Why do toxicologists need an internal standard?

Abuse and misuse of drugs is a huge problem facing our society today. Toxicology reference materials, including internal standards, are essential for professionals tasked with testing for drugs, alcohol, and their breakdown products in seizures, paraphernalia and biological samples.

Quantitation of an analyte is usually accomplished by measuring the response obtained relative to an internal standard (IS). These are used to compensate for loss of analyte during sample preparation and for variation in mass spectrometric analysis (i.e. ionization efficiency and subtle variations in MS analysis). The assumption is that IS losses will be similar to losses of analyte. If a known quantity of IS is added to the unknown sample prior to any manipulations, the ratio of IS to analyte remains constant, because the same fraction of each is lost in any operation (Figure 1).

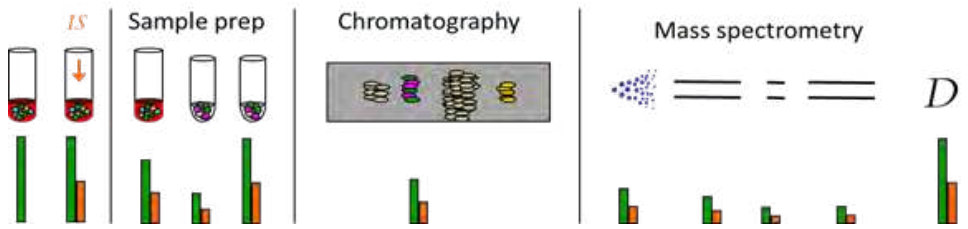
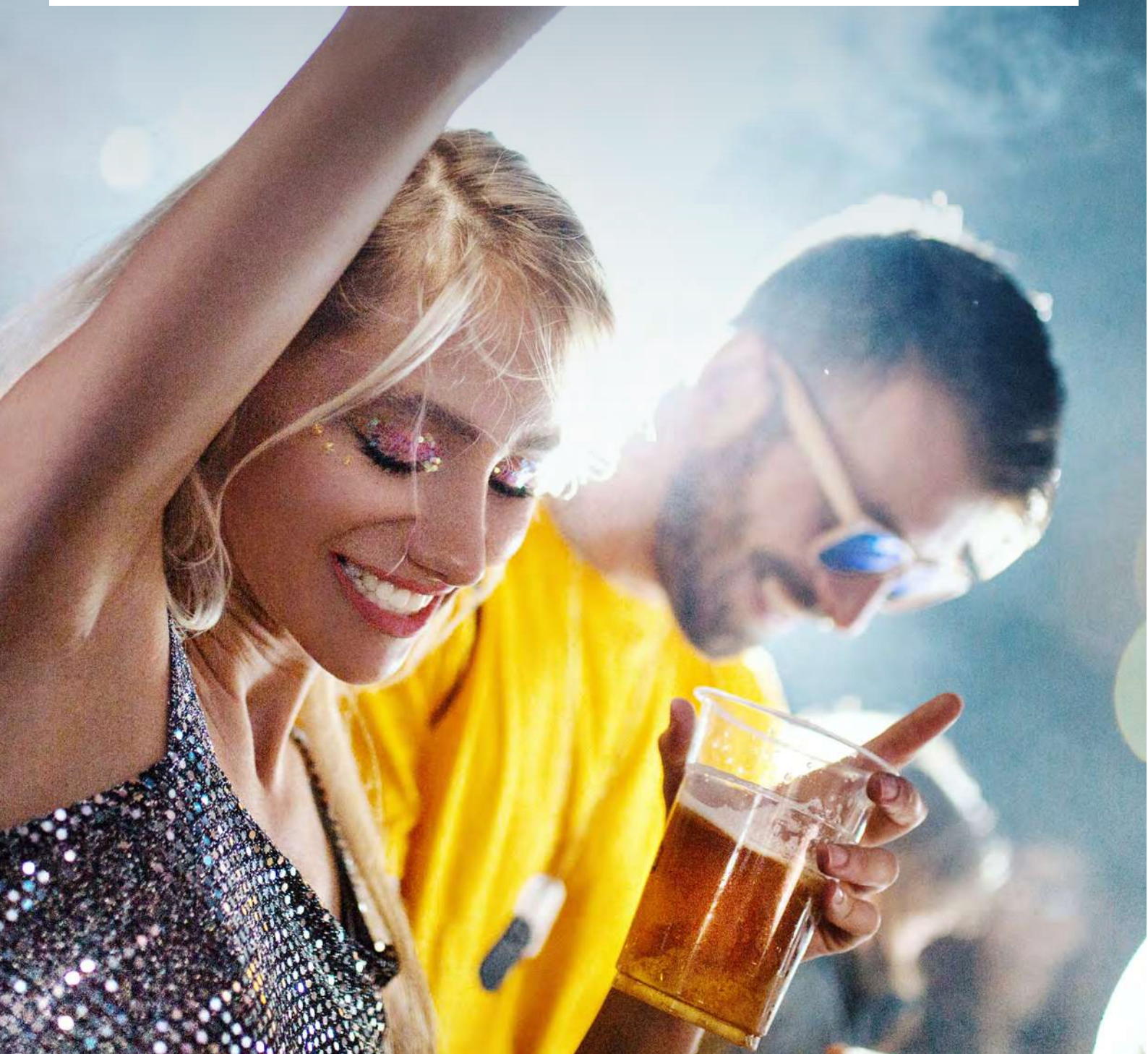


