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A hybrid liquid chromatography–mass spectrometry strategy in a forensic laboratory for opioid, cocaine and amphetamine classes in human urine using a hybrid linear ion trap-triple quadrupole mass spectrometer

Geraldine Dowling*, Liam Regan, Julie Tierney, Michael Nangle

The State Laboratory, Backweston Laboratory Complex, Young's Cross, Celbridge, Co. Kildare, Ireland

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ABSTRACT

A rapid method has been developed to analyse morphine, codeine, morphine-3-glucuronide, 6-monoacetylmorphine, cocaine, benzoylegonine, buprenorphine, dihydrocodeine, cocaethylene, 3,4-methylenedioxyamphetamine, ketamine, 3,4-methylenedioxymethamphetamine, pseudoephedrine, lignocaine, benzylpiperazine, methamphetamine, amphetamine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and methadone in human urine. Urine samples were diluted with methanol:water (1:1, v/v) and sample aliquots were analysed by hybrid linear ion trap-triple quadrupole mass spectrometry with a runtime of 12.5 min. Multiple reaction monitoring (MRM) as survey scan and an enhanced product ion (EPI) scan as dependent scan were performed in an information-dependent acquisition (IDA) experiment. Finally, drug identification and confirmation was carried out by library search with a developed in-house MS/MS library based on EPI spectra at a collision energy spread of 35 ± 15 in positive mode and MRM ratios. The method was validated in urine, according to the criteria defined in Commission Decision 2002/657/EC. At least two MRM transitions for each substance were monitored in addition to EPI spectra and deuterated analytes were used as internal standards for quantitation. The reporting level was 0.05 $\mu g\,mL^{-1}$ for the range of analytes tested. The regression coefficients (r^2) for the calibration curves (0–4 $\mu g\,mL^{-1}$) in the study were $\geq$0.98. The method proved to be simple and time efficient and was implemented as an analytical strategy for the illicit drug monitoring of opioids, cocaines and amphetamines in criminal samples from crime offenders, abusers or victims in the Republic of Ireland. To the best of our knowledge there are no hybrid LC–MS applications using MRM mode and product ion spectra in the linear ion trap mode for opioids, cocaines or amphetamines with validation data in urine.

1. Introduction

The analysis of drugs of abuse such as morphine (MOR), codeine (COD), morphine-3-glucuronide (M-3-G), 6-monoacetylmorphine (6-MAM), cocaine (COC), benzoylecgonine (BENZOYL), buprenorphine (BUPREN), dihydrocodeine (DHC), cocaethylene (COCA), 3,4-methylenedioxyamphetamine (MDA), ketamine, (KET) 3,4-methylenedioxymethamphetamine (MDMA), pseudoephedrine (PSEUDOEPH), lignocaine (LIGNO), benzylpiperazine (BZP), methamphetamine (METHAMP), amphetamine (AMP), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and methadone (METH) in urine is highly important as their illicit use is widespread. The molecular structure of these compounds is shown in Fig. 1. Acute intoxication of these drugs either alone or in combination with other drugs is well documented. Urine is a simple aqueous matrix that has been used frequently and is preferred for screening and identification of illicit drugs because the concentrations of drugs and their metabolites can be reasonably high [1], urine can be easily sampled and testing is non-invasive, the volume of sample is generally high and urine testing provides long detection windows for drug use, from several days for opiates and cocaines up to months for chronic cannabinoid use [2]. The drawbacks of this matrix in regulatory monitoring are that the drug concentrations can be affected due to diurnal fluctuation and the effect of fluid intake, in post-mortem cases urine is not always available and to detect the effect of a drug blood needs to be analysed. The use of drugs analysed in this study alone or in combination with other drugs such as cannabis or alcohol is increasingly popular. 6-MAM is the specific metabolite of heroin in urine [3]. BZP has the reputation of producing amphetamine type effects [4,5] and is banned in Ireland since March 2009. BENZOYL an inactive metabolite of COC has a longer halflife than COC [6] additionally alcohol and COC are widely abused producing COCA having a longer halflife [7,8] than COC. Various adulterants can

* Corresponding author. Tel.: +353 1 505 7000.
E-mail address: Geraldine.Dowling@statelab.ie (G. Dowling).

