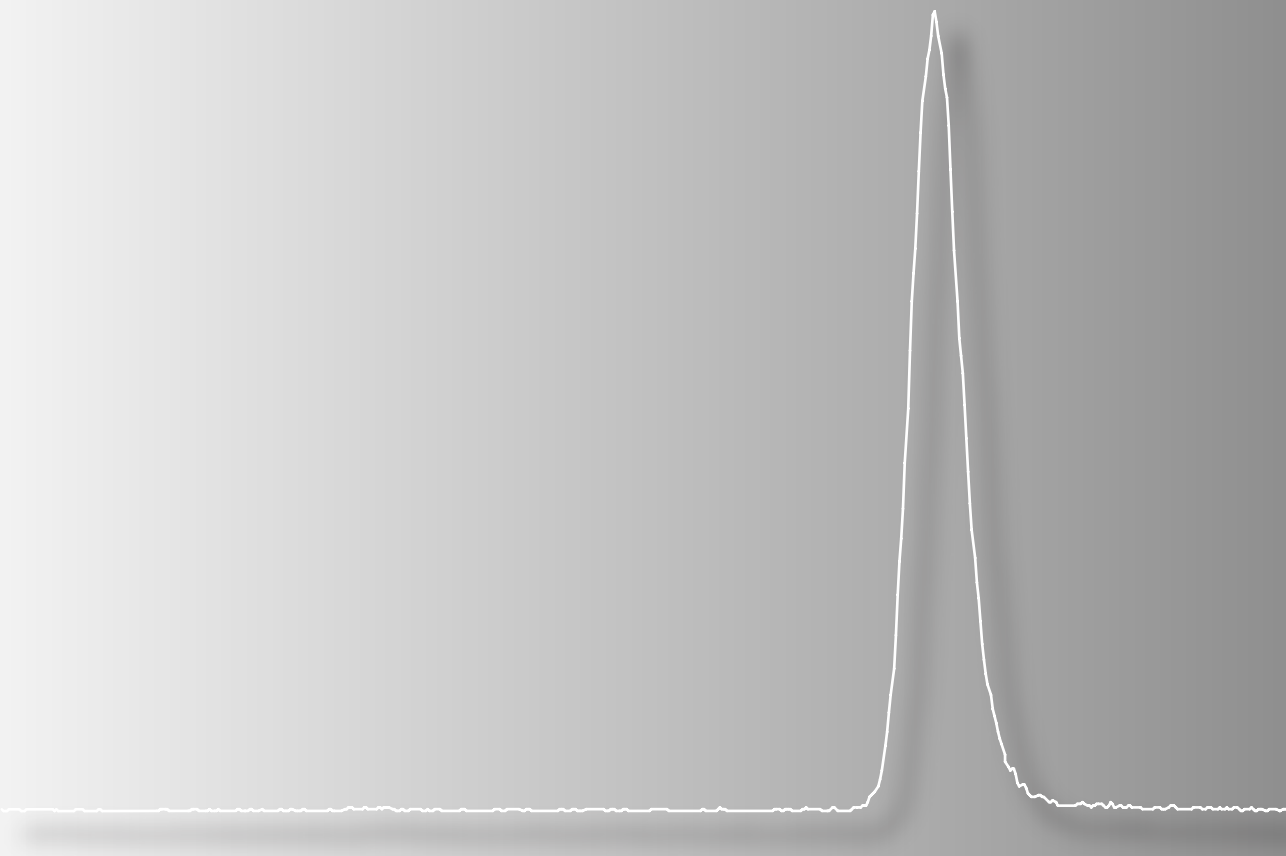
Chapter 6



Internal Standard Studies

6.1

Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography - mass spectrometry: necessity or not?