Figure 1:
Loss and fractionation can occur in the sample preparation, during chromatography, and in the mass spectrometer due to ion suppression.



An ideal internal standard must behave like the analyte of interest during sample preparation and instrumental analysis. It should fulfil the following criteria:

- Not already be present in the sample
- Have similar chemical and physical properties to the analyte
- Extract and derivatise in the same way as the analyte
- Elute close to the peak of interest
- Be free from interferences
- Have a similar response to analyte
- Be stable

The amount of internal standard added should be at a concentration that is close to the mean value for the target analyte and close to critical decision limits. It should also give sufficient response to achieve reproducible measurements.

There are several types of internal standards on the market: unlabelled structural analogues or stable isotope labelled internal standards (SILIS). SILIS behave chemically the same as the analyte, but differ in mass (^{2}H, ^{13}C, ^{15}N, ^{18}O). Combinations with two different isotopes also exist.

^{13}C-labelled IS are the gold standard choice for analytical chemists using LC-MS due to their correction for ion suppression, high precision, and accuracy.

Comparison of deuterated (^{2}H) and ^{13}C-labelled IS

As mentioned above, loss can occur in the sample preparation step and during chromatography, but also in the mass spectrometer due to ion suppression. The first two incidences are addressed by IS with similar chemical properties to the analyte. However, the latter is only compensated by a coeluting IS such as a ^{13}C-labelled IS. Ion suppression occurs since there is competition during electrospray ionization (ESI) between the compound of interest and coeluting compounds, including matrix effects. A compound co-eluting with the analyte may reduce the amount of analyte molecules that can be ionized. This effect is dependent on the concentration of analyte and other compounds, flow rate and injection volume. It is more notable for crude extracts, for example with 'dilute and shoot' sample preparation.

