.
Reference mass:	Leucine enkephalin $[M+H]^+$ =556.2771
Acquisition parameters:	100–1000 m/z; 1 spectrum/second 0.1 second interscan delay
Resolution:	10000 FWHM (W mode)
System 2:	LCT
Ionization mode:	ESI+ at 3 kV
Sample cone voltage:	25V
Desolvation temperature:	150 °C.
Reference mass:	Leucine enkephalin $[M+H]^+$ =556.2771
Acquisition parameters:	100–1000 m/z; 1 spectrum/second 0.1 second inter scan delay
Resolution:	6000 FWHM (V mode)

RESULTS

The structures of the compounds analysed are shown in Figure 3. The required data selection procedure for LCT and LCT Premier is respectively illustrated in Figure 4. The exact mass spectrum obtained for the analysis of metoprolol is presented in Figure 5. The corresponding mass spectrum obtained for D5 labelled metoprolol is shown in Figure 6. From Figure 7 the exact mass spectrum for D3 labelled felodipine is illustrated. In Figures 8 a range of D2 to D8 incorporation can be seen for felodipine. Table 1 illustrates the reproducibility of the degree of incorporation that can be obtained using LC-oa-TOF-MS.

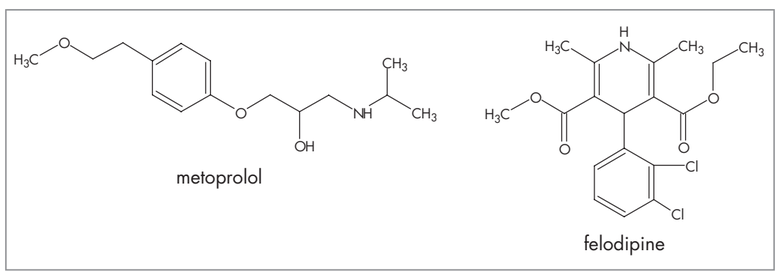

Figure 3. Structures of drug compounds analysed.

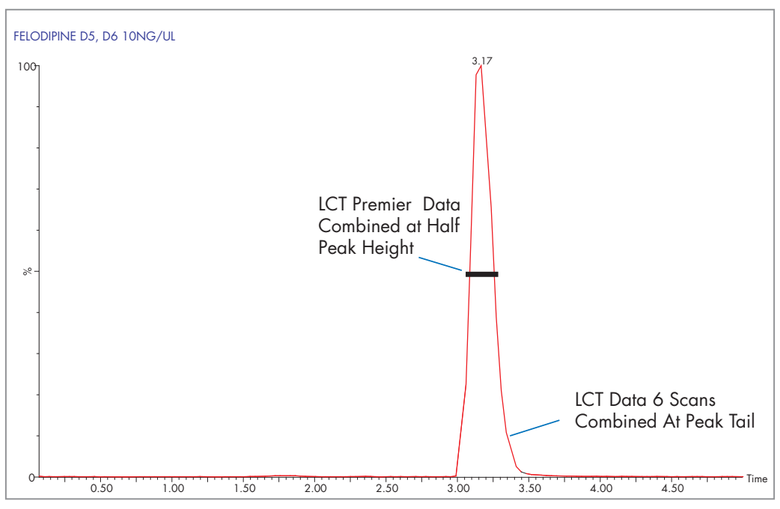

Figure 4. Illustration of data selection procedure required to obtain correct isotope inclusion ratios and exact masses.

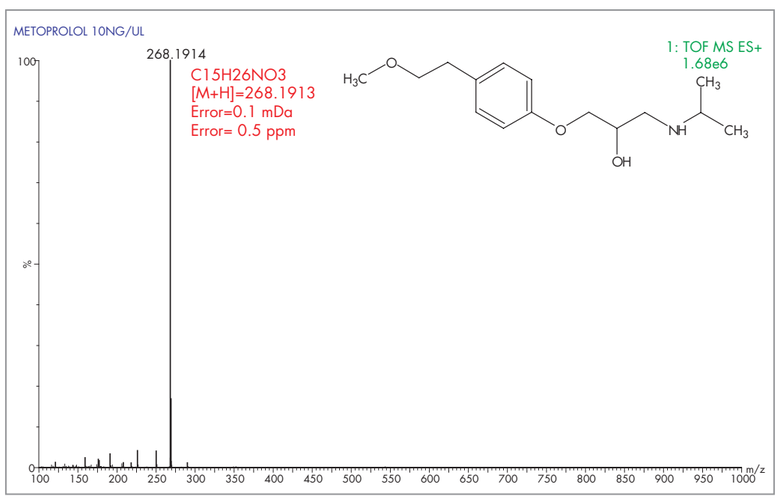

Figure 5. Exact mass spectrum for metoprolol.