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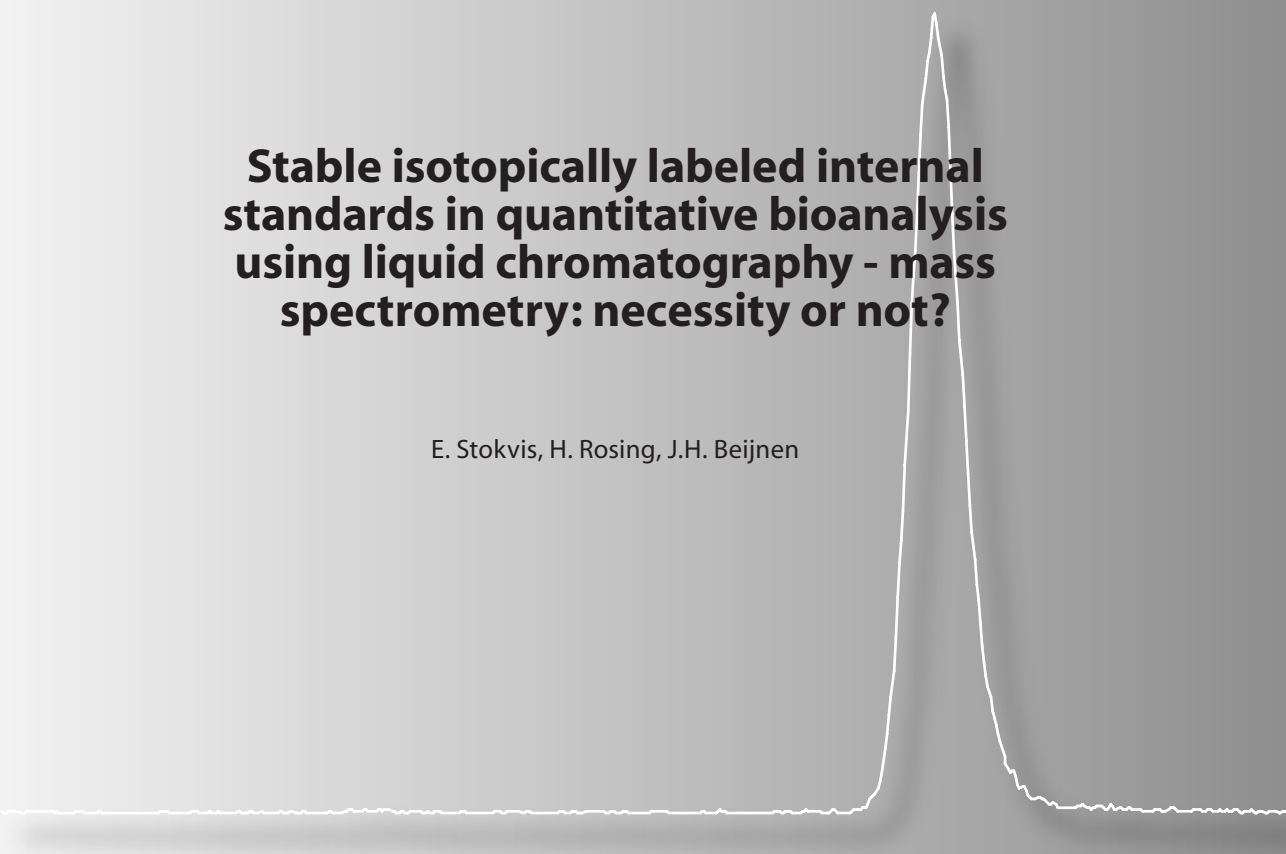


Submitted

Abstract

It appears a general belief that stable isotopically labeled (SIL) internal standards yield better assay performance results for quantitative bioanalytical liquid chromatography mass spectrometry (LC-MS) assays than any other internal standard. In this article we describe our experiences with structural analogues and SIL internal standards and their merits and demerits. SIL internal standards are the first choice, but deuterium labeled compounds may demonstrate unexpected behavior, such as different retention times or recoveries than the analyte. In addition, a SIL internal standard with identical chemical properties as the analyte may cover up assay problems with stability, recovery, and ion suppression. Since SIL internal standards are not always available or very expensive, structural analogues can be used, however with consideration of several issues, which usually display during method validation.

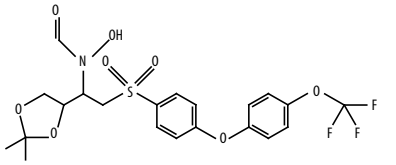
Introduction

The implementation of internal standards in quantitative bioanalysis is an accepted and commonly used procedure. An internal standard (correct name ´processed internal standard`) is meant to correct for variability in dilutions, evaporation, degradation, recovery, adsorption, derivatization, and instrumental parameters such as injection volume, and even more so for gas chromatography (GC) than liquid chromatography (LC) based assays. With the introduction of LC - mass spectrometry (MS) for quantitative bioanalysis, the purpose of an internal standard has become mainly to correct for errors of detection [1].

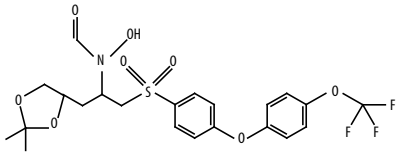
MS detection represents unrivaled sensitivity and selectivity and was therefore a clear candidate for LC based quantitative assays, although MS is not quantitative by nature. When a compound is introduced into the ion source only a portion of the total number of molecules is ionized. This portion (or ionization efficiency) depends largely on the molecular structure of the compound, but, in addition, may vary during day-to-day operation as a result of several parameters that are difficult or nearly impossible to control, such as temperature and pressure of the ion source, and the performance of the detector. Therefore, internal standards are essential in quantitative assays employing MS detection, since instrumental changes are made largely irrelevant because they affect only absolute responses, not ratios [2].