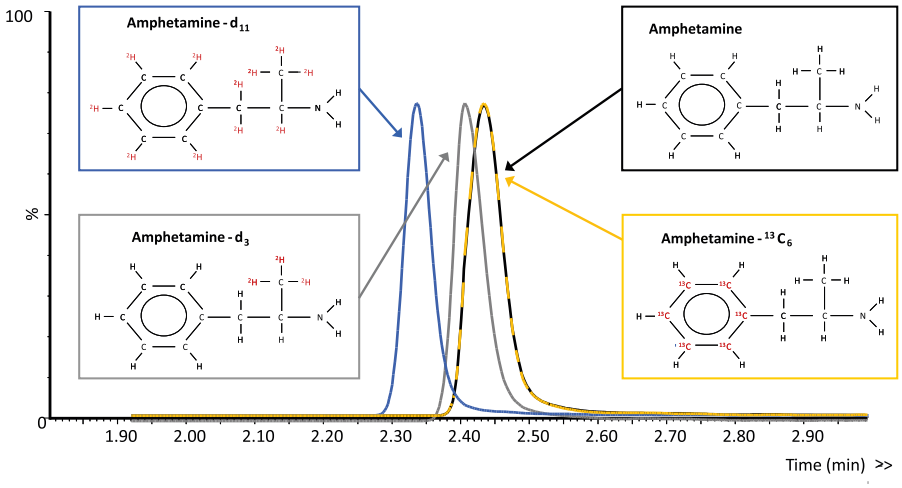


Figure 2:

Amphetamine-$^{13}C_6$ and native amphetamine coelute perfectly on UPLC-(ESI+) MS/MS

Amphetamine-d_3 overlap but do not coelute with the native on UPLC-(ESI+) MS/MS

Amphetamine-d_{11} and amphetamine are almost baseline separated on UPLC-(ESI+) MS/MS

Examples for use of ^{13}C-labelled IS

Data presented by Berg *et al*.[1,2,3] shows that amphetamine-$^{13}C_6$ perfectly coelutes with native amphetamine, thereby correcting for ion suppression. The d_3-labelled amphetamine only partially elutes with the native whilst the d_{11} chromatographically separates from amphetamine due to the difference in physico-chemical properties (Figure 2). When coeluting, factors leading to ion suppression are the same for amphetamine-$^{13}C_6$ as for native. Hence the signal ratio of IS to analyte remains constant and better linearity, accuracy and precision are obtained (Figure 3).

Chiron patented deuterium free, stable isotope labelled hallucinogens and stimulants such as ^{13}C-labelled amphetamines. There is no risk of deuterium exchange with ^{13}C-labelled IS. They are shown to be a highly precise, exactly reproducible, and convenient choice.[4,5]

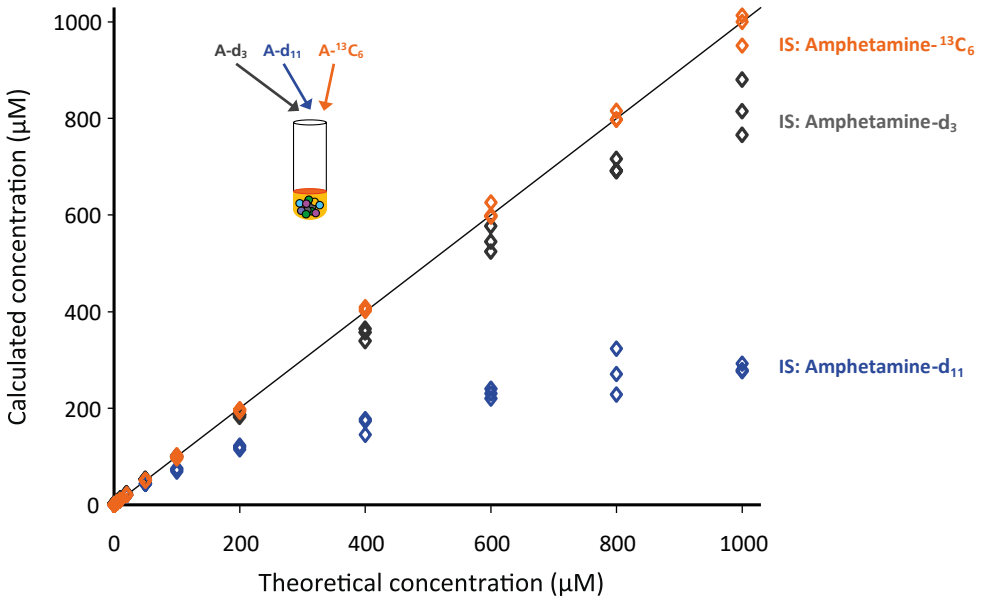


Figure 3:
Comparison of calculated and theoretical amphetamine concentrations in 12 samples. 3 different IS used as shown.

