Measurement of Drugs of Abuse by Liquid Chromatography Tandem Mass Spectrometry using Fully Automated Sample Preparation

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For the analysis of drugs and pharmaceutical compounds in biological matrices, LC-MS/MS analysis is typically preceded by sample preparation which often requires manual steps. In this study, we report a fully automated extraction process directly coupled to an LC- MS/MS system for the determination of amphetamines, cocaine and opiates.

42 target compounds and 20 deuterated internal standards were included in the method. The extraction was carried out by a programable liquid handler (CLAM-2000, Shimadzu) coupled directly to an LC-MS/MS system (Nexera X2 & LCMS-8060, Shimadzu). The acquisition was performed in positive ionisation mode with up to 15 MRM transitions per compound, each with optimised collision energy (Multiple Reaction Monitoring or MRM Spectrum mode) to enable qualitative library searching in addition to quantitation.

This approach is successfully designed to support parallel sample preparation and analysis therefore significantly increasing sample throughput and reducing cycle times.

Figure 1: Sample analysis cycle.

Introduction

Opiates, amphetamines (including analogues) and cocaine are widely used drugs of abuse (DOA) and many laboratories have developed LC-MS/MS procedures to identify and quantify these compounds [1-5]. Such measurements are needed in multiple contexts within clinical and forensic toxicology (suspicion of overdose, monitoring of addicts, driving under the influence of drugs, doping control and pain relief).

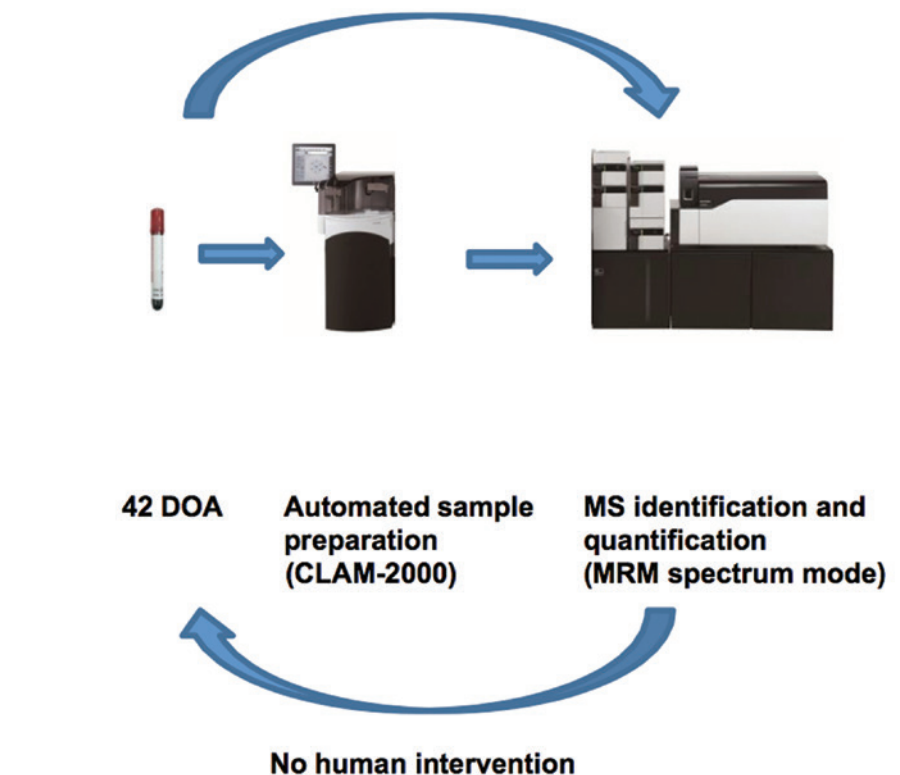To minimise the possibility of false positive or negative results reporting without compromising accuracy, precision and limits of detection, methods were developed to combine the sensitivity of MRM detection with the identification power of MRM spectrum. Contrary to an entire mass spectrum, MRM represents the operation of the mass spectrometer in which a precursor ion is selected and then the abundances of multiple product ions are recorded. In the present study, the method has the capability of following up to 15 MRM transitions per compound and enabling precise, accurate quantitation and library searchable compound identification. Each transition has optimised collision energies for each ion.

Table 1: LC acquisition parameters.

Liquid chromatography		
UHPLC	Nexera LC system	
Analytical column	Restek Raptor Biphenyl (2.7 µm 100 x 2.1 mm)	
Column temperature	40 °C	
Flow rate	0.3 mL/min (0.6 mL/min 11-16.2 min to accelerate column conditioning and equilibration)	
Solvent A	2 mmol/L ammonium formate and 0.002% formic acid	
Solvent B	2 mmol/L ammonium formate and 0.002% formic acid in methanol	
Binary Gradient	Time (mins)	%B
	1.0	10
	2.0	40
	10.5	100
	13.5	100
	13.51	10
	17.0	Stop
Injection volume	3 µL	

Table 2: LC-MS/MS method used to acquire a library searchable data.