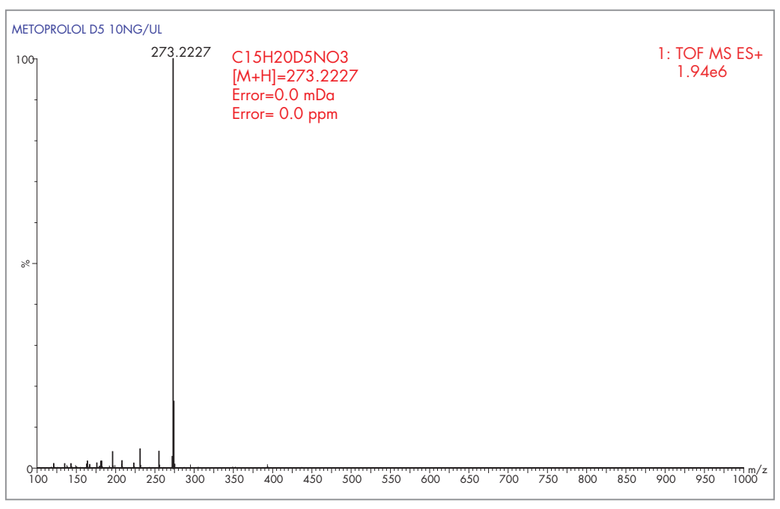

Figure 6. Exact mass spectrum for D5 labelled metoprolol.

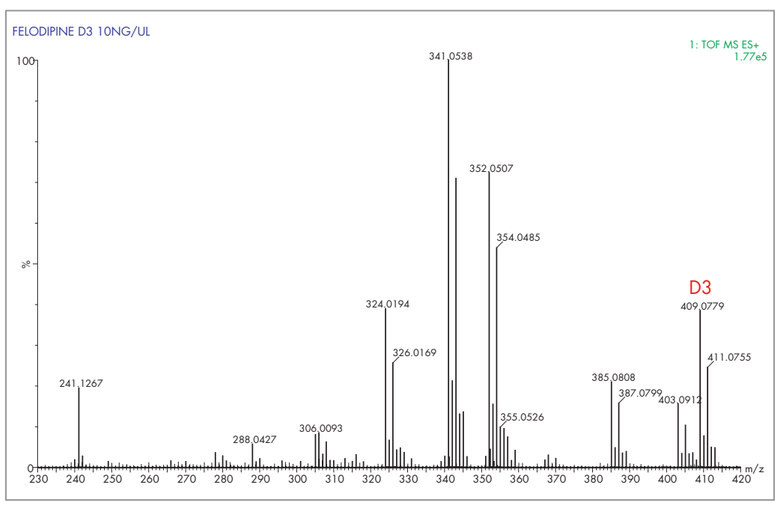

Figure 7. Exact mass spectrum of D3 felodipine.

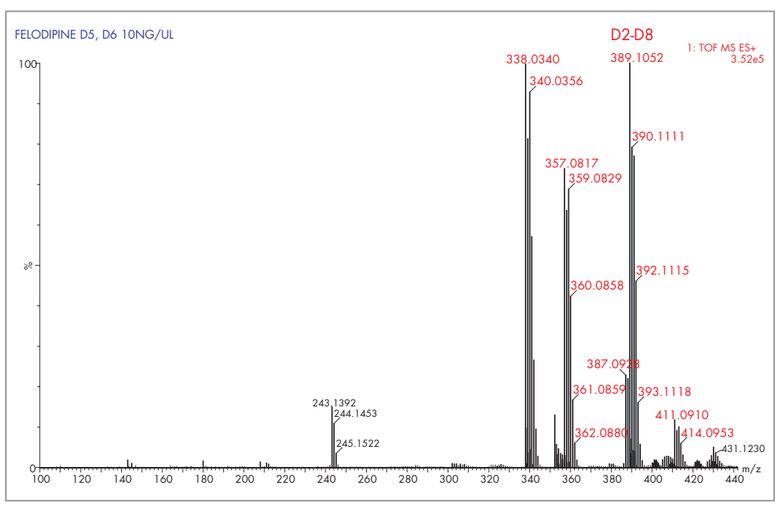

Figure 8. Exact mass spectrum of D5/D6 felodipine.

