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Importance of complete overlapping of analyte and internal standard peaks in eliminating matrix effects with Liquid Chromatography - Mass Spectrometry

Abstract

In the process of a Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS) assay development for two antimicrobial drugs, using their stable isotope labelled analogues as internal standards to correct for matrix/ion suppression effects, an unusually high scatter of data was observed. A systematic observation revealed that the analytes and their stable isotope labelled analogues (internal standards) were not co-eluting completely, and were therefore not experiencing matrix effects to the same extent. When a column with relatively lower resolution ability was used to achieve complete overlapping of the analyte and internal standard peaks, the scatter of LC-MS-MS data was minimised, indicating that the maximum correction of matrix effects by the internal standards occurs when they completely co-elute with the analytes. This work highlights the importance in ensuring complete overlapping of analyte and internal standard peaks in eliminating matrix effects when using stable isotope labeled analogues as internal standards, in LC-MS.

Keywords: LC-MS, Matrix effects, Ion suppression, Stable Isotope Labelled internal standard, co-elution

1

1. Introduction

In liquid chromatography coupled to mass spectrometry (LC–MS), the ion suppression/enhancement effects due to the sample matrix can significantly reduce or enhance the analyte response (1–6); ion suppression is more common than ion enhancement. When the analyte co-elutes with other compounds in the sample, the co-eluting compounds compete with the analyte for either the total available charge or the available surface area of the droplet in the interface of MS detector (6), leading to diminished MS detector signal of the analyte ion. The ion suppression effect has been shown to reduce the accuracy of an assay by as much as 26% (5). Approaches such as removing impurities by sample cleanup has not always been successful in removing the ion suppression effects (7-8). Sample clean-up procedures such as solid phase extraction removes compounds that are dissimilar in physicochemical properties, such as polarity and lipid solubility, to the analyte of interest from the sample. The compounds that are similar to the analyte of interest and that likely co-elute with the analyte are therefore not removed by the sample clean-up methods (9). Clearly, sample clean-up is not necessarily a solution for matrix effects. In addition, it has been shown that even trace levels of compounds in mobile phase solvents can cause ion-suppression (10). Therefore, prevention of matrix effects in LC-MS is often unattainable. The only practical option to obtain LC-MS data that are free from matrix effects is to perform a correction for matrix effects.

Correction for matrix effects is commonly achieved by using a specialized internal standard calibration procedure: The internal standard used must have almost exactly the same physicochemical properties as the analyte so that it will behave the same as the analyte both in the column and in MS detector. This condition is commonly achieved in LC-MS by using a stable isotope labelled (SIL) analogue of the analyte as the internal standard. This internal standard often co-elutes with the analyte but can be resolved from the analyte in LC-MS because of the slight difference in its mass from that of the analyte. As the SIL internal standard is expected to elute exactly at the retention time of the analyte, it was assumed to experience the same matrix/ ion-suppression effects as the analyte in its passage through the MS detector. Therefore, by using the internal standard calibration with a co-

29 eluting SIL internal standards, the analyte peak response is usually corrected for the ion suppression/
30 matrix effects.
31 As each analyte (eluting at a different retention time) is affected by different co-eluting impurities,
32 concentration of each analyte needs to be corrected by using its own co-eluting internal standard.
33 Therefore, a co-eluting SIL internal standard is required for the quantification of each analyte.
34
35 While developing an LC-MS-MS method for the determination of *N*-(3-oxododecanoyl)-L-
36 homoserine lactone (H) and fluconazole (F) using their stable isotope labelled analogues as internal
37 standards, we observed an unusually high scatter in LC-MS data. This manuscript describes the
38 systematic investigation of the reason for this scatter, and how the problem was solved.
39