LC-MS/MS Mass spectrometry	MRM Spectrum mode generating library searchable spectra
Target number of compounds	42 (including 20 ISTDs)
Pause time/dwell time	1 msec./3 to 10 msec.
Ionisation mode	ESI +/-
Polarity switching time	5 msec
Interface temperature	300 °C
Heat block temperature	400 °C
Desolvation line temperature	250 °C
Nebulising gas	3 L/min
Heating gas	10 L/min
Drying gas	10 L/min

Ion intensities from each transition are used to construct an MRM Spectrum that can be used to search against registered library spectra.

To develop an automated generic sample preparation method in clinical toxicology analysis, an automated sample preparation system was coupled to LC-MS/MS system. Once the primary tube is loaded onto the intervention was required. The sample is automatically delivered to a tube containing a filter, to which reagents are added, mixed and then filtered. The extract is finally injected into the LC-MS-MS system.

The procedure was fully validated which included: repeatability, reproducibility, matrix effects, extraction yields, inter- matrix agreement, dilution tests and robustness.

To test its viability, the method was applied to patients' blood or plasma samples and compared against a validated LC-MS/MS method using 2 MRM's for each target compound [5].

Experimental

The sample is automatically delivered to a tube containing a filter, to which reagents are added, mixed and then filtered.

Precisely, 100 µL of acetonitrile were added to a PTFE filter vial (0.45 µm pore size) previously conditioned with 20 µL methanol. Then, 50 µL of plasma (or whole blood) and 10 µL of isotopically labelled internal standards (0.2 mg/L in acetonitrile) were added. The mixture was mixed for 120 seconds (1900 rpm) then filtered by application of vacuum pressure (-60 to -65 kPa) for 120 seconds into a collection vial. Finally, 3 µL of the extract was injected into the LC-MS-MS system.

All compounds were measured by scheduled MRM, with up to 15 transitions per compound throughout the entire scheduled window using 1 msec pause time and 3 to 10 msec dwell time. All transitions were collision energy optimised using flow injection analysis. Chromatographic

peak apex intensity was used to extract ion intensities for construction of an MRM Spectrum.

Validation and Robustness Study

The laboratory of Pharmacology-toxicology of the Limoges University Hospital works towards accreditation by the International Standards Organization (ISO) 15189 standard (accreditation number: 8-2607). These requirements were applied to the present method.

The intra-assay precision and accuracy (n=6) and the inter-assay and accuracy (n=6) were assessed at lower limit of quantitation (LLOQ; 1 or 2,5 or 5, depending on the compound), 50 and upper limit of quantitation (ULOQ 500 ng/mL) after complete extraction procedure and analysis of six different spiked plasma samples (compound-free human plasma) for each level. To assess the inter-assay precision and accuracy, a set of calibrating samples was analysed each day for 6 days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of compound that could be measured with both an intra-assay and inter-assay precision (CV%) and an accuracy (bias) less than 15%.

Calibration standards were prepared by adding automatically appropriate working standard solutions to 50 µL of plasma prior to extraction in order to obtain concentrations ranging from 1 to 500 ng/mL for all compounds (6 levels, 1 or 2,5 or 5 depending on the compound, 10, 50, 100, 200 and 500 ng/mL). Calibration graphs of the compounds of interest-to-internal standard peak-area ratios of the quantification transition versus expected compounds of interest concentration were constructed using a quadratic with 1/x or 1/x^2 weighting regression analysis.

Recoveries were determined at two concentration levels (LLOQ and 500 ng/mL) by comparing the analyte / internal standard peak area ratios obtained after extraction of spiked samples (n=6) with those of DOA-free plasma extracts further spiked with the DOA (n=3).

The effect of dilution was investigated using samples manually spiked at 150% of ULOQ then re-analysed after 2, 4 and 10-fold dilutions.

The absence of carry over was checked.

Matrix agreement was tested for whole blood: the intra-assay precision and accuracy

Table 3: MRM transitions, retention times and the total number of MRM transitions measured using MRM Spectrum mode during the acquisition for 42 compounds and their 20 internal standard compounds. Quantitative data was measured using quantifier ion and reference ion with ion ratio percentage tolerance of 20%. Additional MRM transitions were used for Library identification.