Quantitative detection using MS is further complicated by the effect of matrix components, for instance plasma or urine constituents. When the analyte is introduced into the ion source it will compete for ionization with other compounds introduced into the source simultaneously. Matrix components are infamous for decreasing the analyte signal, so called ion suppression, especially in electrospray ionization (ESI) based MS detection. The degree of ion suppression caused by matrix components may vary largely between matrices. Unfortunately, the degree of ion suppression caused by matrix components also depends on the analyte's structure. This means that if an analyte and internal standard

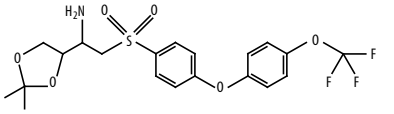
are not sufficiently similar in structure, the ratio of analyte and internal standard detector response may vary as a result of different degrees of ion suppression, thus compromising the quantitation. Therefore, internal standards in quantitative bioanalytical LC-MS assays are either structural analogues or stable isotopically labeled (SIL) analogues of the analyte. Structural analogues may differ in functional groups or backbone structure from the analyte, but rules for their structures have not been defined. SIL internal standards are compounds in which several atoms are replaced by their stable isotopes, such as ^{2}H (D, deuterium), ^{13}C, ^{15}N, or ^{17}O. Labeling with three to eight ^{2}H or ^{13}C atoms or a combination of both is most common. Since a compound and its SIL analogue will theoretically co-elute, it is important that the mass difference between the compounds is at least 3 amu, in order to be able to separate them in the mass analyzer and to prevent "cross-talk". When the difference is less than 3 amu the isotope peaks of the analyte may interfere with the signal of the internal standard. Furthermore, the SIL internal standard should be pure enough to prevent any contributions to the analyte response. SIL internal standards are the preferred internal standards for MS detection since they are chemically identical to the analyte, but unfortunately they are not always available. Interestingly, although it is common knowledge that a SIL internal standard provides better assay performance results than any other internal standard, not many studies have been performed to demonstrate this [3]. On the other hand, several assays have been reported describing the use of an analogous internal standard with excellent results and there are reports describing the disadvantages of SIL internal standards [1,4,5]. In this article we have compared the use of structural analogues and SIL internal standards for several (investigational)



ABT-518



Internal standard



Metabolite

Figure 1. Structures of matrix metalloproteinase inhibitor ABT-518, its analogous internal standard, and a metabolite found in human plasma.

anticancer agents. Merits and demerits of the internal standards in our experiences with these assays are discussed.

Case studies

Assay performance

During the validation and routine use of a bioanalytical assay accuracy and precision are of major importance [6]. Therefore, the comparison of analogous and SIL internal standards was performed initially by means of those two parameters.

For the determination of the matrix metalloproteinase inhibitor ABT-518 an analogous internal standard with an additional internal methylene moiety in the backbone structure was available (Figure 1). This internal standard showed to be appropriate for a correct quantification of the drug in human plasma. A validation was executed according to the FDA guidelines on bioanalytical method validation [6] and results were always within requirements [7]. The assay, however, also included metabolites. One metabolite (Figure 1) is formed after reduction of the N-hydroxy moiety followed by hydrolysis of the amide. For the quantitative determination of this metabolite no dedicated internal standard was available and thus it was attempted to use the same as for the parent drug. This internal standard, however, was not suitable for the determination of the metabolite, which was reflected in the unacceptable validation results (accuracy >15%, precision >15%). In fact, the best results were obtained without the use of any internal standard. From equimolar amounts of ABT-518, its metabolite and internal standard a Q1 spectrum was recorded (Figure 2). Under the same MS conditions response for the metabolite was approximately