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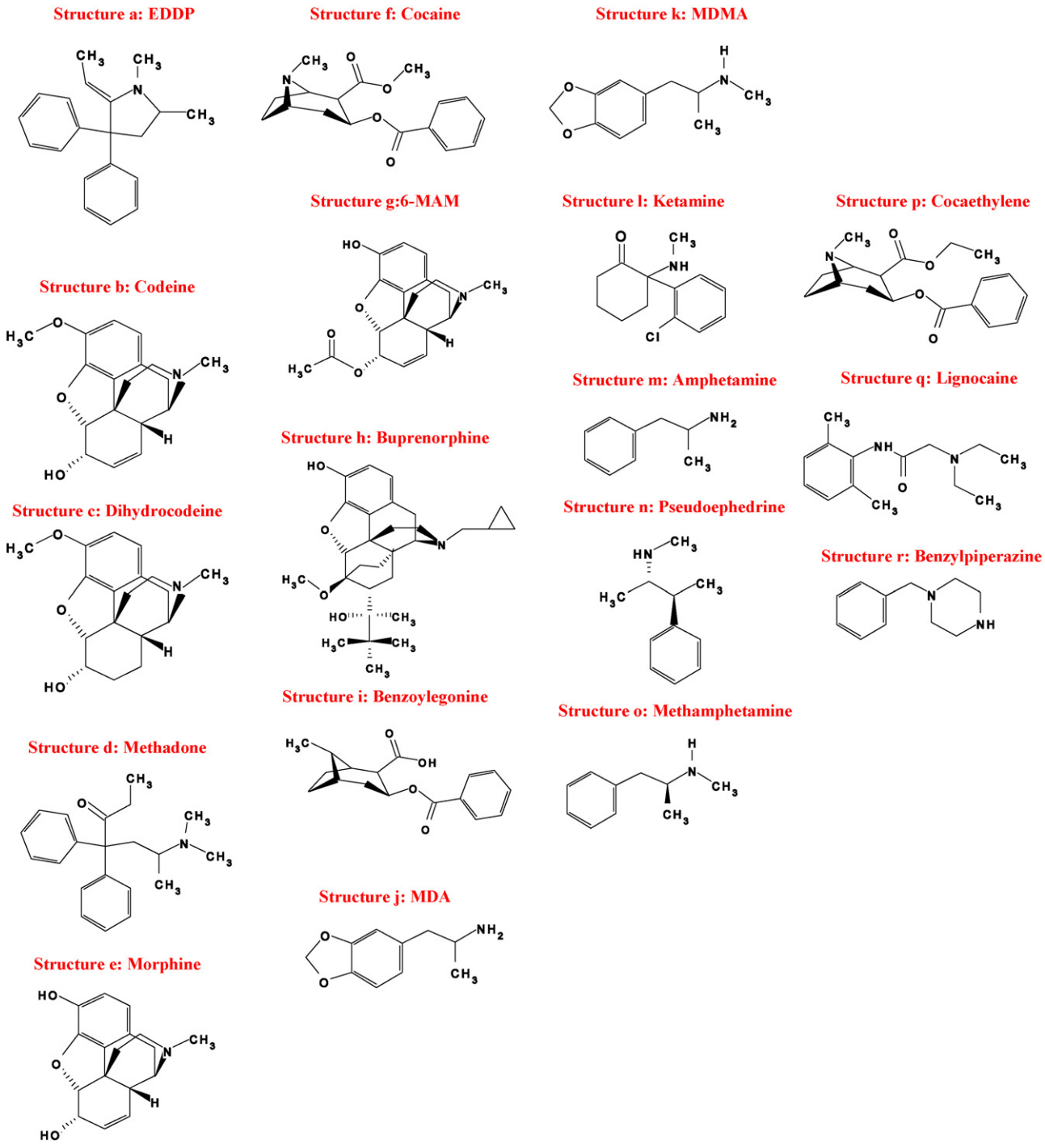

Fig. 1. Structures of the opioid, cocaine and amphetamine classes of drugs.

be added to COC [9,10] or to ectasy tablets [11] but the type of drugs used for recreational use can change rapidly [12,13] with substances like KET becoming popular [14,15].

In our laboratory in the Republic of Ireland, the analysis of opioids, amphetamines and cocaines was carried out using three separate analytical procedures using three fulltime analysts and three different GC–MS instruments.

The procedures were well established and evaluated in a large number of external quality control schemes. GC–MS entails a great deal of sample preparation, requires longer chromatographic runtimes and GC–MS can lack sensitivity for certain drugs.

Work was undertaken in order to evaluate newer technology to improve overall analysis time in the laboratory and expand the classes of drugs for analysis. Sample preparation procedures prior to analysis by LC–MS are generally more simplified. The aim of this study was to develop a fast, simple and reliable sample preparation procedure in urine to analyse 19 drugs representing drugs from the opioid, amphetamine and cocaine classes using a single sample preparation procedure and detection method that can be carried out by a single laboratory analyst. To date the simplest sample preparation procedures in urine in the literature for the determination of drugs included in this study such as AMP, MDA and MDMA [16], MOR, M-3-G, M-6-G, COD, COD-6-glucuronide, ethyl

morphine, M-6-G and 6-MAM [17] and MOR, BENZOYL, 6-MAM, COC, COCA, METH and EDDP [18] were accomplished using direct injection. Alternatively KET has been filtered prior to direct injection [19] with subsequent LC–MS detection. To date drugs of abuse in this study have been analysed in urine by LC-ion trap MS [19–23], LC tandem MS [16–18,24–30] and hybrid LC–MS [31]. Chromatographic runtimes were between 8 and 35 min, respectively. The LC–MS chromatography methods cover only a few analytes usually with long chromatographic runtimes. There was a paucity of studies that used hybrid LC–MS technology in the literature as the majority of studies use LC tandem MS. One such hybrid LC–MS technology is the 4000 QTRAP LC–MS from Applied Biosystems. This study evaluated the possibility of using this technology as a single detection technique to replace three separate GC–MS detection techniques. In the 4000 hybrid linear ion trap-triple quadrupole mass spectrometer, Q3 can be utilised as a quadrupole or a linear ion trap with axial ion injection [32]. Operation as a triple quadrupole mass spectrometer is useful when high sensitivity and selectivity of MRM transitions is needed for example in quantitative analysis or targeted screening. Operation as a linear ion trap mass spectrometer is necessary when higher sensitivity in fullscan experiments is required. In EPI mode, Q1 is used to filter the precursor ions. Q2 acts as a collision cell to generate fragments while Q3 working in ion trap mode is used to scan product ions. This results in triple quadrupole MS like fragmentation pattern but with higher sensitivity. The QTRAP has a built in collision energy spread (CES) feature which allows collection of data at the different collision energies in one EPI spectrum.