| Compounds | Precursor ion | | Product ion | | | | | | Retention time (min) | Number total of MRM |
| | m/z | Q1 pre-bias (V) | Quantitation | | | Reference | | | | |
			m/z	Collision energy (V)	Q3 pre-bias (V)	m/z	Collision energy (V)	Q3 pre-bias (V)		
2-Cl	308.00	-11.0	290.85	-14.0	-13.0	275.85	-24.0	-29.0	5.03	15
2C-B	260.05	-10.0	242.90	-12.0	-11.0	227.85	-22.0	-23.0	4.58	15
3,4-Methylenedioxypyrovalerone	276.15	-10.0	126.10	-14.0	-12.0	175.00	-12.0	-17.0	5.18	15
4-MTA	182.10	-12.0	117.10	-21.0	-11.0	165.05	-12.0	-10.0	4.45	15
6-acetylmorphine	328.35	-12.0	164.95	-39.0	-16.0	211.00	-27.0	-21.0	3.61	15
6-acetylmorphine-D3	331.35	-24.0	165.15	-43.0	-17.0	211.10	-27.0	-21.0	3.61	2
Amphetamine	136.10	-10.0	91.00	-22.0	-17.0	119.05	-15.0	-20.0	3.42	8
Amphetamine-D5	141.10	-15.0	93.10	-18.0	-17.0	124.15	-14.0	-13.0	3.42	2
Anhydroecgonine methyl ester	182.10	-12.0	118.00	-23.0	-11.0	91.05	-29.0	-20.0	3.17	15
BDB	194.10	-13.0	135.00	-20.0	-13.0	177.05	-12.0	-17.0	4.06	8
Benzoylecgonine	290.15	-11.0	168.05	-10.0	-16.0	77.00	-29.0	-13.0	4.57	15
Benzoylecgonine-D3	293.15	-14.0	171.20	-20.0	-17.0	77.05	-56.0	-13.0	4.57	2
Buprenorphine	468.30	-16.0	54.95	-52.0	-20.0	396.00	-41.0	-26.0	7.31	15
Buprenorphine-D4	472.30	-13.0	59.10	-50.0	-22.0	88.10	-50.0	-16.0	7.31	2
Cocaethylene	318.15	-20.0	196.00	-10.0	-20.0	76.95	-32.0	-30.0	5.49	15
Cocaethylene-D3	321.15	-12.0	199.25	-21.0	-22.0	85.20	-32.0	-16.0	5.49	2
Cocaine	304.15	-11.0	182.00	-10.0	-20.0	76.95	-30.0	-29.0	4.94	15
Cocaine-D3	307.15	-22.0	185.15	-19.0	-20.0	85.25	-31.0	-15.0	4.94	2
Codeine	300.15	-11.0	215.00	-25.0	-22.0	151.95	-62.0	-28.0	3.56	15
Codeine-D3	303.15	-14.0	215.25	-26.0	-20.0	181.20	-37.0	-17.0	3.56	2
Dextromethorphan	272.20	-10.0	171.00	-20.0	-17.0	215.05	-12.0	-14.0	6.43	15
Dihydrocodeine	302.20	-11.0	198.95	-33.0	-19.0	127.95	-64.0	-23.0	3.56	15
Dihydrocodeine-D3	305.20	-15.0	199.15	-35.0	-21.0	128.30	-55.0	-25.0	3.56	2
Ecgonine methylester	200.15	-12.0	182.05	-18.0	-18.0	82.05	-26.0	-13.0	0.97	15
Ecgonine methylester-D3	203.15	-14.0	185.25	-18.0	-13.0	85.20	-26.0	-30.0	0.97	2
EDDP	278.20	-10.0	234.00	-17.0	-20.0	249.05	-13.0	-16.0	6.95	15
EDDP-D3	281.20	-19.0	234.30	-31.0	-16.0	249.35	-25.0	-17.0	6.95	2
Ephedrine-D3	169.15	-17.0	151.25	-14.0	-16.0	91.20	-33.0	-17.0	3.28	2
Ethylmorphine	314.20	-12.0	152.00	-65.0	-14.0	165.00	-42.0	-16.0	4.04	15
Hydrocodone	300.15	-11.0	198.95	-31.0	-20.0	127.90	-59.0	-22.0	3.82	15
Hydromorphone	286.15	-10.0	185.00	-30.0	-19.0	157.00	-42.0	-15.0	3.24	15
MBDB	208.15	-20.0	134.95	-6.0	-20.0	50.95	-60.0	-19.0	4.28	9
m-CPP	197.10	-12.0	118.10	-34.0	-11.0	154.00	-20.0	-15.0	4.38	15
MDA	180.10	-12.0	105.05	-21.0	-22.0	163.05	-13.0	-16.0	3.65	15
MDA-D5	185.10	-13.0	110.15	-22.0	-11.0	168.15	-13.0	-18.0	3.65	2
MDEA	208.15	-11.0	163.00	-13.0	-15.0	105.00	-25.0	-10.0	4.11	11
MDEA-D5	213.15	-23.0	163.15	-14.0	-30.0	105.20	-28.0	-18.0	4.11	2
MDMA	194.10	-13.0	163.05	-15.0	-28.0	105.05	-25.0	-18.0	3.84	12
MDMA-D5	199.10	-21.0	165.15	-15.0	-18.0	107.15	-25.0	-11.0	3.84	2
Mephedrone	178.10	-13.0	145.05	-20.0	-14.0	160.05	-15.0	-10.0	3.99	15
Methadone	310.20	-18.0	310.20	-8.0	-21.0	76.95	-30.0	-13.0	7.60	14
Methadone-D9	319.20	-20.0	268.25	-20.0	-20.0	105.05	-25.0	-20.0	7.60	2
Methamphetamine	150.15	-10.0	91.00	-22.0	-20.0	119.05	-16.0	-21.0	3.63	8
Methcathinone	164.10	-30.0	131.05	-21.0	-23.0	146.05	-16.0	-30.0	3.43	13
Methiopropamine	156.10	-11.0	97.00	-23.0	-10.0	58.00	-12.0	-23.0	3.39	15
Methylphenidate	234.15	-20.0	84.00	-8.0	-20.0	91.0	-46.0	-17.0	4.71	7
Morphine	286.15	-10.0	152.00	-60.0	-15.0	201.00	-27.0	-20.0	3.11	15
Morphine-D3	289.15	-14.0	152.10	-59.0	-26.0	201.15	-26.0	-21.0	3.11	2
Naloxone	328.15	-12.0	310.00	-21.0	-21.0	212.00	-39.0	-22.0	3.60	14
Naloxone-D5	333.15	-12.0	315.20	-20.0	-22.0	258.10	-29.0	-27.0	3.60	2
Naltrexone	342.15	-12.0	324.05	-22.0	-15.0	270.05	-28.0	-28.0	3.75	14
Naltrexone-D3	345.15	-16.0	327.15	-22.0	-23.0	270.15	-28.0	-29.0	3.75	2
Norbuprenorphine	414.25	-28.0	54.90	-63.0	-24.0	83.05	-50.0	-14.0	5.50	15
Norephedrine	152.10	-10.0	134.05	-15.0	-13.0	115.05	-25.0	-11.0	3.02	11
Norfenfluramine	204.10	-14.0	159.00	-20.0	-15.0	109.05	-40.0	-18.0	4.16	15
Noroxycodone	302.15	-11.0	199.00	-37.0	-20.0	196.95	-26.0	-20.0	3.56	15
Noroxycodone-D3	305.15	-22.0	287.15	-17.0	-20.0	190.10	-25.0	-20.0	3.56	2
Norpseudoephedrine	152.10	-10.0	134.05	-15.0	-13.0	115.05	-25.0	-11.0	3.12	11
Oxycodone	316.15	-12.0	298.00	-20.0	-20.0	240.95	-29.0	-24.0	3.73	14
Oxycodone-D3	319.15	-23.0	301.10	-19.0	-21	259.10	-26.0	-27.0	3.73	2
Pholcodine	399.25	-14.0	114.05	-36.0	-11.0	381.05	-25.0	-18.0	3.20	5
Ritalinic acid	220.15	-14.0	84.10	-22.0	-14.0	56.05	-44.0	-22.0	4.14	10