Alcohol biomarkers

Recently, Chiron developed phosphatidylethanol-$^{13}C_3$ ammonium salt (PEth-$^{13}C_3$). PEth is an abnormal cell membrane phospholipid with two fatty acid chains. It is a marker for alcohol consumption since it is formed in the presence of ethanol by conversion of phosphatidylcholine to phosphatidylethanol via the action of phospholipase D. Once formed, PEth incorporates into the phospholipid membranes of blood and tissue cells. It has a half-life of 4-5 days in blood, which means that repeated or chronic exposure leads to accumulation, giving rise to a wide window of detection. A patent application on the topic was filed. [6]

Other applications for ^{13}C labelled internal standards

In close collaboration with Chiron, Brockbals *et al*.[7] have developed a fast, single injection method for quantification of cocaine using ICAL-GC-MS. Three isotopically labelled ISs were used for internal calibration: cocaine-d_3, cocaine-$^{13}C_6$, and cocaine-$^{13}C_6d_3$. Validation provided excellent results and included precision, robustness, linearity, and accuracy, by comparison to reference standards and a validated method.

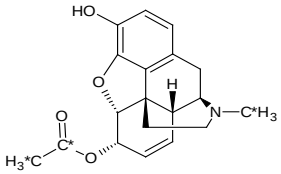
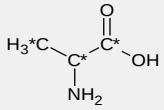
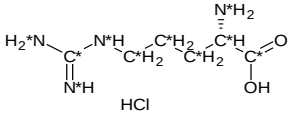
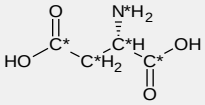
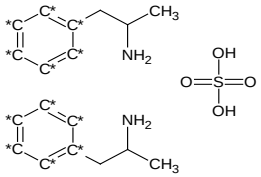
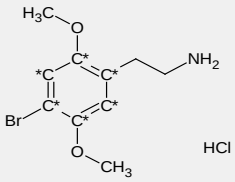
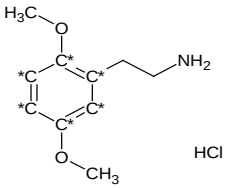
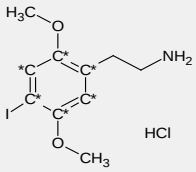
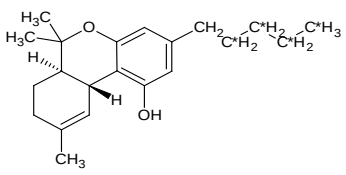
In conclusion, ^{13}C-labelled IS are superior to deuterium labelled, since they not only correct for loss due to sample preparation and chromatography but also ion suppression giving better accuracy and precision. ^{13}C-labelled IS are still limited on the market. However, Chiron offers a variety of ^{13}C-IS and we are continuously increasing our portfolio.

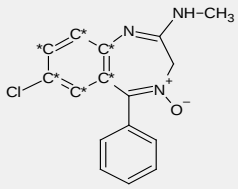
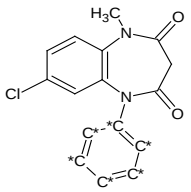
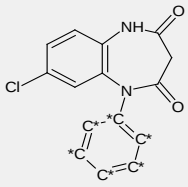
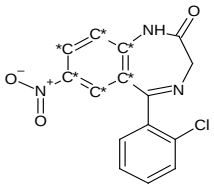
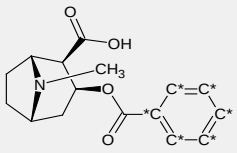
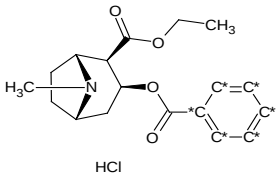
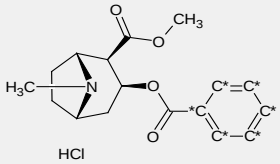
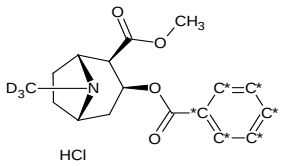
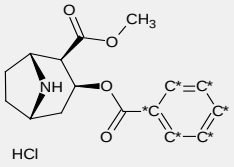
References:

1. T.Berg, D.H.Strand, *J.Chromatogr.*A, 1218 (2011) 9366-9374.
2. T.Berg, M.Karlsen, Å.M.Leere Øiestad, J.E.Johansen, H.Liu, D.H.Strand, *J.Chromatogr.*A, 1344 (2014) 83-90.
3. Å.M.Leere Øiestad, T.Berg, E.Eliassen, T.Wiklund., K.Sand, E.Leere Øiestad, *J. Liq. Chromatogr. Relat. Technol.*, 41:7 (2018) 391-400.
4. Johansen, J.E. Liu, H. Karlsen, M.S. (2016). *US Patent No. 9,435,816 B2*. Deuterium free, stable Isotope labeled 2-phenylethylamine hallucinogens and/or stimulants, methods of their preparation and their use (also patented as AU2012288743B2/ CA2841937A1/WO2013014287A2).
5. Karlsen, M.S. Liu, H. Johansen, J.E. (2016). *US Patent No. 9,347,961 B2*. Test Kit for the Quantitative Determination of Narcotic Drugs (also patented as AU2013291925B2/ CA2878670A1/WO2014013063A1).
6. European Patent Application 20196913.6.
7. L.Brockbals, M.Karlsen, J.Ramsey, B.Miserex, *Forensic Toxicol.*, 35 (2017) 153-161.

Chiron produce a wide range of ^{13}C-labelled internal standards for analysis of drugs of abuse as well as pharmaceuticals. They are available in neat form, as calibrated solutions, and as multicomponent mixes.

Chiron`s ^{13}C-labelled IS portfolio for toxicology includes amino acids, antibiotics, antiepileptics, antiretrovirals, amphetamines, benzodiazepines, cocaines, drugs for sleep disorders, ethanol related compounds, GHB, methylenedioxyphenethylamines, NPS, and opiates.

Chiron No.	Structure	Name	CAS
		6-Acetylmorphine (6-MAM)	
10147.19		6-Acetylmorphine-13C3 (13C2-acetyl, N-methyl-13C)	1538555-51-9
		Amino acids	
12174.3		DL-Alanine-13C3	144476-54-0
13363.6		L-Arginine-13C6,15N4 hydrochloride	202468-25-5
12175.4		L-Aspartic acid-13C4-15N	202468-27-7
		Amphetamines	
9676.9		DL-Amphetamine-13C6 hemisulphate (2:1, ring-13C6)	1419916-44-1 (free base)
		2C-B	
10098.10		2C-B-13C6 hydrochloride (ring 13C6)	1419925-85-1 (free base)
		2C-H	
10100.10		2C-H-13C6 hydrochloride (ring 13C6)	N/A
		2C-I	
10102.10		2C-I-13C6 hydrochloride (ring 13C6)	N/A
		Cannabinoids (Phyto)	
10200.21		(-)-trans-Δ9-THC-13C4 (pentyl-2,3,4,5-13C4)	1972-08-3 (unlabelled)

Chiron No.	Structure	Name	CAS
		Chlordiazepoxide	
10767.16		Chlordiazepoxide-13C6 (7-chlorobenzo-13C6)	58-25-3 (unlabelled)
		Clobazam	
10363.16		Clobazam-13C6 (phenyl-13C6)	1782501-70-5
10365.15		Norclobazam-13C6 (phenyl-13C6)	1784141-52-1
		Clonazepam	
10758.15		Clonazepam-13C6 (7-nitrobenzo-13C6)	1538556-00-1
		Cocaine	
10327.16		Benzoylecgonine-13C6 (benzoyl-13C6)	1538555-74-6
10328.18		Cocaethylene-13C6 hydrochloride (benzoyl-13C6)	N/A
10326.17		Cocaine-13C6 hydrochloride (benzoyl-13C6)	1538555-68-8 (free base)
10815.17		Cocaine-13C6,d3 hydrochloride (benzoyl-13C6, N-methyl-d3)	N/A
10329.16		Norcocaine-13C6 hydrochloride (benzoyl-13C6)	N/A