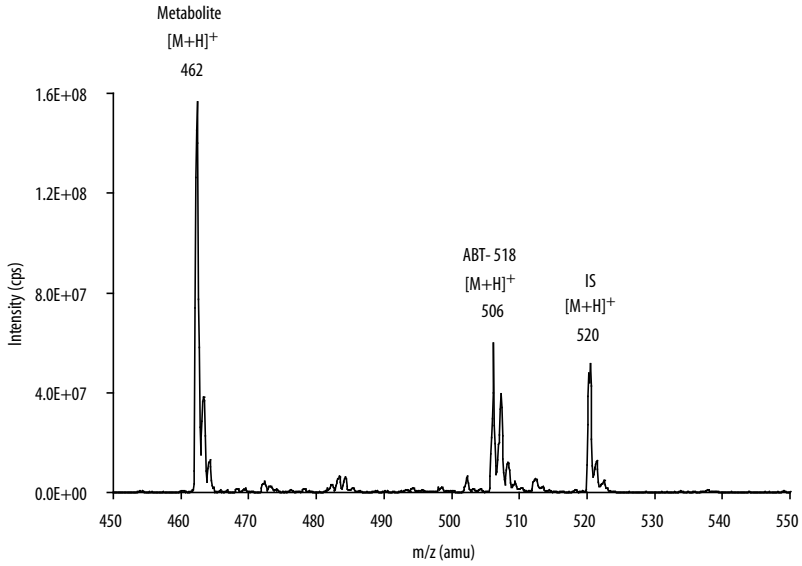


Figure 2. Q1 spectrum of ABT-518, the analogous internal standard (IS) and a human metabolite recorded from equimolar amounts.

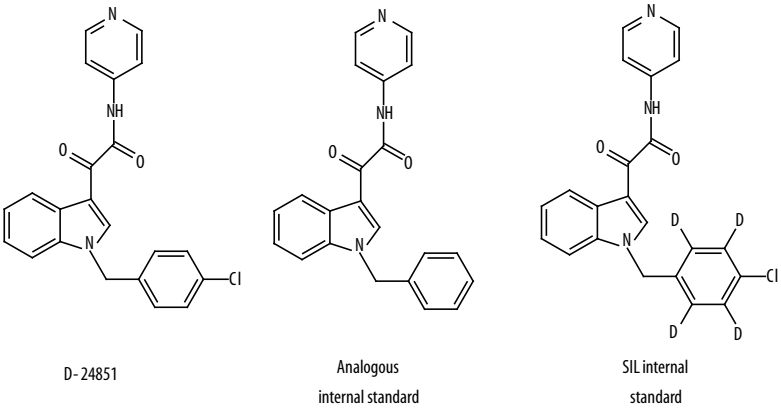


Figure 3. Structures of tubulin inhibitor D-24851, the analogous internal standard, and the SIL internal standard.

four times higher than for the internal standard, which can be attributed to the higher proton affinity of the amine moiety in the metabolite than of the amide group present in the internal standard molecule. The parent drug and internal standard, however, show similar ionization efficiencies [7].

During development of an assay for the tubulin inhibitor D-24851 (Figure 3) only an analogous internal standard was available lacking the chlorine atom at the benzyl moiety. This internal standard appeared inappropriate for use in the assay as is demonstrated in Table 1 (accuracy >15%, precision >15%). Quantitation without the use of an internal standard yielded even better results. On the other hand, for an LC-UV assay for the determination of D-24851, this internal standard was suitable (unpublished data). This suggests that the problems with accuracy and precision are caused by ion suppression rather than differences in extraction recovery. Apparently, the presence of the chlorine atom has a profound influence on the analyte's charge distribution which results in unacceptable

Table 1. Accuracy and precision data from calibration standards of D-24851 analyzed using different internal standards

	Analogous internal standard			**No internal standard**			**SIL internal standard**		
Conc. (ng/mL)	**Accuracy (%)**		**Precision (%)**	**Accuracy (%)**		**Precision (%)**	**Accuracy (%)**		**Precision (%)**
	1	2		1	2		1	2	
1.00	12.9	-22.9	25.3	11.9	4.95	4.90	-2.92	-0.604	1.64
5.00	13.9	-22.9	26.0	-24.6	-3.37	15.0	4.23	10.5	4.42
25.0	23.8	-13.5	26.4	2.38	-4.37	4.77	6.05	5.65	0.285
101	14.9	-7.72	16.0	5.94	-1.88	5.53	5.74	9.77	2.85
252	27.8	-14.3	29.7	26.2	-2.78	20.5	0.403	0.403	0.00
1010	-13.2	-30.5	12.3	-12.9	-6.93	4.20	-11.5	-8.06	2.42
Sum.	106	112	136	83.8	24.3	54.9	30.8	35.0	11.6

Conc. Concentration; Sum. Absolute summation of the relative accuracy and precision values