A method by Mueller et al. [31] analyses 301 drugs qualitatively in blood and urine by 3200 QTRAP hybrid LC–MS with a chromatographic runtime of 30 min. Urine although mentioned in the manuscript data or validation results were not given. In addition one MRM transition was monitored in the study therefore MRM ratios cannot be calculated if a situation arises and an EPI scan does not trigger successfully and re-injection of the sample would be necessary. Three EPI scans were also utilised in the study at three separate collision energies (CE) which increase the duty cycle. The advantage of the study by Mueller et al. is the capability to monitor a large number of compounds simultaneously in urine and blood however substances such as COCA, EDDP, BNZY and LIGNO were not analysed. The consumption of alcohol and COC is extremely popular and the detection of COCA is important in forensic toxicology because COCA has a longer detection window than COC [7,8]. This study describes the first hybrid triple quadrupole linear ion trap method with MRM as survey scan and IDA and EPI scan as dependent scan for the determination of COCA, EDDP, BNZY and LIGNO. Furthermore this is the first time that the 19 forensically important drugs have been analysed simultaneously running two MRMs and a single EPI experiment in positive mode in urine providing information on high and low mass fragments in a single injection to the best of our knowledge. Drug identification was carried out by library searching with an in-house developed MS/MS library based on EPI spectra at a single CES of 35 ± 15 in positive mode. Validation of the method was based on Commission Decision 2002/657/EC [33], a validation protocol used in the field of veterinary drug residue monitoring. The decision states criteria on the agreement of retention times, base peak and diagnostic ions and relative abundances between the standards and the samples are essential. The decisions are based on the calculation of identification points (IPs) which depend on the analytical technique. In the case of banned substances the minimum number of IPs for a methodology to be considered reliable has to be equal to 4 or higher. This is achieved by monitoring a minimum of 4 selected ions per compound when working with single MS and a minimum of two selected reaction monitoring (SRM) transitions per compound in tandem MS. Thus running two MRM transitions gives 4 IPs and also obtaining an EPI spectrum gives additional confirmatory information in this study. In this paper a fast, simple and reliable method is described for the simultaneous analysis of the 19 analytes in urine.

Table 1
LC gradient profile for determination of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO.

Time (min)	Component A (%)	Component B (%)
0.0	95	5
1.5	95	5
6.0	5	95
8.0	0	100
8.1	0	100
10.0	0	100
10.5	95	5
12.5	95	5

Component A: water:methanol (95:5, v/v + 25 mM ammonium acetate). Component B: methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid).

2. Experimental

2.1. Materials and reagents

LC–MS grade water, methanol and propan-2-ol (HPLC) were obtained from Reagecon and formic acid was obtained from BDH (Merck, UK). Ammonium acetate was obtained from Sigma–Aldrich. MOR, M-3-G, COCA, COD, DHC, 6-MAM, METH, BUPREN, EDDP, COC, BENZOYL, LIGNO, MOR-d_6, COD-d_6, DHC-d_6, METH-d_9, M-3-G-d_3, BENZOYL-d_8, COC-d_3, METHAMP, KET, MDA, MDMA, PSEUDOEPH, BZP, AMP, METHAMP-d_{14}, MDA-d_5, MDMA-d_5, and AMP-d_{11} were purchased from LGC Standards (LGC, UK). Commercially prepared primary stock standards in solution were purchased from LGC Standards available in concentrations ranging from 100 to 1000 $\mu g\,mL^{-1}$ except for BZP. A stock solution of BZP standard was prepared in methanol at a concentration of 1000 $\mu g\,mL^{-1}$. A working internal standard solution of MOR-d_6, COD-d_6, DHC-d_6, METH-d_9, M-3-G-d_3, BENZOYL-d_8, COC-d_3, METHAMP-d_{14}, MDA-d_5, MDMA-d_5, AMP-d_{11} was prepared at a concentration of 2 $\mu g\,mL^{-1}$ (stable for 6 months). A intermediate standard solution (stable for 6 months) of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO was prepared at a concentration of 10 $\mu g\,mL^{-1}$ (stable for 6 months). Standard fortification solutions (stable for 6 months) were prepared in methanol at a concentration of 2.5 $\mu g\,mL^{-1}$ from the 10 $\mu g\,mL^{-1}$ intermediate stock solution and at a concentration of 0.25 $\mu g\,mL^{-1}$ from the 2.5 $\mu g\,mL^{-1}$ stock. All standards were stored at 4 °C in the dark. Injection solvent was water:methanol (1:1, v/v).

2.2. LC–MS/MS conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). The drugs were chromatographed on a 5 μm Phenomenex HYPURITY C_8 column (4.6 mm × 100 mm) (AGB, Ireland) and the column temperature was maintained at 30 °C. A gradient was applied with water and methanol (95:5, v/v + 25 mM ammonium acetate) (A) and methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid) (B) (Table 1). The total runtime was 12.5 min with a flow rate of 0.8 $mL\,min^{-1}$. The injection volume was 20 μL. The mass spectrometer used was a QTRAP™ 4000 with a TurbolonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software.

Table 2
MS/MS parameters for determination of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO.