40 **2. Experimental**

41 **2.1 Instrumentation**

42 Compounds were separated using an Agilent 1100 LC binary pump and Agilent 1100 autosampler
43 (Agilent Technologies, Santa Clara, CA, USA). The columns used were: Zorbax Extend-C18, 3.5µm
44 80Å (2.1 x 50mm) HPLC column (Agilent) for method 1, and Synergy 2µm Fusion RP 100Å (2.0 x
45 20mm) HPLC column (Phenomenex, Torrance, CA, USA) for method 2. An API 3000 tandem mass
46 spectrometer with a turbo ion spray interface and the software program Analyst 1.5 (Applied
47 Biosystems, Foster City, CA, USA) were used for detection and quantification.
48

49 **2.2 Materials**

50 Fluconazole (F) and *N*-(3-oxododecanoyl)-L-homoserine lactone (H) were purchased from Sigma (St
51 Louis, MO, USA). The stable isotope analogue of *N*-(3-oxododecanoyl)-L-homoserine lactone, *N*-
52 (12,12,12-d_3-3-oxododecanoyl)- L-homoserine lactone (Hd), was synthesized by Dr S. R. Chhabra
53 (Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK). The stable isotope
54 analogue of fluconazole, fluconazole-d_4 (Fd), was purchased from BOC Sciences (Shirley, NY,
55 USA). All solvents used were of HPLC grade.
56

57 **2.3 Preparation of Samples and standard solutions**

58 Stock solutions of analytes and deuterated analytes were prepared in acetonitrile and stored at -20°C.
59 Standard solutions for a six-point calibration curve were prepared to match analyte concentrations in
60 1000x dilution of samples (as described below), using 100µL of combined internal standard solution
61 (300µM *N*-(12,12,12-d_3-3-oxododecanoyl)- L-homoserine lactone and 40µM fluconazole-d_4 in 10%
62 acetonitrile aqueous solution), appropriate volumes of 100µM Fluconazole, appropriate volumes of
63 100µM and 3.36mM *N*-(3-oxododecanoyl)-L-homoserine lactone, and 10% acetonitrile to make the
64 final volume up to 1000µL. The concentrations of calibration standards were 10, 20, 30, 40, 50, 60
65 µM *N*-(3-oxododecanoyl)-L-homoserine lactone; 1, 2, 4, 6, 8, 10 µM fluconazole; 30µM *N*-(12,12,12-
66 d_3-3-oxododecanoyl)- L-homoserine lactone (in all six standards) and 4 µM fluconazole-d_4 (in all six
67 standards) in 10% acetonitrile.

68

69 **2.4 Preparation of mobile phase**

70 Mobile phase A consisted of deionised water containing 0.1% (v/v) formic acid, and mobile phase B
71 consisted of acetonitrile with 0.1% (v/v) formic acid. Both solutions were filtered through a 0.45µm
72 polytetrafluoroethene (PTFE) filter (Millipore, Bedford, MA, USA) before use.

73

74 **2.5 Chromatographic Conditions**

75 Separation was carried out at an ambient temperature of approximately 25°C. The flow rate was
76 200µL/min with an injection volume of 10µL. Following injection, analytes were separated using
77 gradient elution: Method 1 - mobile phase composition was changed from 10% B to 100%B during
78 the first 15 minutes, then held at 100%B for 2 minutes, before returning to 10% B from 17 to 20
79 minutes; the original composition of 10%B was maintained for the final eight minutes prior to the
80 next injection, Method 2 - mobile phase composition was kept at 12%B for the first 6 minutes,
81 changed from 12% B to 55%B during the next 3 minutes, held at 55%B for the next 6 minutes, then
82 changed to 100% over the next 2 minutes, held at 100% for 1 minute before returning to 12% B over
83 2 minutes; the original composition of 12%B was maintained for the final six minutes to equilibrate
84 the column prior to the next injection.