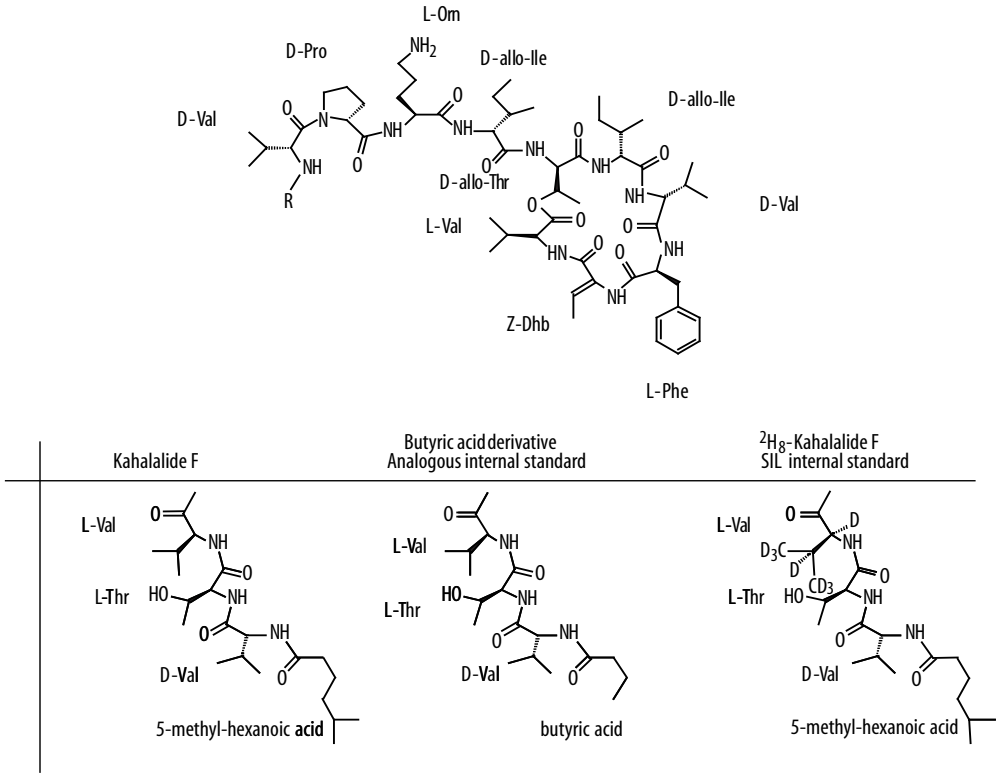


Figure 4. Structures of depsipeptide kahalalide F, the analogous internal standard, and the SIL internal standard.

differences in ionization efficiency between analyte and internal standard. The quadruply deuterated SIL internal standard yielded excellent results (Table 1).

For the LC-MS/MS assay of the depsipeptide marine anticancer agent kahalalide F, which contains a 5-methyl-hexanoic acid conjugated to the N-terminal, a butyric acid analogue was available as internal standard (Figure 4) [8]. The assay was validated according to the FDA guidelines and all data were within requirements. When a SIL D_8-internal standard became available, it was implemented into the assay. Re-validation results fulfilled all FDA requirements. The data was also statistically evaluated to determine whether the implementation of the SIL internal standard had influenced the assay performance. Calculated concentrations were documented for calibration standards and quality control samples as determined using the two methods. A Levene's test for equality of variances was performed followed by an independent samples t-test to compare the means. The mean bias was 96.8% for the use of the analogous internal standard with a standard deviation of 8.6% (n=284) and 100.3% for the SIL internal standard with a standard deviation of 7.6% (n=340). The Levene's test showed that the variance using the SIL internal standard was significantly lower (p=0.02) than with the use of the butyric acid analogue, indicating that the precision of the method has significantly improved by implementation of the SIL

internal standard. In addition, the accuracy of the assay has improved significantly, since the bias using the SIL internal standard did not deviate significantly from the true value of 100% (p=0.5) while the bias using the analogous internal standard did (p<0.0005).
The results of these studies suggest that when analyte and analogous internal standard differ in functional groups, the internal standard is less likely to be appropriate than when the difference is in the carbon backbone of the molecule. Atoms such as oxygen, nitrogen, sulfur, halogens, etc, are more likely to alter a compound's charge distribution, and as a result ionization efficiency, than carbon-hydrogen moieties. Statistics showed that the performance of the kahalalide F assay improved significantly after substitution of a well functioning analogous internal standard with a SIL internal standard. These experimental data underline the theoretical superiority of SIL internal standards over analogous internal standards.