Compound	Transition	Declustering potential [V]	Collision energy [eV]	Collision cell exit potential [V]
MOR	286.0 > 151.9	106	83	10
	286.2 > 128.1	106	85	20
COD	300.0 > 151.9	101	95	10
	300.0 > 115.2	101	103	6
DHC	302.0 > 199.07	96	47	16
	302.2 > 128.2	96	89	8
6-MAM	328.0 > 165.0	121	55	12
	328.2 > 211.3	121	37	16
METH	310.0 > 265.0	56	21	22
	310.0 > 105.2	56	43	6
M-3-G	462.0 > 286.0	106	43	16
COC	304.2 > 182.1	36	29	12
	304.2 > 77.0	36	89	12
BENZOYL	290.2 > 167.9	46	29	10
	290.2 > 77.3	46	79	4
EDDP	278.6 > 234.3	60	35	4
	278.6 > 186.2	60	50	4
BUPREN	468.3 > 165.4	136	125	12
	468.3 > 165.4	136	125	12
COCA	317.0 > 82.0	80	45	5
	317.9 > 196.3	80	29	28
BZP	177.0 > 91.0	30	35	15
	177.0 > 65.0	30	65	20
METHAMP	150.0 > 91.0	60	30	4
	150.0 > 65.0	60	50	4
LIGNO	235.1 > 86.2	71	25	14
	235.1 > 58.0	71	53	10
PSEUDOEPH	166.0 > 148.0	60	20	4
	166.0 > 91.0	60	50	4
AMP	136.0 > 91.0	60	20	4
	136.0 > 65.0	60	50	4
KET	238.0 > 125.0	60	35	4
	238.0 > 220.0	60	20	4
MDA	180.1 > 103.0	60	20	4
	180.1 > 133.0	60	20	4
MDMA	194.1 > 163.0	31	33	2
	194.1 > 105.2	31	17	4
MOR-d6	292.06 > 152.0	116	81	12
COD-d6	306.0 > 152.1	101.6	95	10
DHC-d6	308.0 > 202.0	111	49	16
COC-d3	307.2 > 185.0	56	29	10
BENZOYL-d8	298.2 > 171.0	58	29	12
M-3-G-d3	465.2 > 289.0	116	45	16
METH-d9	319.0 > 268.1	76	23	6
METHAMP-d14	164.1 > 130.0	60	20	4
AMP-d11	147.0 > 130.0	60	15	4
MDA-d5	185.0 > 110.0	16	31	4
MDMA-d5	199.1 > 165.1	31	17	4

Note: Matrix-matched curves were used for quantification and deutrated internal standards were used as internal standards for all compounds.

2.3. MS/MS/EPI parameters

The analysis was performed using positive ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Two transitions were used and the collision energy was optimised as shown (Table 2). The MRM MS/MS detector conditions were as follows: ion mode electrospray positive; curtain gas 25 psi; ion spray voltage 5000 V; temperature 650 °C; ion source gas 1 50 psi; ion source gas 2 50 psi; interface heater on; entrance potential 10 V; resolution Q1 unit; resolution Q2 unit; collision-activated dissociation CAD gas = medium.

The strongest MRM transition and the CES spectra at 35 ± 15 for each substance were chosen from the enhanced product ion spectra (EPI mode) to set up the libary. The IDA scan intensity threshold was set at 500 counts per second (cps). The dependent scan was an EPI scan which was carried out at the CES conditions before switching back to MRM mode. The resulting EPI spectra were then searched against the mass spectral libary. The set up of the libary was achieved as follows: the LC parameters described above were utilised and the injection volume was 20 μL: concentration of each substance was 0.1 μg mL^{-1}. Turbo ion spray source in EPI scan mode with 60 V declustering potential. Q1 resolution was unit. Dynamic fill time of the trap (Q3) was set. Curtain gas 25 psi; ion spray voltage 5000 V; temperature 650 °C; ion source gas 1 50 psi; ion source gas 2 50 psi; CAD medium; CES 35 ± 15 V.

2.4. Urine samples

Urine obtained for use as negative controls was separated into 50 mL aliquots and stored at −20 °C. The urine was analysed in previous batches and urine found to contain no detectable residues of opioids, cocaines and amphetamines was used as negative controls.

2.5. Sample preparation

Urine samples (100 μL) were aliquoted into 15 mL polypropylene tubes. The urine aliquots were fortified with internal standard at levels corresponding to 0.1 μg mL^{-1} by adding a 100 μL portion of a 2 μg mL^{-1} mix solution of MOR-d_6, COD-d_6, DHC-d_6, METH-d_9, M-3-G-d_3, BENZOYL-d_8, COC-d_3, METHAMP-d_{14}, MDA-d_5, MDMA-

d_5 and AMP-d_{11}. Samples were fortified at levels corresponding to 0.1, 0.5 and 1 μg mL^{-1} by adding 40 μL of a 0.25 μg mL^{-1} fortification solution and 20 and 40 μL portions of a 2.5 μg mL^{-1} fortification solution. After fortification, samples were held for 15 min prior to the next analytical step. Methanol:water (1:1, v/v) (1800 μL) was added to the urine samples and vortexed (30 s), centrifuged (3568 × *g*, 5 min, 4 °C) and the supernatant was transferred to an autosampler vial. An aliquot (20 μL) was injected on the LC column.

2.6. Matrix-matched calibration

Matrix-matched calibration curves were prepared and used for quantification. Control urine previously tested and shown to contain no residues was prepared as above (2.4). Control urine sample (10 mL) was diluted with methanol:water (1:1, v/v) to 200 mL. A single urine sample was used for each calibration standard level. Urine samples (mL) were aliquoted into 50 mL polypropylene tubes and samples were fortified with internal standard at levels corresponding to 0.1 μg mL^{-1} by adding a 100 μL portion of a 2 μg mL^{-1} mix solution of MOR-d_6, COD-d_6, DHC-d_6, METH-d_9, M-3-G-d_3, BENZOYL-d_8, COC-d_3, METHAMP-d_{14}, MDA-d_5, MDMA-d_5 and AMP-d_{11}.