Table 4: Clinical validation results for quantitation of 42 compounds. Percentage recovery from automated sample preparation in plasma. Inter and Intra-assay average accuracy and precision were within acceptable tolerance of 85-115% and better than 15% relative standard deviation (RSD).

Compound	Associated internal standard	Si (LLOQ)	LLOQ (ng/mL)	Recovery [%] (%RSD) LLOQ (n=6)	Recovery [%] (%RSD) 500 ng/mL (n=6)	Intra-assay precision [%] (%RSD) LLOQ (n=6)	Intra-assay precision [%] (%RSD) 50 ng/mL (n=6)	Intra-assay precision [%] (%RSD) 500 ng/mL (n=6)	Inter-assay precision [%] (%RSD) LOQ (n=6)	Inter-assay precision [%] (%RSD) 50 ng/mL (n=6)	Inter-assay precision [%] (%RSD) 500 ng/mL (n=6)	Dilution test [%] (%RSD) (1.5*ULOQ)/10 (n=3)	Dilution test [%] (%RSD) (1.5*ULOQ)/4 (n=3)	Dilution test [%] (%RSD) (1.5*ULOQ)/2 (n=3)
2-CI	Cocaine-D3	85	2,5	103.1 (4.2)	112.5 (3.1)	102.2 (6.3)	95.5 (3.4)	88.4 (0.9)	99.9 (4.0)	102.7 (5.3)	105.2 (6.6)	88.6 (9.6)	108.2 (1.7)	102.1 (3.7)
2C-B	Cocaine-D3	89	2,5	112.0 (11.7)	111.8 (3.2)	93.7 (8.4)	86.0 (2.2)	94.3 (0.6)	102.7 (7.6)	96.9 (3.9)	102.5 (4.2)	85.4 (5.0)	104.1 (4.1)	101.4 (3.9)
3,4-Methylenedioxypyrovalerone	Cocaine-D3	98	1	92.3 (10.7)	106.8 (2.9)	103.8 (3.5)	101.5 (2.0)	85.3 (0.9)	113.6 (6.7)	102.5 (4.5)	103.9 (4.7)	89.9 (11.1)	113.3 (0.7)	104.5 (3.0)
4-MTA	Cocaine-D3	96	2,5	111.6 (6.7)	106.6 (2.3)	94.8 (6.3)	88.5 (3.1)	92.6 (1.2)	109 (9.6)	102.6 (6.6)	106.1 (4.2)	94.9 (3.2)	103.9 (3.9)	95.8 (2.2)
6-acetylmorphine	6-acetylmorphine-D3	94	5	93.4 (9.8)	91.4 (4.4)	95.6 (9.7)	99.1 (4.1)	86.8 (2.8)	96.0 (7.4)	97.8 (5.2)	107.8 (9.9)	90.9 (14.2)	112.2 (2.0)	104.7 (8.6)
Amphetamine	Amphetamine-D5	90	5	95.6 (9.4)	102.8 (2.1)	104.7 (8.9)	112.0 (4.2)	85.3 (0.7)	103.8 (9.1)	96.1 (6.0)	104.6 (5.3)	89.0 (10.4)	107.3 (5.6)	103.6 (4.6)
Anhydroecgonine methyl ester	Morphine-D3	94	5	114.5 (10.4)	110.6 (8.7)	114.0 (2.8)	107.4 (7.0)	104.2 (3.8)	93.7 (5.6)	97.0 (2.0)	108.9 (7.5)	91.2 (1.2)	111.2 (5.0)	101.0 (4.3)
BDB	MDEA-D5	95	5	77.1 (12.2)	100.9 (0.9)	93.6 (9.0)	96.7 (2.1)	95.1 (1.0)	101.3 (8.8)	99.6 (2.3)	107.5 (10.2)	89.4 (6.2)	104.5 (3.1)	100.6 (4.3)
Benzoylecgonine	Benzoylecgonine-D3	94	1	103.7 (2.1)	105.8 (3.0)	117.5 (3.5)	85.7 (0.7)	86.0 (1.4)	113.9 (7.6)	96.4 (2.2)	101.5 (4.1)	87.9 (7.7)	111.2 (3.0)	106.8 (2.3)
Buprenorphine	Buprenorphine-D4	96	2,5	106.5 (13.4)	107.5 (1.8)	95.4 (10.6)	101.8 (5.0)	104.7 (3.4)	107.2 (9.0)	97.0 (3.2)	104.1 (10)	85.7 (5.9)	110.5 (5.0)	107.0 (12.7)