Stability

Although a positive effect of the implementation of a SIL internal standard is probably most appreciated in terms of accuracy and precision, there may be other reasons for the preference of SIL internal standards. When the butyric acid analogue of kahalalide F was used as internal standard, stability of the drug in the processed extract was limited to utmost 16 h at both ambient and refrigerated temperatures because the analogous internal standard demonstrated a higher rate of degradation in the processed extract than kahalalide F, resulting in an overestimation of the analyte concentration [8]. Following the

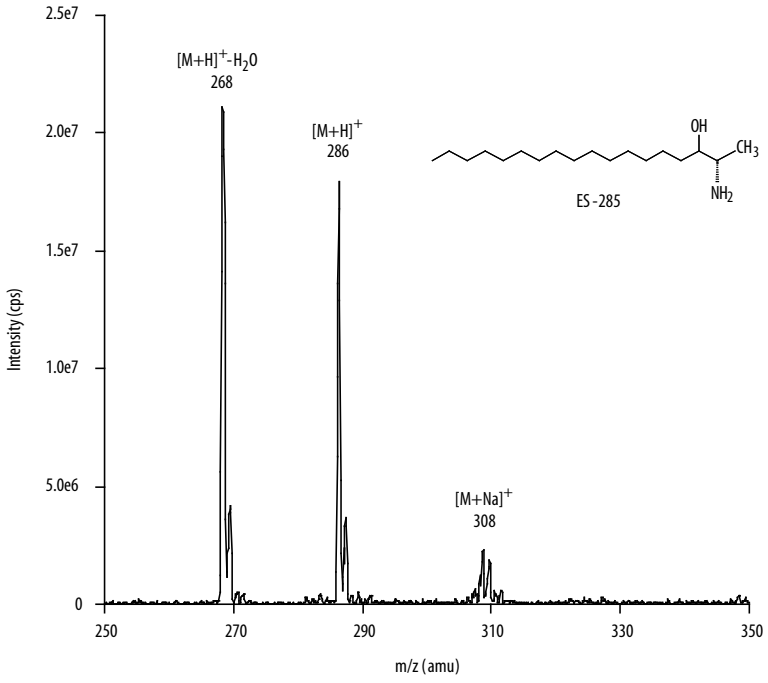


Figure 5. Q1 spectrum of ES-285

implementation of the more stable SIL internal standard, the time of stability in the final extract could be prolonged to at least 5 days at ambient temperatures and at least 40 days at refrigerated temperatures.

Unusual mass transitions

Anticancer agent ES-285 (2-amino-3-hydroxy octadecane) is a molecule with a lipid-like structure. For quantitation of ES-285 the transition from the molecular ion to a fragment ion corresponding to the elimination of water from ES-285 was monitored in MRM mode. The only peak in the product ion mass spectrum corresponded to this fragment ion. This transition was not considered to be robust for a correct quantification, especially since this ion is also present in the Q1 spectrum of ES-285 and thus formed also in the source (Figure 5). In fact, the dehydrated ES-285 peak is the base peak in the Q1 spectrum, which indicates that elimination of a water molecule from ES-285 happens readily. As a result of this the peak area ratio of ES-285 and dehydrated ES-285 present in Q1 may vary too much as a result of small changes in instrument parameters which hampers a correct quantitation of ES-285. Selected ion monitoring (SIM) was tested as an alternative for MRM monitoring of the described transition, but was discarded due to sensitivity problems. Fortunately, a triply deuterated SIL internal standard became available, which allowed the mass transition from the molecular ion to the fragment ion corresponding to the elimination of water to be used. Plasma was spiked with ES-285 and after processing, a sample was analyzed 60 times, and variation in the ratio (by means of the relative standard deviation) was only 4.9%. It was believed that this robustness could not have been obtained using an analogous internal standard [9].

Potential metabolites

An important factor to consider in the selection of an analogous internal standard is whether this compound may be formed *in vivo* by metabolic reactions or degradation of the drug. For instance, for the determination of the marine anticancer agent ET-743 (Trabectedin, Yondelis™) a structural analogue, ET-729, was available, corresponding to *N*-desmethyl-ET-743. ET-743, however, may undergo N-dealkylation to form ET-729. Therefore, many clinical plasma samples were analyzed without addition of an internal standard for the presence of ET-729. ET-729 was not detected and could therefore be utilized as internal standard in the bioanalytical assay of ET-743 [10].

Deuterium labeled internal standards

It is generally assumed that an analyte and SIL internal standard have equal physico-chemical properties. Deuterated compounds, however, may show unexpected results. Both Wieling and Kato et al. report on different retention times for the analyte and deuterated internal standard from reversed phase LC (Figure 6) [1,4]. In both cases the deuterated internal standard eluted first. Wieling's explanation for this phenomenon is that deuteriums have a stronger binding with carbon atoms than hydrogens, thereby introducing small differences in physico-chemical properties [1]. Kato et al. describe that they only observed differences in retention times when a neutral eluent was applied (ammonium