Calibration standard levels were fortified at levels corresponding to 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 μg mL^{-1} by adding 0, 20, 40, 100 μL portions of a 0.25 μg mL^{-1} fortification solution and 20, 40, 80 and 160 μL portions of a 2.5 μg mL^{-1} standard solution of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO. After fortification, samples were held for 15 min prior to the vortexing and centrifugation procedure as described above (2.5). The concentration of the drugs (μg mL^{-1}) was determined from the matrix-matched calibration curves. The calibration curves were calculated by linear regression, plotting the response factor (peak area analyte/internal standard peak area of the strong transition as a function of analyte concentration).

2.7. Method validation

For estimation of accuracy, blank urine samples were fortified with MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO at 0.1, 0.5 and 1.0 μg mL^{-1}. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility were calculated. The decision limit (CCα) of the method was calculated according to the ISO 11843 calibration curve procedure using the intercept (value of the signal, *y*, where the concentration, *x* is equal to zero) and 2.33 times the standard error of the intercept for a set of data with six replicates at three levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα. Carryover was investigated by analysing a blank solvent before and after each injection during validation and routinely in each analytical batch. The stability of standard solutions was evaluated by quantifying levels in an external quality control material over a 6-month period as a QC is ran with every batch routinely. Short-term stability of extracts was performed by analysing extracts held at 4 °C for 48 h. Matrix effects were investigated by infusion of all analytes (2.5 μg mL^{-1}) by an external syringe pump to a tee-connector at 10 μL mL^{-1} between the electrospray probe and the outlet of the analytical column with simultaneous injection of methanol:water (1:1, v/v). Subsequently five different blank matrices diluted in methanol:water (1:1, v/v) were injected on the analytical column. The specific ion transitions of the analytes in different blank matrices were recorded and any signal decreasing or increasing at the retention time of the investigated analyte was compared with the methanol:water (1:1, v/v) injection.

3. Results and discussion

3.1. Preliminary experiments

In this study an analytical strategy was developed to analyse urine samples to detect drugs of abuse. The LC–MS/MS method using MRM mode and product ion spectra in the linear ion trap mode (Q3) was developed to provide unequivocal confirmatory data for the analysis of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO. The ionisation of all drugs was studied in positive mode. The optimum conditions (declustering potential, collision energy, collision cell exit potential) were determined for each drug and the best diagnostic ions for MS/MS analysis were obtained and can be seen in Table 2. For a method to be deemed confirmatory four identification points must be obtained. In MRM (multiple reaction monitoring) mode this is achieved by monitoring one precursor ion (parent mass) and two daughter ions (corresponding to strong and weak ion) which is a suitable confirmatory method in accordance with 2002/657/EC [33]. Precursor and product ions for each analyte of interest were determined by direct infusion of single analyte solutions (1 μg mL^{-1} in methanol:water 1:1, v/v). Chromatographic tests were carried out using a 5 μm HYPURITY C_8 column (4.6 mm × 100 mm). All analytes were eluted at a flow rate of 800 μL min^{-1} and a runtime of 12.5 min per injection with good peak shape when using a mobile phase of water:methanol (95:5, v/v + 25 mM ammonium acetate) (A) and methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid) (B). The formic acid was used to assist the ionisation of the analytes in positive mode and improve peak shape by reducing peak tailing. The ammonium acetate assists with separation of the analytes. In the initial stages of development carryover was observed but a wash program was set up in the autosampler. No carryover problem was noted during validation and during routine use of the method when solvent blanks are analysed before and after samples. In MRM mode the possibility of crosstalk of analytes with internal standards was evaluated after tuning by injection of standards on column singularly. Crosstalk occurs if two compounds co-eluting have similar fragment ions in two successive transitions in an MRM method. The degree of the problem depends on the instrument speed as the fragment ions pass through the collision cell rapidly enough to exit the cell before the same fragments of the next compound come in. However this can cause dead time making the scan cycle time significantly longer leading to fewer datapoints across the chromatographic peak. No issue with crosstalk was identified during the evaluation. A EPI experiment was set up in the Analyst 1.5 software. The strong MRM transition was chosen upon completion of tuning in MRM mode. The IDA scan intensity threshold was set at 500 cps (counts per second) in the instrument method. The dependent scan was an EPI scan set at 35 CES ± 15. One drawback of the Analyst 1.5 software in data dependent mode is that the software only allows a single preselected CE or CES for all analytes in an EPI experiment. It would be better if the software manufacturers in the future would allow individual DP and CE settings per compound in EPI dependent scan mode. The resulting EPI spectra were searched against a mass spectra library. In Fig. 2 a chromatogram of COCA is shown. In Fig. 3 the library spectrum of COCA and also the acquired library spectrum of COCA at a CES of 35 ± 15 including the library search fit values are shown. The fit value (Fit) gives information about the resemblance of the signals in the reference spectrum with those in the unknown