Cocaethylene	Cocaethylene-D3	96	1	92.4 (7.1)	95.1 (2.4)	116.0 (1.4)	87.4 (0.8)	86.7 (0.8)	115.1 (7.8)	94.3 (3.1)	104.2 (7.0)	86.9 (1.6)	110.0 (4.1)	108.1 (5.1)
Cocaine	Cocaine-D3	96	1	83.8 (0.3)	99.5 (2.4)	100.7 (3.0)	92.7 (1.2)	87.3 (0.6)	99.7 (5.1)	100.2 (3.0)	95.1 (5.8)	85.8 (3.1)	108.9 (3.6)	93.9 (3.8)
Codeine	Codeine-D3	96	2,5	99.9 (12.4)	96.9 (7.2)	104.5 (8.3)	87.0 (1.3)	89.7 (6.1)	100.5 (13.9)	95.7 (8.0)	99.0 (10.1)	89.0 (1.3)	106.2 (4.8)	99.4 (3.1)
Dextromethorphan	Cocaine-D3	97	1	94.8 (6.1)	107.3 (2.6)	106.0 (4.5)	103.7 (2.1)	89.1 (1.9)	106.3 (6.3)	107.6 (5.0)	103.7 (7.2)	88.2 (11.9)	114.2 (5.0)	103.2 (3.0)
Dihydrocodeine	Dihydrocodeine-D3	93	2,5	105.8 (6.0)	108.9 (8.1)	94.9 (8.7)	98.9 (2.3)	86.4 (2.8)	101.1 (9.3)	95.6 (5.1)	104.5 (7.7)	88.1 (7.4)	111.0 (5.7)	105.8 (3.9)
Ecgonine methylester	Ecgonine methylester-D3	93	1	89.6 (11.2)	110.6 (15)	104.8 (8.9)	90.5 (1.1)	92.1 (6.6)	108.6 (16.1)	97.5 (5.2)	103.5 (6.5)	89.5 (2.8)	112.4 (5.5)	98.9 (4.6)
EDDP	EDDP-D3	92	1	102.8 (1.5)	91.1 (2.6)	117.2 (4.3)	85.8 (1.3)	86.1 (2.5)	113.0 (12.4)	97.0 (4.3)	100.4 (5.9)	85.4 (8.6)	109.1 (3.4)	105.5 (4.3)
Ethylmorphine	MDEA-D5	91	2,5	78.2 (16.4)	81.5 (5.1)	82.7 (11.2)	90.7 (2.8)	85.5 (1.7)	102.4 (13.3)	98.9 (3.5)	98.9 (3.5)	85.9 (4.3)	104.8 (1.1)	100.3 (4.2)
Hydrocodone	MDMA-D5	88	1	78.3 (3.1)	78.2 (4.2)	115.0 (5.8)	85.5 (3.6)	89.0 (2.5)	109.5 (12.8)	98.2 (3.8)	102.6 (8.8)	85.9 (3.6)	108.7 (6.6)	100.7 (5.1)
Hydromorphone	Morphine-D3	96	5	84.3 (15.0)	84.1 (4.2)	89.1 (4.1)	91.7 (1.5)	85.6 (1.7)	103.6 (11.4)	92.6 (5.1)	110.3 (5.4)	91.0 (4.9)	104.4 (5.6)	95.0 (4.5)
MBDB	Benzoylecgonine-D3	97	1	71.5 (6.2)	87.0 (3.6)	103.1 (5.0)	90.3 (1.5)	85.1 (1.4)	103.5 (9.9)	100.2 (2.6)	95.9 (4.1)	89.2 (7.2)	114.8 (2.6)	99.1 (2.0)
m-CPP	Benzoylecgonine-D3	93	2,5	101.1 (6.9)	96.0 (2.3)	84.8 (7.6)	90.8 (3.0)	86.8 (2.8)	112.6 (6.8)	94.6 (6.0)	105.7 (6.9)	89.9 (13.5)	113.3 (1.4)	109.3 (7.7)
MDA	MDA-D5	91	5	79.4 (1.5)	97.3 (5.8)	94.3 (10)	101.7 (3.9)	86.0 (3.4)	90.2 (6.6)	95.3 (3.6)	101.7 (9.5)	87.9 (5.7)	105.7 (7.5)	106.1 (14.4)
MDEA	MDEA-D5	91	1	77.3 (12.0)	87.8 (3.7)	112.1 (3.7)	88.2 (3.2)	88.3 (1.4)	112.9 (7.6)	97.6 (2.6)	103.3 (5.4)	86.0 (6.4)	105.6 (3.5)	103.2 (2.2)
MDMA	MDMA-D5	81	5	81.8 (6.5)	78.7 (6.2)	103.7 (3.2)	103.6 (2.3)	85.6 (1.2)	91.4 (7.8)	99.6 (4.1)	103.9 (7.3)	85.1 (2.1)	112.1 (5.5)	101.4 (4.7)
Mephedrone	MDEA-D5	86	1	73.8 (19.6)	87.3 (5.1)	106.9 (7.7)	94.8 (4.1)	85.8 (2.8)	104.2 (9.2)	99.9 (3.5)	100.4 (1.9)	85.1 (5.5)	109.2 (2.2)	102.4 (2.7)