Chiron No.	Structure	Name	CAS
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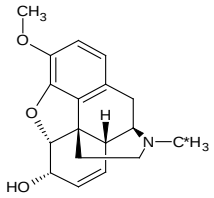
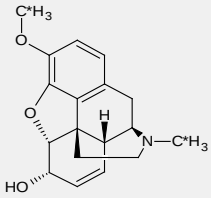
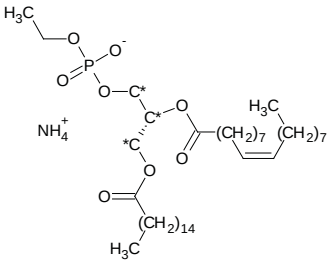
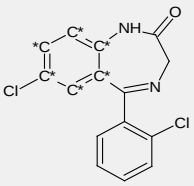
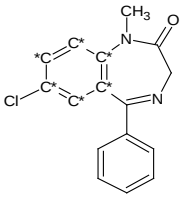
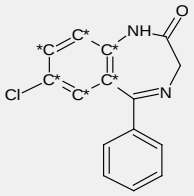
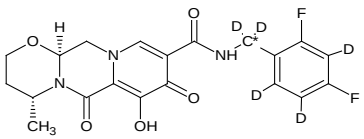
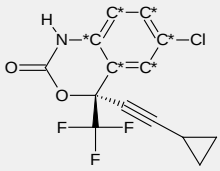
10151.18		Codeine-13C1 (N-methyl-13C)	1315159-97-7 (free base)
10146.18		Codeine-13C2 (N-methyl-13C, methoxy-13C)	1538555-55-3 (free base)
		Ethanol metabolites	
13776.39		Phosphatidylethanol-13C3 ammonium salt	N/A
		Delorazepam	
11431.15		Delorazepam-13C6 (7-chlorobenzo-13C6)	N/A
		Diazepam	
10799.16		Diazepam-13C6 (7-chlorobenzo-13C6)	1538556-15-8
10800.15		Nordiazepam-13C6 (7-chlorobenzo-13C6)	1538556-10-3
		Dolutegravir	
10600.20		Dolutegravir-13C,d5 (N-benzyl-13C,d5)	1051375-16-6 (unlabelled)
		Efavirenz	
10612.14		(+)-(S)-Efavirenz-13C6 (ring-13C6)	1261394-62-0