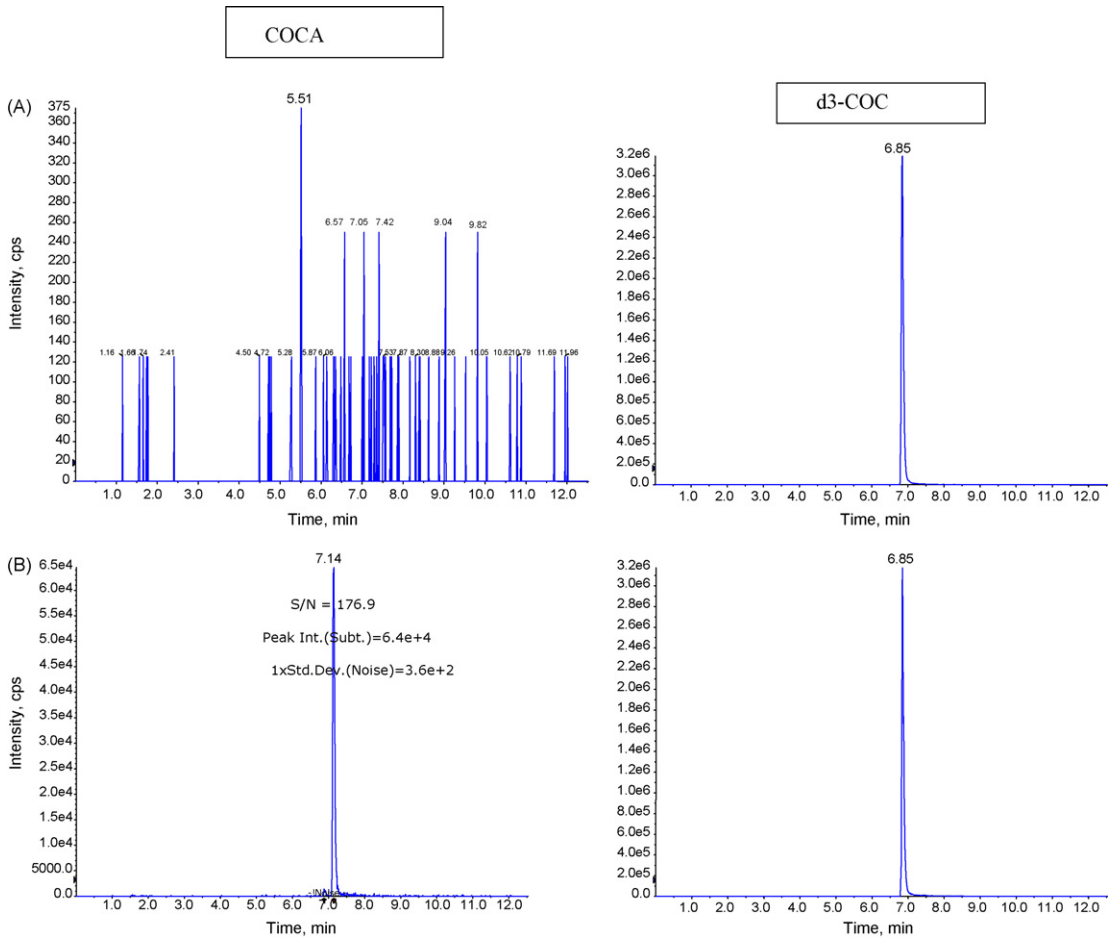

Fig. 2. Chromatogram of negative control urine fortified with 0.1 μg mL^{-1} of d_3-cocaine (A) and fortified with 0.1 μg mL^{-1} of cocaethylene and at 0.1 μg mL^{-1} with internal standard d_3-cocaine (B).

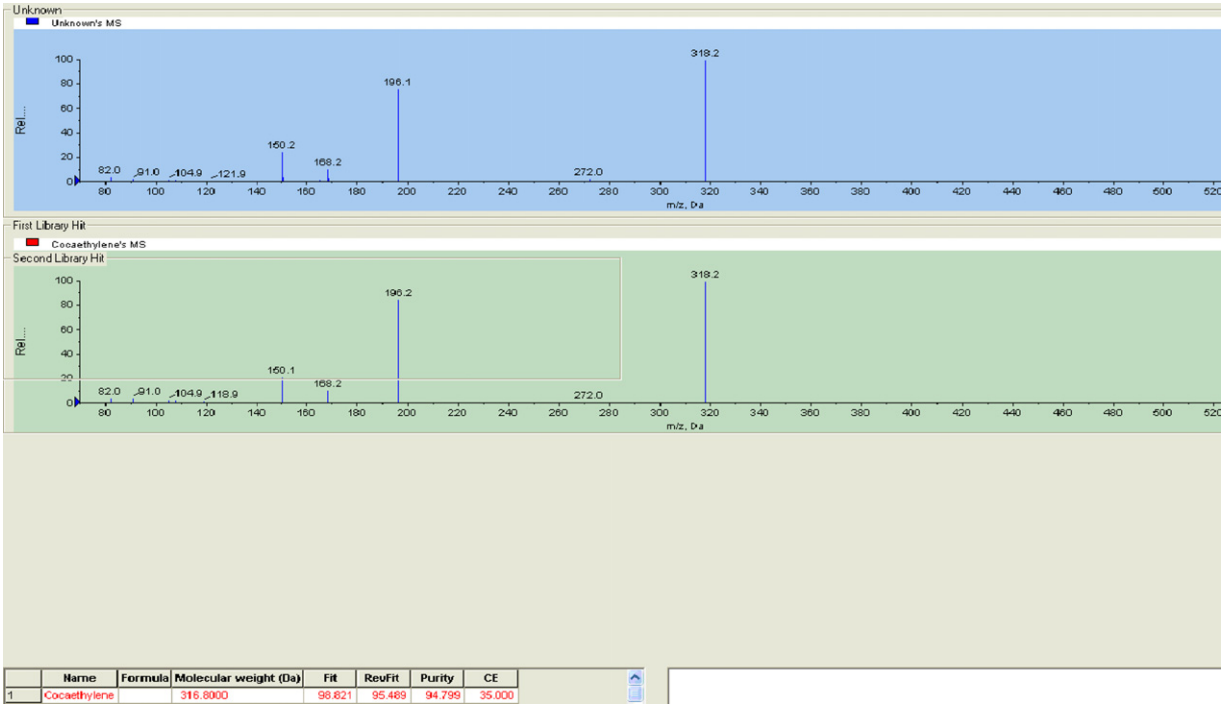

Fig. 3. Spectrum of COCA.