Methadone	Methadone-D9	98	5	83.9 (4.1)	89.4 (3.4)	105.1 (7.0)	93.6 (0.8)	85.5 (0.4)	106.2 (7.7)	101.7 (3.2)	103.6 (8.3)	85.1 (10.6)	111.3 (2.2)	103.4 (3.5)
Methamphetamine	Dihydrocodeine-D3	93	1	92.3 (17.0)	114.0 (3.3)	106.0 (8.0)	109.7 (2.0)	86.2 (1.7)	111.7 (11.4)	99.4 (6.3)	101.7 (6.8)	88.3 (1.7)	114.5 (5.7)	107.1 (2.1)
Methcathinone	Amphetamine-D5	97	2,5	80.1 (15.0)	85.7 (8.4)	84.1 (3.5)	99.4 (1.75)	87.3 (5.5)	102.1 (6.0)	96.4 (8.4)	105.4 (6.5)	88.8 (9.8)	101.8 (6.3)	99.2 (6.9)
Methiopropamine	Amphetamine-D5	95	2,5	94.2 (17.0)	103.1 (6.9)	90.1 (5.3)	101.6 (4.3)	87.5 (3.1)	110.3 (7.9)	92.4 (3.8)	95.8 (6.2)	89.6 (8.0)	106.6 (5.9)	104.7 (4.9)
Methylphenidate	Cocaine-D3	99	2,5	81.3 (4.4)	90.7 (0.9)	101.8 (3.7)	89.6 (2.2)	85.6 (3.3)	103.0 (6.3)	99.6 (5.0)	98.4 (8.4)	88.0 (2.7)	112.5 (4.1)	90.5 (2.2)
Morphine	Morphine-D3	95	5	65.8 (12.7)	77.9 (9.6)	88.9 (10.2)	94.0 (2.8)	87.3 (2.2)	102.7 (12.5)	98.0 (4.7)	107.1 (9.3)	92.6 (4.5)	110.5 (3.1)	100.6 (5.9)
Naloxone	Naloxone-D5	92	2,5	103.4 (4.4)	102.6 (10.3)	88.9 (10.2)	94.0 (2.8)	87.3 (2.2)	102.7 (12.5)	98.0 (4.7)	107.1 (9.3)	85.2 (8.7)	108.3 (4.9)	101.0 (8.0)
Naltrexone	Naltrexone-D3	91	1	95.9 (13.9)	85.8 (1.8)	115.9 (6.3)	86.9 (2.0)	88.7 (1.5)	119.9 (5.4)	93.4 (4.6)	100.7 (8.4)	85.2 (8.7)	102.7 (0.6)	97.9 (1.6)
Norbuprenorphine	Buprenorphine-D4	86	5	81.4 (12.3)	88.9 (8.7)	100.2 (14.9)	100.1 (2.9)	100.1 (2.9)	96.1 (10.7)	92.9 (6.6)	98.0 (3.8)	87.4 (11.6)	92.7 (9.4)	89.6 (7.6)
Norephedrine	Ephedrine-D3	98	5	109.7 (12.4)	101.2 (14.3)	90.4 (12.2)	87.6 (2.4)	89.4 (2.6)	100.0 (13.9)	99.7 (11.1)	108.0 (13.9)	105.7 (4.9)	104.1 (5.4)	101.3 (5.3)
Norfenfluramine	MDEA-D5	83	1	72.8 (16.9)	90.4 (3.3)	105.8 (5.6)	86.7 (3.4)	85.7 (2.4)	110.5 (8.1)	97.6 (3.0)	104.3 (6.3)	85.8 (6.5)	106.7 (3.3)	102.0 (4.0)
Noroxycodone	Noroxycodone-D3	80	2,5	109.3 (8.0)	106.2 (4.4)	104.5 (14.5)	86.9 (5.0)	85.1 (2.2)	104.1 (13.4)	93.7 (4.6)	103.9 (11.2)	85.6 (1.7)	112.2 (1.7)	107.0 (5.0)
Norpseudoephedrine	Ephedrine-D3	91	2,5	90.2 (4.2)	92.8 (6.9)	94.6 (9.7)	89.8 (8.4)	96.8 (7.4)	101.1 (15.2)	101.2 (6.5)	107.6 (10.8)	103.7 (4.3)	110.9 (5.3)	98.5 (4.3)
Oxycodone	Oxycodone-D3	96	1	85.8 (9.8)	112.7 (3.8)	86.7 (5.1)	92.3 (2.0)	85.6 (4.5)	113.2 (6.7)	98.1 (5.1)	102.7 (5.1)	86.2 (4.9)	108.3 (6.8)	104.6 (1.1)
Pholcodine	Morphine-D3	98	2,5	69.7 (14.3)	90.7 (10.3)	105.8 (5.9)	92.1 (4.5)	91.7 (4.9)	104.3 (9.0)	93.8 (6.2)	108.6 (6.5)	87.1 (4.2)	105.6 (3.3)	96.0 (5.3)
Ritalinic acid	MDEA-D5	96	5	99.6 (11.2)	95.4 (8.3)	93.2 (2.9)	103.3 (2.0)	90.3 (2.6)	97.2 (9.5)	99.1 (3.4)	106.0 (5.2)	93.1 (6.2)	112.6 (3.8)	110.5 (3.8)