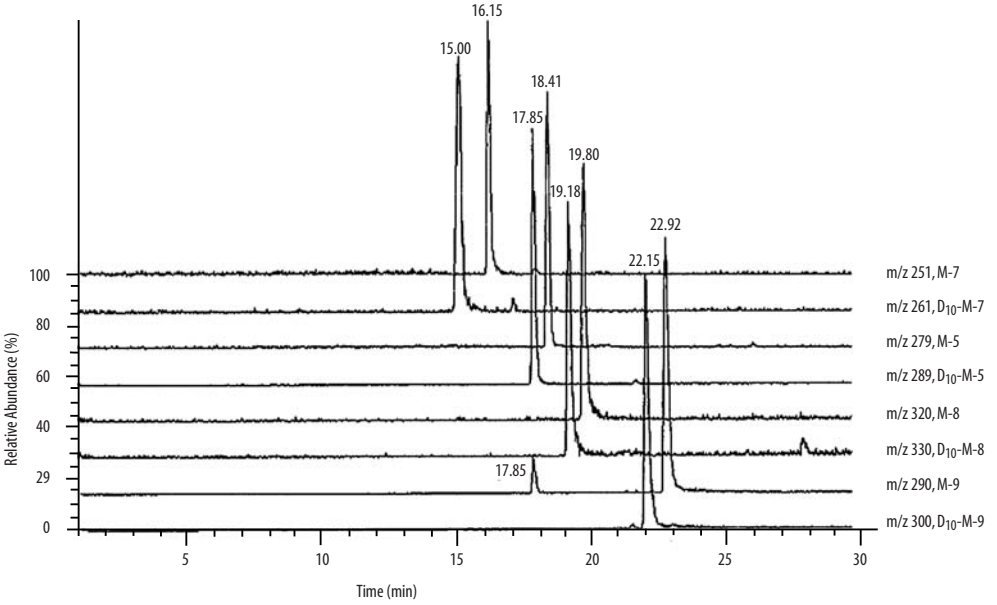


Figure 6. Reversed phase LC separation of pibutidine metabolites and their deuterium-labeled analogues [4].

Table 2. Hydrogen/deuterium exchange of the $^{13}CD_3$ and $^{13}C_7$-labeled rofecoxib [5]

Experiment	**Content (%) of unlabeled and labeled rofecoxib**				
	Unlabeled	**$^{13}CH_3$**	**$^{13}CDH_2$**	**$^{13}CD_2H$**	**$^{13}CD_3$**
A. $^{13}CD_3$ rofecoxib					
1. Standard in cyclooctane					>99.5
2. Standard in acetonitrile	0.24	17.33	7.15	9.66	65.62
3. Spiked in human plasma, stored:					
a. 0 h	0.50	15.39	4.39	4.19	75.53
b. 3 h	0.27	15.28	4.22	4.43	75.80
c. 6 h	0.28	19.76	4.11	4.91	70.94
	Unlabeled	**$^{13}C_5$**	**$^{13}C_6$**	**$^{13}C_7$**	
B. $^{13}C_7$ rofecoxib					
1. Standard in cyclooctane	0.0	0.2	5.6	94.2	
2. Standard in acetonitrile	0.0	0.24	5.43	93.95	
3. Spiked in human plasma, stored:					
a. 0 h	0.0	0.24	5.40	94.36	
b. 3 h	0.0	0.11	5.56	94.32	
c. 6 h	0.0	0.24	5.46	94.33	

acetate (20 mM, pH 7.0)–methanol-acetonitrile). When an acidic solvent system was used, co-elution of analyte and deuterated internal standard was established [4].

Another phenomenon observed by Wieling for deuterated internal standards was a different extraction recovery in the case of haloperidol and deuterated haloperidol, the latter's recovery being 35% lower [1]. This difference may also result from differences in physico-chemical properties but, in addition, may be due to exchange of part of the deuterium atoms by hydrogen atoms. Normally, deuterium atoms that are covalently linked to carbon atoms are not easily exchanged, however Chavez-Eng et al. describe deuterium exchange for the deuterated internal standard of rofecoxib (Figure 7a and b; Table 2) [5]. For comparison the sample was dissolved in cyclooctane and analyzed using gas chromatography. The results demonstrated the purity of the standard and thus that the presence of partially or unlabeled internal standard observed in other solutions is not due to a contamination of the reference standard. The SIL internal standard ($^{13}CD_3$-rofecoxib) demonstrated a loss of deuterium in acetonitrile solutions, which is according to the authors probably due to the traces of water usually present in acetonitrile [5]. Exchange into the $^{13}CH_3$ species was mainly observed. In plasma, exchange was observed to a lesser extend, but was still substantial. After six hours of incubation of $^{13}CD_3$-rofecoxib in human plasma at room temperature, the amount of $^{13}CH_3$-rofecoxib has increased 28%. These results clearly indicated that $^{13}CD_3$-rofecoxib was not suitable as an internal standard in the bioanalytical assay. Another SIL internal standard for rofecoxib was available, $^{13}C_7$-rofecoxib (Figure 7c). Similar experiments were performed as described for the deuterated internal standard and the $^{13}C_7$-labeled internal standard did not show

