Norwegian Institute of Public Health

Experiences with ^{13}C and ^{2}H labeled internal standards for the reversed phase UPLC-MS/MS analyses of drugs in biological samples

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BACKGROUND

Reversed phase (RP) LC-MS/MS is commonly used for qualitative and quantitative detections of drugs in human biological samples. Stable isotope labeled internal standards (SIL ISs) are often used to ensure these detections. Ideally an SIL IS behaves identically as its corresponding analyte during sample preparation and LC-MS/MS analysis.

SIL ISs used for drug analysis are usually deuterium (^{2}H) labeled. However, ^{2}H labeled ISs and their corresponding analytes are not always co-eluting, which in turn reduces the IS`s ability to correct for ion suppression effects. There is a greater difference in physical-chemical properties between H isotopes than between isotopes of other elements. ^{13}C-labelled ISs are more similar to their corresponding analytes than ^{2}H labeled ISs and therefore expected to behave more similarly during LC-MS/MS analysis.

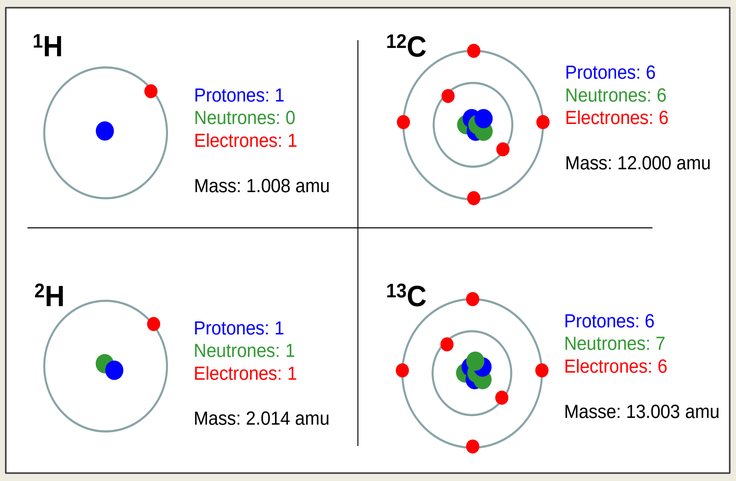

Fig.1 Two hydrogen and two carbon isotopes

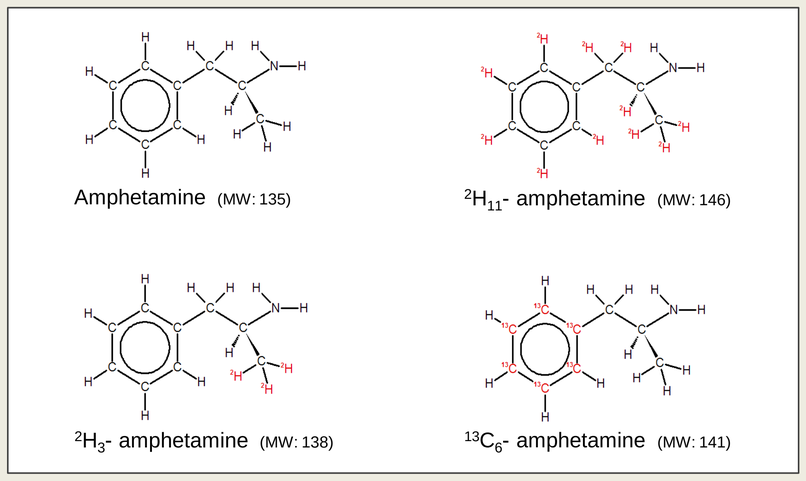

Fig.2 Molecular structure of amphetamine and different SIL ISs

EXPERIMENTAL

Sample preparation:
Urine samples (0.5 mL) were added ISs and then prepared by filtration on Whatman Mini Uniprep filters (0,2µm pores). Two microliters of the prepared samples were analysed by UPLC-MS/MS.

Chromatographic separation:
Instrument: Waters Acquity UPLC
Column: Waters Acquity BEH C_{18} (2.1x50mm), 1.7µm particles
Mobile-phase: A: 5mM NH_4-formate (pH 10.2)
B: Methanol
Gradient profile used was similar to the gradient earlier described in published article*

MS/MS analyses:
Instrument: Waters Quattro Premier Xe
Positive ESI with 2 MRM ions for analyte and ISs
Cone voltage, collision energy and other MS/MS parameters were optimized.

SIL ISs:
$^{13}C_6$-amphetamines were obtained from Chiron AS.
^{2}H-labelled ISs were purchased from Cerilliant.

RESULTS

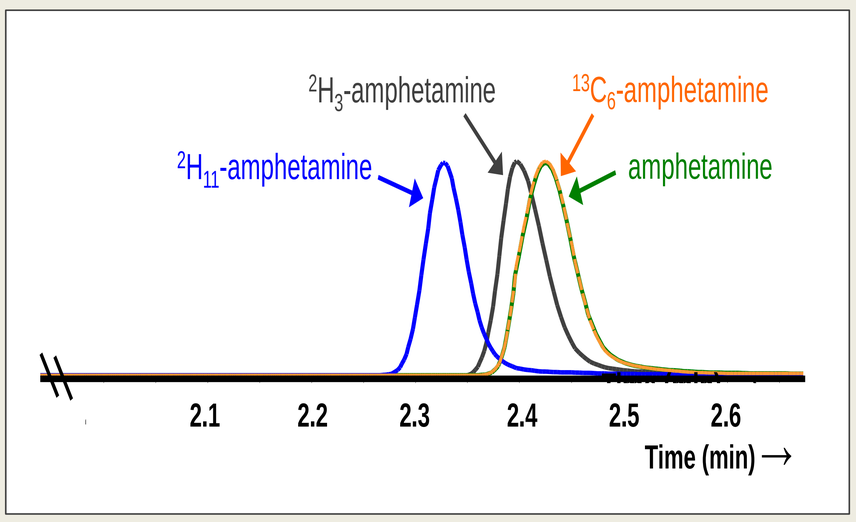

Fig.3 Chromatographic separation of amphetamine and different SIL ISs

Fig. 3 shows:

- $^{13}C_6$-amphetamine and amphetamine were perfectly co-eluting
- $^{2}H_3$-amphetamine and amphetamine were overlapping, but not perfectly co-eluting
- $^{2}H_{11}$-amphetamine and amphetamine were almost baseline separated.

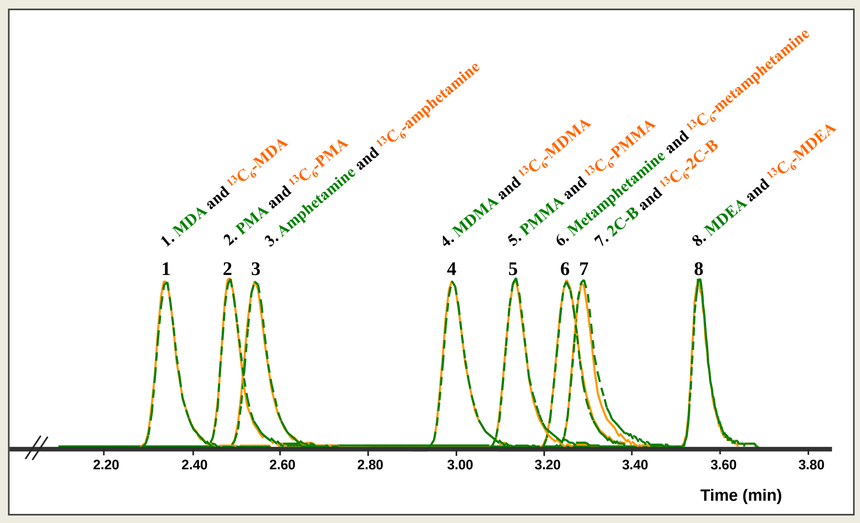

Fig.4 Chromatographic separation of amphetamines and their ^{13}C labeled ISs

Fig. 4 shows:

- All eight ^{13}C labeled ISs were perfectly co-eluting with their analytes.

Example: The importance of co-eluting ISs in UPLC-MS/MS analysis of drugs

Table 1 below shows the calculated amphetamine concentrations in two unknown samples. Three different ISs were added to both the unknown samples and the calibrators.

Table 1. Calculated amphetamine concentrations in two samples

	UPLC-MS/MS			GC-MS
Sample	IS: $^{2}H_{11}$-amphetamine	IS: $^{2}H_3$-amphetamine	IS: $^{13}C_6$-amphetamine	IS: $^{2}H_{11}$-A
Unknown sample 1	20.6 µM	5.0 µM	4.0 µM	4.1 µM
Unknown sample 2	4.2 µM	1.5 µM	1.2 µM	1.2 µM

Table 1 shows:

- Using $^{2}H_{11}$-amphetamine and/or $^{2}H_3$-amphetamine as IS lead to too high amphetamine UPLC-MS/MS concentrations (red pen), due to ion suppression of the ISs.
- Using $^{13}C_6$-amphetamine as IS the correct amphetamine concentrations were determined (green pen). A comparative method (GC-MS) was used to confirm and determine the correct amphetamine concentrations of the two samples.

Experiences with ^{13}C and ^{2}H labeled ISs:

At NIPH about 40 different ^{2}H and 10 different ^{13}C labeled ISs are used for the analysis of drugs in urine, blood and saliva by RP UPLC-MS/MS, and the experiences are based on using these SIL ISs.

- All ^{13}C labeled ISs used co-elute with their corresponding analytes, using both low pH and high pH mobile phases.
- Generally the ^{2}H labeled ISs elute slightly earlier than their corresponding analytes (data not shown).
- Increasing the number of ^{2}H substitutes increases the resolution between the ^{2}H labeled IS and their analogues, but other factors like molecular structure and mobile phase pH may also play a role.
- Improved ability to correct for ion suppression effects have been observed using $^{13}C_6$-amphetamine and $^{13}C_6$-methamphetamine , compared to using ^{2}H labeled ISs, for the analyses of amphetamine and methamphetamine in unknown urine samples by UPLC-MS/MS (data not shown).*
- Increased linear range has been observed when ^{13}C labeled ISs have been used for the analysis of amphetamine and methamphetamine in urine by UPLC-MS/MS, compared to using ^{2}H labeled ISs (data not shown).*

*/ Ref: Berg and Strand, JCA 1218 (2011), 9366-9374

CONCLUSIONS

^{13}C labeled ISs co-elute with their analytes in RP LC-MS/MS analyses and are therefor better suited to correct for ion suppression effects than ^{2}H labeled ISs.

When the number of ^{2}H substitutes in ^{2}H labeled ISs increases the chromatographic resolution between the analyte and the IS normally increases.