were assessed by preparing six replicates at LLOQ and at 500 ng/mL in whole blood from 6 different individuals and calculated with a calibration standard prepared in plasma. Precision CV and bias were set less than 15% to successfully validate.

Matrix effects were evaluated in whole blood and plasma, for the 42 molecules. Six different whole blood and plasma samples were tested. In each case, potential ion suppression or enhancement was explored by comparing the signal observed for the molecule of interest (whole blood or plasma spiked at 50 ng/mL) to that obtained with Milli Q water/acetonitrile (1/2).

A robustness study was performed to evaluate the acceptable quantitative accuracy that could be provided by a calibration curve. Freshly prepared control standards (5 and 50 ng/mL) were quantified with freshly prepared calibration standards over a 4 week period. Control sample data were first processed using calibration standards prepared on the same day as the control samples and then re-processed using calibration standard data which are up to 4 weeks old.

Results

Automated sample preparation was performed in 8 minutes followed by chromatographic separation of the DOA in about 9 min
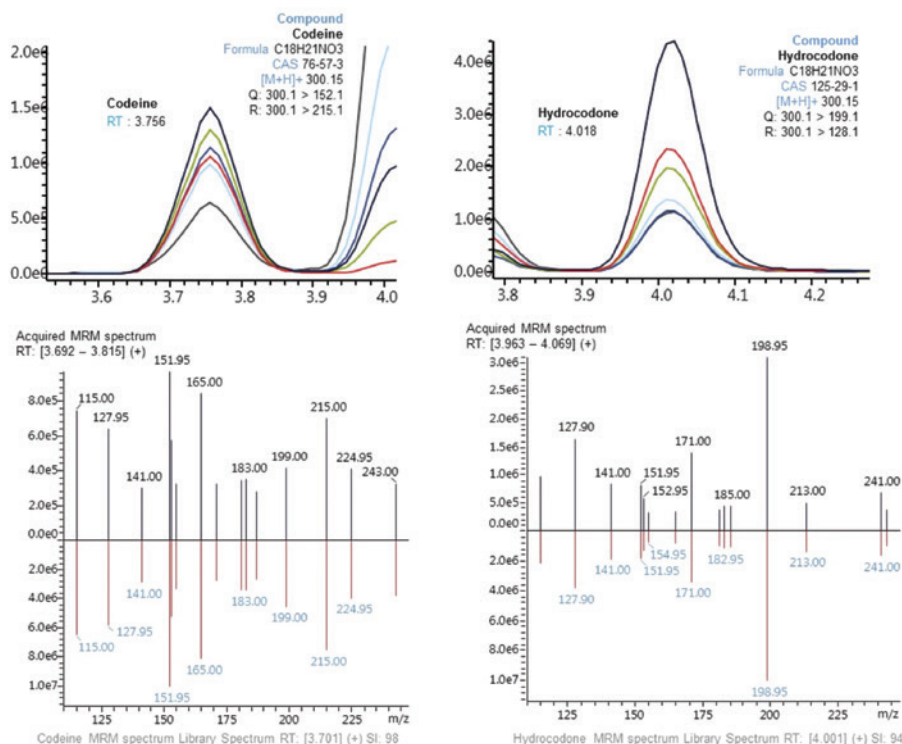
Figure 2: MRM spectrum mode MS/MS data for codeine and hydrocodone extracted from plasma samples using the CLAM-2000 extraction protocol. The identification of isobaric compounds such as codeine and hydrocodone in plasma samples was confirmed by matching acquired MRM spectrum data with a reference library generated using certified materials.
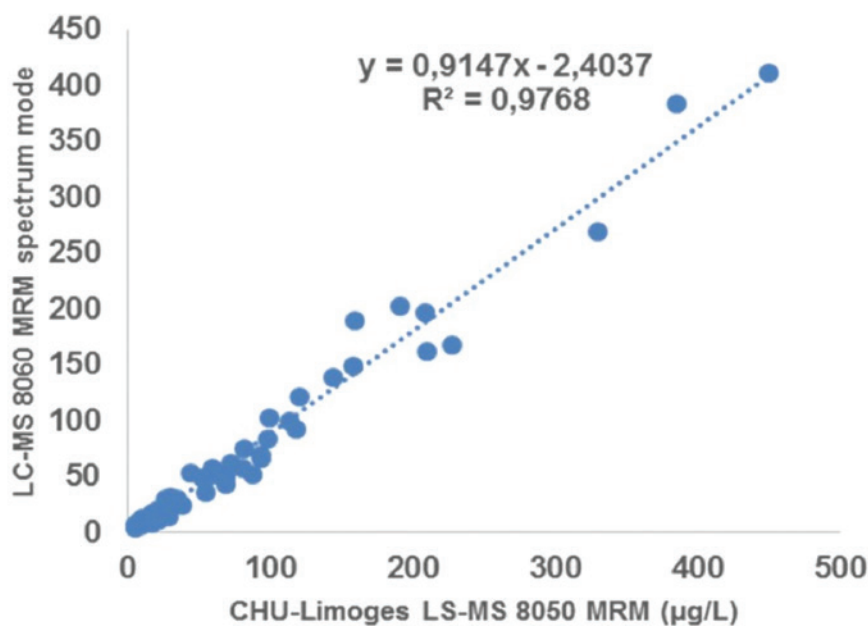