85

86 **2.6 Mass Spectrometry conditions**

87 Multiple Reaction Monitoring (MRM) was used in positive ion mode. The transitions of 298 m/z ion
88 → 102 m/z ion (for H) and 301 m/z ion → 102 m/z ion (for Hd); and 307 m/z ion → 238 m/z ion (for
89 F) and 311 m/z ion → 242 m/z ion (for Fd) were monitored for each chromatographic run. The MS
90 parameters were optimised for each analyte to obtain the highest sensitivity. The optimised values for
91 H were: orifice/ declustering potentials (DP) of 101V, ring/ focusing potentials (FP) of 370V,
92 collision energy (CE) of 19V, and collision exit potential (CXP) of 8V. The optimised values for F
93 were: orifice/ declustering potentials (DP) of 56V, ring/ focusing potentials (FP) of 330V, collision
94 energy (CE) of 23V, and collision exit potential (CXP) of 16V.
95 An ion spray voltage (IS) of 5000V and entrance potential (EP) of 10V were used. Curtain gas
96 (CUR), nebuliser gas (NEB) and the collision gas (CAD) flows were maintained at 12, 8 and 8 L/min
97 respectively. The temperature of the ion spray was maintained at 400ºC. A dwell time of 1000 msec
98 was used for all transitions. Resolution of both Q1 and Q3 were 1amu.

99

100

101 **3. Results and Discussion**

102 An unusually high scatter was observed with both H and F data obtained from the developed method
103 (method 1). A systematic study was therefore carried out to identify the reasons behind this
104 phenomenon. A combination standard containing H, Hd, F and Fd was injected and LC-MS-MS was
105 run six consecutive times, using method 1, which was the method in use at the time. The upper
106 chromatogram of Figure 1 shows peaks for H and F along with their co-eluting internal standards Hd
107 and Fd. The uppermost row of graphs in Figure 2 shows the individual peak areas of H and F, Hd and
108 Fd, and the peak area ratio of analyte/internal standard for H and F (all presented as percentage
109 deviation from the third of the six runs). It is clear from Figure 2 that the internal standards did not
110 correct for the differences in elution and detection conditions. The chromatograms (Figure 1 –top)
111 further revealed that there were slight differences in the retention times between analytes and internal
112 standards. The differential retention times between analytes and their deuterated analogues has been

113 previously observed (11-12), and attributed to the small difference in lipophilicity of the internal
114 standard and the analyte, due to deuteration. The difference is more pronounced with the F/Fd pair as
115 Fd has four deuterium atoms whereas Hd has only three. Due to the differences in the retention times,
116 the elution and detection conditions experienced by the analyte and the internal standard differ, and
117 the resultant ion-suppression effects experienced by the two compounds are dissimilar. This explains
118 the scatter in the data after correction with the internal standard (the ratio plot on the top right graph in
119 figure 2). The percent standard deviations for data in ratio plot 1 are: 6.67% (H/Hd), and 26.2%
120 (F/Fd), and those for ratio plot 2 are: 1.35% (H/Hd), and 1.37% (F/Fd).
121
122 In order to force the two peaks to co-elute, various gradients were investigated. However, the
123 differences in lipophilicities and the resolution capability of the column were too high to achieve co-
124 elution simply by changing the elution conditions. Therefore, a column with lower resolution capacity
125 was used to promote the overlap but still achieve similar retention of both analytes. By using an
126 appropriate gradient with the new column (method 2), we achieved better overlap of peaks (bottom
127 chromatogram in Figure 1). The bottom row of graphs in Figure 2 shows the effect of peak
128 overlapping: although there is observable scatter in individual peak areas in the first two graphs in the
129 second row, there is insignificant scatter in the third graph (the ratio of analyte to internal standard)
130 indicating the intended function of the internal standard. The extent of overlap in Figure 1 (top vs
131 bottom) is suggested as the direct cause of the extent of scatter in the last column of Figure 2 (top *vs.*
132 bottom). Thus, it is clear that despite the seemingly very small difference in peak overlap, its impact
133 on the accuracy and precision of the data is very significant.
134 It is, therefore, advisable to ensure precise co-elution by periodic surveillance of chromatograms
135 obtained in routine LC-MS methods as well as in method development since slight changes in column
136 and eluents/solvents may affect the extent of co-elution. It is also recommended to examine and
137 ensure the linearity of analyte and the internal standard (separately) responses, within the
138 concentration ranges expected, when using analyte/internal standard response ratio for calibrations
139 (13).