Chiron No.	Structure	Name	CAS
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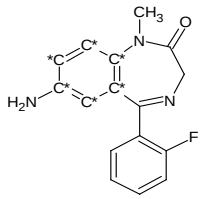
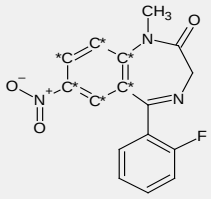
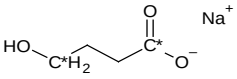
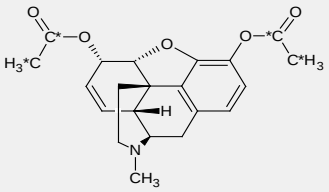
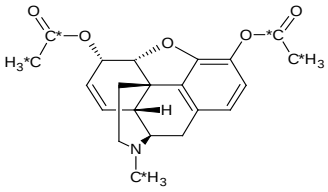
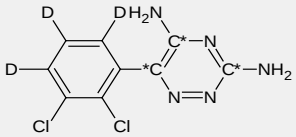
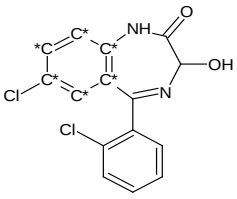
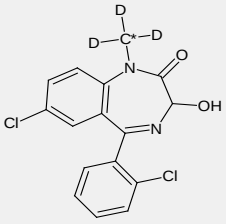
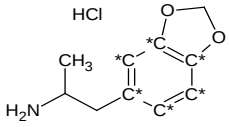
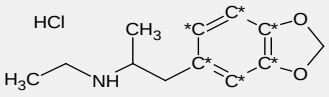
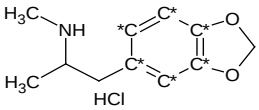
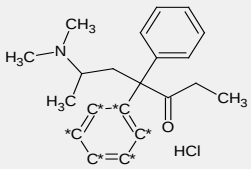
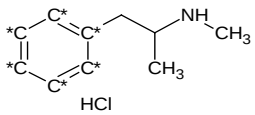
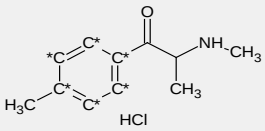
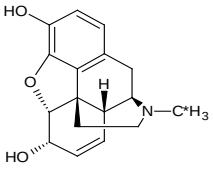
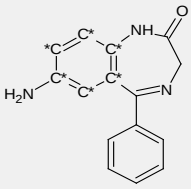
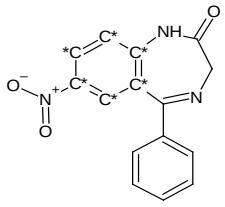
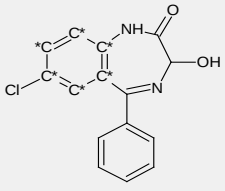
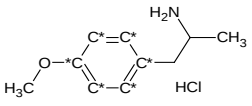
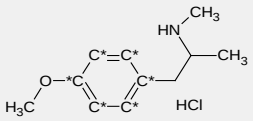
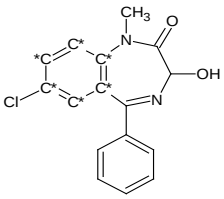
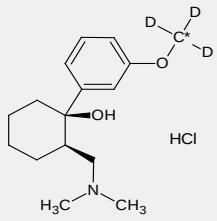
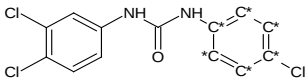
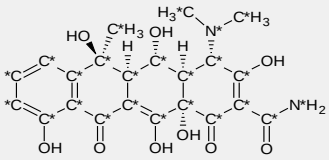
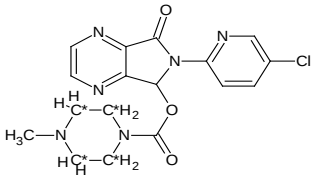
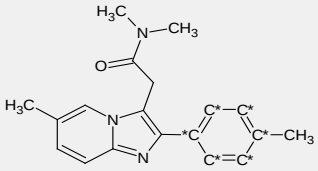
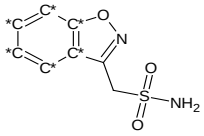
11364.16		7-Aminoflunitrazepam-13C6 (7-aminobenzo-13C6)	1538556-09-0
11363.16		Flunitrazepam-13C6 (7-nitrobenzo-13C6)	1538556-06-7
		Gammahydroxybutyrate	
10324.4		Sodium gammahydroxybutyrate-1,4-13C2	502-85-2 (unlabelled)
		Heroin	
10148.21		(-)-Heroin-13C4 (diacetyl-13C2)	1538555-47-3
10149.21		(-)-Heroin-13C5 (diacetyl-13C2, N-methyl-13C1)	1538555-48-4
		Lamotrigine	
13768.9		Lamotrigine-13C3,d3, Major	1246815-13-3
		Lorazepam	
11172.15		Lorazepam-13C6 (7-chlorobenzo-13C6)	846-49-1 (unlabelled)
		Lormetazepam	
14002.16		Lormetazepam-13C,d3	1285932-00-4
		MDA (3,4-Methylenedioxyamphetamine)	
10022.10		(±)-MDA 13C6 hydrochloride (ring-13C6)	1536075-11-2