spectrum. The reverse fit (Reverse Fit) gives information on the resemblance of the signals in an unknown spectrum with those in the reference spectrum. The purity (Purity) is a combination of both other values. For COCA in Fig. 3, the purity value was greater than 94%. The stability of the solution standards was evaluated by quantifying levels in an external QC material over a 6-month period as the QC material was analysed with every batch routinely and is within specification. The short-term stability of sample extracts was acceptable when extracts were stored at 4 °C for 48 h. In the initial stages of method development matrix suppression was witnessed at the beginning of the chromatographic runtime and as a result the eluent was diverted to waste initially to reduce the bulk of matrix components. To further reduce any possible matrix effects deuterated internal standards were used. During the evaluation period 233 samples were analysed by this LC–MS procedure and an established GC–MS procedure and results were in good agreement. Often in forensic toxicology cases it can be difficult for the pathologist to obtain large sample sizes. Preconcentration of urine during method development was not required based on the sensitivity achieved by the QTRAP method thus the urine samples (100 μL) were diluted 20-fold in methanol:water (1:1, v/v) and a good peak shape was achieved. It was envisaged that urine samples would not require extensive clean-up due to its low protein and high aqueous content. The first advantage of the sample preparation procedure is a small sample volume is required. The second advantage is that the 19 drugs can be analysed rapidly and simultaneously using simple dilution with methanol:water (1:1, v/v). The third advantage is that the sample preparation procedure reduces the workload in monitoring for these substances in any laboratory and as a result a single analyst is capable of preparing a matrix-matched curve and 50 samples in a single day. A fourth advantage is the savings in cost as there is no need to purchase costly hydrolysis reagents for the detection of drugs that are extensively metabolised. The first advantage of the hybrid LC–MS detection method developed in this study is the fast runtime of 12.5 min per injection allowing detection of 19 forensically important drugs. The second advantage is that high and low concentrations of the drugs in urine samples can be identified, quantified and confirmed simultaneously in a single injection using EPI spectra. High levels of drugs can be detected routinely using EPI spectra and the samples do not need to be re-injected as EPI spectra can be used to unambiguously confirm overdose cases in a straightforward manner. The disadvantage of using MRM ratios only is that the sample will require dilution as a result of detector saturation and re-injection. The method has been used since 2009 to replace three other analytical strategies in our laboratory for detection of opioid, cocaine and amphetamine drugs in urine. In addition full laboratory information management system (LIMS) connectivity of the analytical strategy has been achieved using Analyst 1.5 software as part of routine monitoring of forensic toxicology samples.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [33] covering specificity, calibration curve linearity, accuracy, precision, decision limit (CCα) and detection capability (CCβ).

3.2.1. Specificity

The technique of liquid chromatography hybrid triple quadrupole linear ion trap (QTRAP) mass spectrometry itself offers a very high degree of selectivity and specificity. To establish the selectivity/specificity of the method, urine samples (30) were fortified with the above drugs and also non-fortified samples were analysed. Interfering peaks were observed at the retention time of some of the analytes in the chromatograms of the non-fortified samples but the response was negligible when compared to the reporting level.

3.2.2. Linearity of the response

The linearity of the chromatographic response was tested with matrix-matched curves using 8 calibration points in the concentration range of 0–4.0 μg mL^{-1}. In routine monitoring 25 analytical batches were analysed and the regression coefficients (r^2) for all the calibration curves were ≥0.98.

3.2.3. Accuracy

The accuracy (n = 18) of the method determined using human urine samples fortified at 0.1, 0.5 and 1.0 μg mL^{-1} in three separate assays was 84–113%.

3.2.4. Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (0.1, 0.5 and 1.0 μg mL^{-1}) was less than 12% (Table 3).

3.2.5. CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1% is applied. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of $1 - \beta$, were β = 5%. CCα and CCβ were calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with six replicates at three levels (0.1, 0.5 and 1.0 μg ml^{-1}). CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times that standard error of the intercept). The full list of CCα and CCβ values are shown in Table 4. In our methodology CCα values of 0.03–0.05 μg mL^{-1} and CCβ values of 0.04–0.09 μg mL^{-1} were obtained. Although CCα and CCβ values are widely adopted in the field of veterinary drug residues. The use of these parameters was investigated as an alternative approach to limit of detection and limit of quantification in this study. Based on the calculated CCα level in this validation study samples were analysed to verify that the method can detect the calculated CCα level. In our methodology CCα values of 0.03–0.05 μg mL^{-1} were obtained during validation. The lowest level in our matrix-matched calibration curve standard is 0.05 μg mL^{-1}. This standard give acceptable signal-to-noise ratios for each compound and is used routinely as the reporting level.