140 To obtain a significant mass resolution and to prevent cross-talk, the masses between the analyte and
141 the internal standard must differ at least by 3 amu (12). As observed in our study, replacement of three
142 H atoms by deuterium can change the lipophilicity of the molecule to a significant extent depending
143 on the column.
144 The problem of differential lipophilicity that affects the change in retention on reversed phase can be
145 minimized by using alternative (to deuterated) stable isotope labelled analogues such as C-13, N-15 or
146 O-17 as internal standards (11-12). As analysts who develop LC-MS methods are well aware,
147 sourcing a SIL internal standard is a challenge, and is often expensive. Most common types of SIL
148 internal standards available are deuterated analogues. The only other effective alternative to using SIL
149 internal standards, to correct for matrix effects in LC-MS, is to use the standard addition with internal
150 standardization (14).
151
152 **4. Conclusion**
153 The effect of incomplete co-elution of the analyte and SIL internal standard on the scatter and the
154 accuracy of the LC-MS data was studied. The compound deuteration affects the retention of analytes
155 on reversed phase chromatography, causing the analyte and its deuterated analogue to separate
156 slightly. This separation lead to incomplete co-elution of the analyte and SIL internal standard, and
157 consequently differential matrix effects on the analyte and the internal standard, giving rise to
158 scattered and inaccurate results with internal standard calibration. Using a column with reduced
159 resolution to achieve co-elution of analyte and the deuterated internal standard proved to be an
160 effective method in overcoming the problem.
161
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164

165

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185

186

187 **Figure Captions**

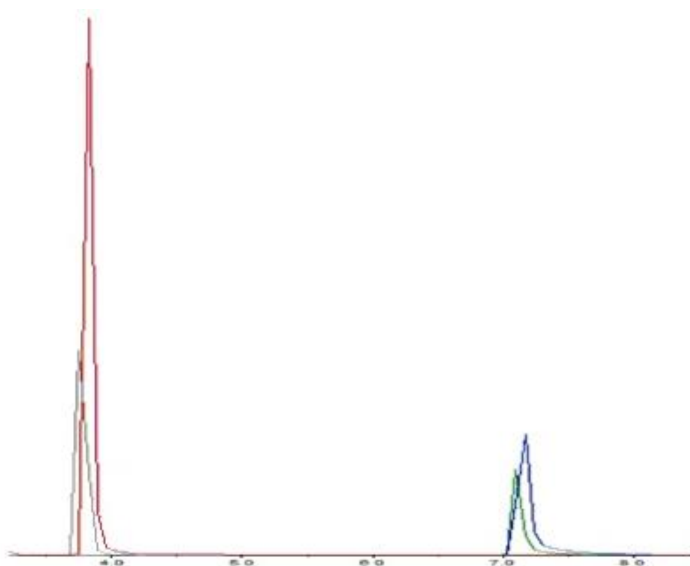
188 **Figure 1**: Chromatograms (scales adjusted to comparable peak sizes) showing the extent of co-elution
189 of Fluconazole with its deuterated analog (early peaks), and Homoserine Lactone with its deuterated
190 analog (late peaks). Top chromatogram is with method-1 and the bottom with method-2. Top
191 chromatogram shows that the (deuterated) internal standards have slightly longer retention times than
192 the analytes F and H.