Figure 3: Regression analysis comparing the results from 43 patient blood or plasma samples acquired using the LCMS-8060 MRM spectrum mode method with library searching to a conventional LCMS-8050 MRM method.

were higher than 0.99 for all the compounds. Depending on the molecule, the LLOD and the LLOQ were set at 1, 2.5 or 5 ng/mL. No matrix effects (n=6) were reported in our conditions. Dilution tests (n=3) also reported good results.

The quantification of the control standards (5 and 50 ng/mL) with calibration curve acquired up to one month before the injection of the controls produced accuracy variation between 70 and 130%. The maximum CV value was 13.0% for the control at 5 ng/mL and 14.9% at 50 ng/ml. Correct accuracy was also obtained for the quantification of the control standard with calibration curve acquired up to one month after the injection of the controls. The maximum CV was 13.4% for the control at 5 ng/ml and 14.2% at 50 ng/mL.

Figure 2 illustrates the approach for 2 isobaric compounds.

Application of the Whole Procedure to Patient Sample(s)

The whole automated sample preparation and LC-MS/MS analysis was tested by comparing quantitative results from 43 patients samples (plasma or whole blood) prepared by the automated technique with those from a pre-existing validated method using an LCMS-8050 system, using QuEChERS salts extraction method, routinely used in the lab [5]. The automated sample preparation method was measured by MRM Spectrum mode whereas the LCMS-8050 system measured samples using a conventional MRM method. Patient blood or plasma samples were obtained from a diverse range of backgrounds commonly encountered in the laboratory including routine drug testing, DUID or emergency overdose.

Figure 3 shows a global agreement in terms of quantitation of these compounds.

Conclusion

We report a fully automated LC-MS/MS analysis method for the detection and determination of DOA in blood with the inclusion of library identification using MRM Spectrum mode.

The implementation of automation for all or part of the analysis process eliminates human errors made by manual preparation and saves time in the laboratory enabling technicians to perform other manual tasks while the system performs the analysis automatically.

(with an additional 9 min for column wash and equilibration) with retention time from 0.97 min for ecgonine methylester to 7.9 minutes for methadone. About 26 minutes were needed to obtain the first result and then, extraction and separation were performed in parallel with the system producing a result every 18 minutes. Table 3 summarises the results of the optimisation of MRM transitions acquisition. Up to 15 MRM transitions were obtained for a targeted compound.

The results of the validation study are summarised in Table 4. Acceptance criteria were obtained for all explored parameters. Concerning the intra-assay and the inter-assay (n=6) precision and accuracy, the CV values were less than 15% (except for benzoylecgonine, cocaethylene, EDDP and naltrexone for which values less than 20% were reported at the LLOQ). Using quadratic models with a 1/x or 1/x² weighting regression, the correlation coefficients of the calibration curves (LLOQ to 500 ng/mL)

We have developed a method where no human intervention was necessary when the primary tube was loaded on board the system. Sample preparation was synchronised with the LC-MS/MS system resulting in no time being lost whilst maintaining the ability to prepare the sample on- line and direct injection immediately after preparation.

A spectral acquisition method was used that allows a reconstruction of a spectrum containing all the specific transitions of a molecule. Unlike other previously published approaches where two or three collision energies were applied to all molecules in a method using product ion scanning, the collision energy for up to 15 transitions per molecule have been optimised. This approach makes it possible to obtain extremely specific and rich spectral information. Furthermore, no threshold triggering was applied, so all MRMs were measured during the entire scheduled acquisition period. Therefore, even at very low signal intensities an MRM Spectrum could be generated. By using very fast dwell and pause times the burden of measuring additional MRM transitions did not alter the sensitivity compared to the standard 2-3 transition approach and the 42 molecules were all validated to the requirements of ISO 15189 accreditation. Validation included: specificity, sensitivity and robustness of this method for

the analysis of 42 DOA and we compared its performance with that of a method accredited in the laboratory in a panel of samples obtained from patients. Investigation in to the system stability and robustness by repeat calibration curve analysis demonstrated excellent reproducibility.

With inclusion of spiked deuterated standards in unknown samples for quality control purposes we estimated our results could be quantified with an uncertainty of less than 20% using a calibration curve dating up to one month. In the case of emergency patient sample analysis, quantifying a concentration from an unknown sample to this level of accuracy with such speed may mean that lifesaving treatment might be administered within a time frame which is normally not possible with conventional sample treatment and analysis.

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