Chiron No.	Structure	Name	CAS
		MDEA (3,4-Methylenedioxy-N-ethylamphetamine)	
10024.12		(±)-MDEA-13C6 hydrochloride (ring-13C6)	1538556-40-9
		MDMA (3,4-Methylenedioxymethamphetamine)	
10023.11		(±)-MDMA-13C6 hydrochloride (ring 13C6)	1536079-50-1
		Methadone	
10377.21		(±)-Methadone-13C6 hydrochloride (ring-13C6)	1095-90-5 (unlabelled)
		Methamphetamines	
9677.10		DL-Methamphetamine-13C6 hydrochloride (ring13C6)	1419916-80-5
		Methylmethcathinones	
11866.11		4-Methylmethcathinone-13C6 hydrochloride (benzene ring-13C6)	1419921-62-2 (free base)
		Morphine	
10150.17		Morphine-13C1	1538555-49-5
		Nitrazepam	
10912.15		7-Aminonitrazepam-13C6 (7-aminobenzo-13C6)	4928-02-3 (unlabelled)
10391.15		Nitrazepam-13C6 (7-nitrobenzo-13C6)	146-22-5 (unlabelled)
		Oxazepam	
11031.15		Oxazepam-13C6 (7-chlorobenzo-13C6)	1538556-21-6
		PMA (p-Methoxyamphetamine)	
10025.10		(±)-PMA-13C6 hydrochloride (ring-13C6)	1538556-41-0

Chiron No.	Structure	Name	CAS
		PMMA (p-Methoxymethamphetamine)	
10026.11		(±)-PMMA-13C6 hydrochloride (ring 13C6)	1538556-44-3
		Temazepam	
11173.16		Temazepam-13C6 (7-chlorobenzo-13C6)	1538556-24-9
		Tramadol	
10940.15	 rel. stereochemistry	Tramadol-13C,d3 hydrochloride	36282-47-0 (unlabelled)
		Triclocarban	
13340.13		Triclocarban-13C6	1216457-76-9
		Oxytetracycline	
10653.22		U-[13C22 15N2]-Oxytetracycline	N/A
		Zopiclone	
11360.17		(±)-Zopiclone-13C4 (piperazinyl-13C4)	43200-80-2 (unlabelled)
		Zolpidem	
10252.19		Zolpidem-13C6 (tolyl-13C6)	1538556-27-2
		Zonisamide	
10366.8		Zonisamide-13C6 (ring-13C6)	1435934-68-1

Labelled Pharmaceutical Mix 36

Chiron No.	Name	CAS	Concentration
S-5225-K-10ME	Labelled Pharmaceutical Mix 36 in methanol		
9158.9	DL-Amphetamine-d5 hydrochloride (alpha-methyl-d3-ethylamine-1,2-d2)	N/A	1000 µg base/mL
9161.1	DL-Methamphetamine-d5 hydrochloride (N-methyl-d3-ethylamine-1,2-d2)	60124-81-4	
9165.11	(±)-MDMA-d5 hydrochloride (alpha-methyl-d3-ethylamine-1,2-d2)	N/A	
9163.10	(±)-MDA-d5 hydrochloride (alpha-methyl-d3-ethylamine-1,2-d2)	136765-42-9 (free base)	
10025.10	(±)-PMA-13C6 hydrochloride (ring-13C6)	1538556-41-0	
11176.12	(±)-MDEA-d5 hydrochloride (Ethyl-d5)	1286588-92-8	
9412.13	Ritalinic acid-d9 hydrochloride (piperazine-d9) (erythro+threo)	1276197-13-7	
9829.17	Cocaine-d3 hydrochloride (N-methyl-d3)	N/A	
11312.16	Benzoylecgonine-d3 tetrahydrate (N-methyl-d3)	5928-96-1 (unlabelled)	

Cocaine IS mix

Chiron No.	Name	CAS	Concentration
S-4862-ASS-ME	Cocaine IS mix in methanol		
9829.17	Cocaine-d3 hydrochloride (N-methyl-d3)	N/A	1.0 mg base/mL
10326.17	Cocaine-13C6 hydrochloride (benzoyl-13C6)	1538555-68-8 (free base)	0.4 mg base/mL
10815.17	Cocaine-13C6-d3 hydrochloride (benzoyl-13C6, N-methyl-d3)	N/A	0.2 mg base/mL

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