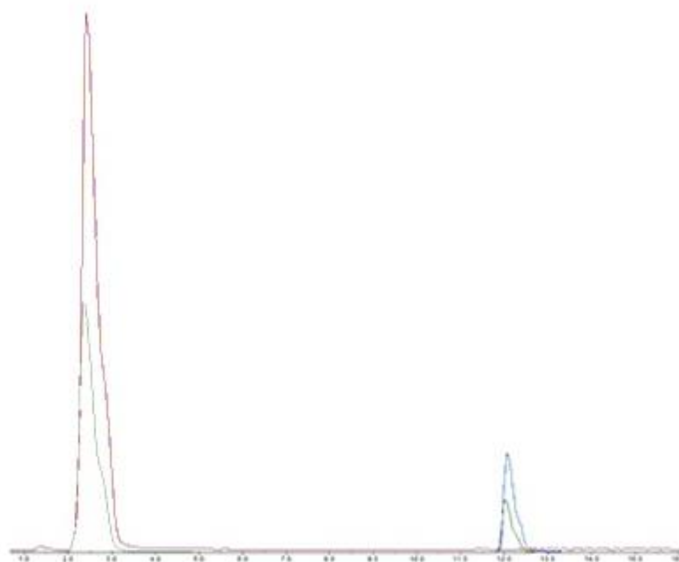
193

194 **Figure 2**: Peak areas (first 2 graphs of both top and bottom), and peak area ratios of analyte/internal
195 standard (third graphs of both top and bottom) of H (Homoserine Lactone), Hd (deuterated
196 Homoserine Lactone –internal standard), F (Fluconazole) and Fd (deuterated Fluconazole –internal
197 standard), presented as percentage deviation from the respective peaks in the 3rd chromatogram of six
198 successive chromatograms run using identical injections of a standard solution containing H, Hd, F
199 and Fd. H and Hd are presented as solid bars (first bar in each pair of bars); F and Fd are presented as
200 patterned bars (second bar in each pair). The top row of figures contain data using method-1 and the
201 bottom raw using method-2. The percent standard deviations for data in ratio plot 1 are: 6.67%
202 (H/Hd), and 26.2% (F/Fd), and those for ratio plot 2 are: 1.35% (H/Hd), and 1.37% (F/Fd).

203

204 **Figure 1**





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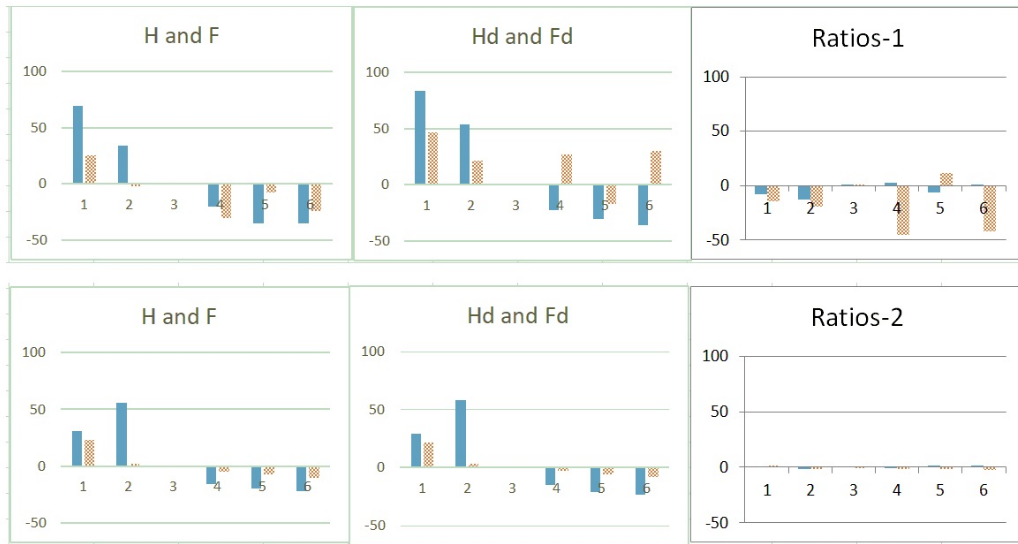
211 **Figure 2**



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