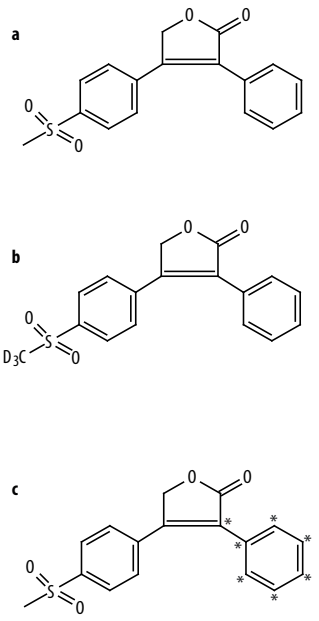


Figure 7. Structures of rofecoxib (a) and the $^{13}CD_3$ (b) and $^{13}C_7$ (c) labeled internal standards. The asterisks correspond to ^{13}C atoms.

degradation into the unlabeled or partially labeled species. ^{13}C labeling is clearly preferred over D incorporation.
The results of this study suggest that SIL internal standards can not be seen as one class of compounds. Deuterium labeled internal standards may demonstrate unwanted behavior, that can compromise a correct quantitation of the analyte.

Matrix effects
It is considered very important that an analyte and its analogous internal standard co-elute in order to ensure the same amount of matrix effect for the two compounds. Otherwise, different degrees of ion suppression may be imposed on the two compounds. Interestingly, Sancho et al. demonstrated in a mechanistic study on xenobiotics that co-elution of analyte and analogous internal standard in the presence of high matrix levels is less appropriate for a correct quantitation than non co-elution when matrix levels are low [11]. This unexpected effect may result from high matrix levels that enlarge any differences in ionization efficiency between the two analogous compounds, while this is minimized with low matrix levels. The data described by Fu et al. in their manuscript investigating the influence of matrix effects on the determination of the HIV protease inhibitor indinavir support this theory. They state that when structural analogues are utilized as internal standard, they may only partially compensate for variable ionization effects caused by matrix components when minimum sample preparation or little chromatographic separation of the compounds from matrix components is applied [3]. These findings may suggest that an analogous internal standard is less suitable for high throughput analyses.
On the other hand, Jemal et al. demonstrated that low background matrix levels are essential as well for a correct quantitation of melvalonic acid using a SIL internal standard in urine [12]. In one batch of urine the matrix effect was 26% higher for the deuterated internal standard than for the analyte, which could only be corrected by developing a method that displayed low matrix levels. The observed problems with the consistency of the analyte/internal standard response ratio may result from different chemical properties for deuterated and their unlabeled species, as was described above.

Miscellaneous
For quantitative LC-MS/MS a triple quadrupole mass analyzer in the MRM mode is commonly used. This mode ensures unrivaled sensitivity and selectivity. However, it is important to realize that much more is happening than can be seen in the ion chromatogram. Internal standards in general, but especially SIL internal standards can effectively cover-up analytical problems with for instance instability or ion suppression and recovery. For a bioanalytical assay it is essential to determine ion suppression as a part of the validation. Preferably, ion suppression, but also extraction recovery, are determined by comparison of absolute peak areas, since ratios can theoretically be 100% even though the absolute ion suppression may be >90%, which may cause sensitivity problems. Thus, apart from optimal assay performance, sample clean-up is also very important, e.g. for assay sensitivity.
Stability of the analyte and internal standard in the processed extract can give similar

problems. When employing an appropriate internal standard the ratio of analyte and internal standard responses may demonstrate stability, however, the absolute areas may have decreased so dramatically that the LLOQ level falls below the detection limit of the assay.

Conclusion

In this article several merits and demerits of analogous and SIL internal standards in several bioanalytical quantitative LC-MS assay have been discussed. SIL internal standards are preferred for a correct quantity determination using LC-MS and LC-MS/MS assays. ^{13}C, ^{15}N or ^{17}O labeled compounds may be more appropriate that deuterium labeled compounds. However, SIL internal standards are not always available or very expensive, especially when seeking exclusively non-deuterium labeled SIL internal standards. Then, structural analogues can be used, with consideration of the structural similarities between the internal standard and the analyte.

Finally, it is important to realize, especially for less experienced operators that when using SIL internal standards problems with for instance stability, extensive ion suppression or low extraction recoveries may not be observed in the MRM mode, but may cause sensitivity problems. It is advised to determine recovery and ion suppression during the validation of the method.

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