		LCT PREMIER						LCT	
		% INCORPORATION							
m/z	Incorporation	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 1	Run 2
386	2D	0	0	0	0	0	0	0	0
387	3D	12	12	12	13	12	13	11	14
388	4D	10	9	9	10	9	10	10	11
389	5D	45	46	45	45	45	45	42	42
390	6D	27	27	27	27	27	27	27	24
391	7D	6	5	6	6	6	6	7	7
392	8D	0	0	0	0	0	0	1	3

Table 1. Example of reproducibility of the calculated isotope inclusion for D5/D6 felodipine acquired using DRE functionality on the LCT Premier.

DISCUSSION

As shown in Figure 4 the procedure required to obtain the correct isotope distribution using the LCT was possible but could be challenging, due to having to select a section of the chromatographic peak where saturation of the detector was not taking place. If selected correctly the correct isotope ratios and exact masses could be obtained. This procedure could be time consuming, but reliable data would result. Using the LCT Premier's extended dynamic range functionality, saturation of the detector does not take place until very high ion counts, so it is possible to simply combine the whole of the chromatographic peak to produce a mass spectrum with the correct exact mass and isotope distribution. In this case the data was averaged at half height. The power of exact mass is illustrated in Figures 5 and 6, where the exact mass measurement of metoprolol is illustrated where the correct elemental composition was obtained within 0.5 ppm and a combined ion intensity of 1.7 million ion counts is shown. The exact mass spectrum of D5 labelled metoprolol is shown in Figure 6, where 0 ppm error has been obtained showing that the required isotope incorporation has been achieved, since the exact mass of protonated D5 metoprolol is m/z 273.2777. In this example the combined ion intensity is almost 2 million ion counts.

As can be seen in Figure 7, where the resultant mass spectrum of D3 felodipine is shown, the isotope patterns produced can be more complex because of the chlorine isotope. Figure 8 illustrates further the complex nature of the data that can be generated via isotope incorporation, here D5 and D6 incorporation was required. As can be seen from Figure 8, a range of incorporation has been achieved from D2 to D10. With the enhanced dynamic range no saturation of the detector has taken place. As well as the parent ion isotope cluster, two other isotope clusters were also observed due to felodipine being prone to thermal degradation. Thermal degradation was reduced by using a desolvation temperature of 120 °C for the LCT Premier, this compared to 150 °C used for the data acquisition illustrated for the LCT.

The reduced ion statistics of the LCT data is believed to be responsible for the minor differences in the levels of incorporation determined between the LCT Premier and LCT. Using the isotope model function within MassLynx™ Sofware the percentage theoretical isotope contribution was calculated and used to determine the actual isotope incorporation. Excellent reproducibility for the percentage incorporation determined is shown in Table 1. Six consecutive measurements were made using the LCT Premier, and they agree well with the results obtained using the LCT for the analysis of the same samples. In the future, this process may be further simplified if a desirable novel software development allowed for the automatic calculation of isotope incorporation. This would be achieved by automatic correction of the intensities by subtraction of contribution from A+1, A+2, A+3 etc contributions from non-labelled and previous labelled isotopes.

CONCLUSIONS

- Determination of accurate mass using DRE is much simplified compared to non-DRE mode; simple combination of the scans half the peak height in DRE mode can be utilised, whereas only scans at the slope of the peak can be used in non-DRE mode because of saturation at much lower concentrations.
- The isotope incorporation determined has been shown to be reproducible.
- Isotope incorporation can be determined with increased reliability, reproducibility and simplicity due to the enhanced dynamic range of the oa-TOF technology used.
- The highly specific nature of exact mass measurement enables elemental compositions to be easily and rapidly determined, giving complete confidence in producing the correct identification of the isotope incorporation.
- Mass measurement errors of <3 ppm have been obtained routinely.
- Combined ion intensities in the order of 2 million ion counts without detector saturation have been obtained.