3.2.6. Measurement uncertainty

According to SANCO/2004/2726 rev 1 the within-laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [34]. For the calculation of the extended uncertainty a safety factor is required. The within-laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα, corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different urine sourced from different humans it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method was estimated at 32, 26, 29, 27, 18, 16, 35, 30, 19, 28, 16, 20, 30, 18, 25, 17, 13, 31, and 20% for MOR, COD, M-3-G, 6-MAM, COC, BENZOYL, BUPREN, DHC, COC, MDA, KET, MDMA, PSEUDOEPH, LIGNO, BZP, METHAMP, AMP, EDDP and METH. This was determined by calculating the within-laboratory reproducibility of the method,

Table 3
Intra- and inter-assay variation for accuracy of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO.

Analyte	Fortification level (ng mL^{-1})	Accuracy (%)	Within run CV (%)	Between run CV %)	Total CV %)
MOR	0.1	93	9.165	10.08	13.624
	0.5	105	6.928	2.000	7.211
	1.0	97	7.211	7.416	10.344
Combined variance	0.1, 0.5, 1.0				10.7
COD	0.1	96	5.657	7.874	9.695
	0.5	91	0.051	0.000	7.211
	1.0	97	6.708	6.403	9.274
Combined variance	0.1, 0.5, 1.0				8.8
DHC	0.1	101	6.600	2.324	6.957
	0.5	94	6.000	0.000	6.000
	1.0	94	6.245	13.000	14.422
Combined variance	0.1, 0.5, 1.0				9.9
6-MAM	0.1	89	10.724	5.532	12.066
	0.5	113	8.000	0.000	8.000
	1.0	105	5.831	0.000	5.831
Combined variance	0.1, 0.5, 1.0				9.0
METH	0.1	97	6.000	0.000	6.000
	0.5	93	4.000	2.000	4.480
	1.0	94	4.470	7.070	8.370
Combined variance	0.1, 0.5, 1.0				6.5
M-3-G	0.1	92	10.2	0.000	10.2
	0.5	102	6.928	0.000	6.928
	1.0	97	7.746	7.681	10.909
Combined variance	0.1, 0.5, 1.0				9.5
COC	0.1	93	4.123	0.000	4.123
	0.5	99	4.472	3.464	5.657
	1.0	99	5.831	5.000	7.681
Combined variance	0.1, 0.5, 1.0				6.0
BENZOYL	0.1	102	4.000	0.316	4.012
	0.5	102	4.000	0.000	4.000
	1.0	101	7.141	0.000	7.141
Combined variance	0.1, 0.5, 1.0				5.3
EDDP	0.1	98	6.900	1.000	7.000
	0.5	84	6.320	6.000	8.720
	1.0	89	5.290	12.850	13.890
Combined variance	0.1, 0.5, 1.0				10.3
BUPREN	0.1	100	8.124	15.649	17.632
	0.5	103	4.000	5.292	6.633
	1.0	101	5.477	4.123	6.856
Combined variance	0.1, 0.5, 1.0				11.6
COCA	0.1	93	4.123	0.000	4.123
	0.5	97	4.899	4.899	6.928
	1.0	96	6.856	1.414	7.000
Combined variance	0.1, 0.5, 1.0				6.2
BZP	0.1	94	5.400	11.000	12.200
	0.5	97	3.460	2.000	4.000
	1.0	96	4.690	4.900	6.780
Combined variance	0.1, 0.5, 1.0				8.4
METHAMP	0.1	99	5.500	0.000	5.500
	0.5	96	3.460	2.000	4.000
	1.0	98	4.900	4.900	6.930
Combined variance	0.1, 0.5, 1.0				5.6
LIGNO	0.1	97	3.700	1.900	4.200
	0.5	102	6.320	5.660	8.480
	1.0	105	3.610	0.000	3.610
Combined variance	0.1, 0.5, 1.0				5.9
PSEUDOEPH	0.1	96	11.800	0.000	11.800
	0.5	97	6.640	0.000	6.640
	1.0	92	8.190	6.480	10.440
Combined variance	0.1, 0.5, 1.0				9.9
AMP	0.1	100	4.900	1.300	5.100
	0.5	100	2.820	2.000	3.460
	1.0	100	4.580	0.000	4.580
Combined variance	0.1, 0.5, 1.0				4.4
KET	0.1	99	4.700	4.800	6.700

Table 3 (*Continued*)

Analyte	Fortification level (ng mL^{-1})	Accuracy (%)	Within run CV (%)	Between run CV %)	Total CV %)
	0.5	104	2.000	3.460	4.000
	1.0	99	4.583	0.000	4.580
Combined variance	0.1, 0.5, 1.0				5.2
MDA	0.1	96	5.292	12.256	13.349
	0.5	101	4.000	0.000	4.000
	1.0	98	4.583	7.071	8.426
Combined variance	0.1, 0.5, 1.0				9.4
MDMA	0.1	100	3.300	2.000	3.900
	0.5	97	3.460	7.220	8.000
	1.0	99	5.480	5.660	7.870
Combined variance	0.1, 0.5, 1.0				6.8

followed by multiplication of the within-laboratory reproducibility by the safety factor of 3.0.

3.3. Evaluation

The method developed in this study has been used to evaluate the presence of MOR, COD, M-3-G, 6-MAM, COC, BENZOYL, BUPREN, DHC, COC, MDA, KET, MDMA, PSEUDOEPH, LIGNO, BZP, METHAMP, AMP, EDDP and METH in human urine in the Republic of Ireland in 2009. In monitoring for these substances at our laboratory drug identification was carried out by libary search with a developed in-house MS/MS libary based on EPI spectra at a collision energy spread (CES) of 35 ± 15. Additionally routinely it was possible to detect the precursor ion and two daughter ions (within a single injection) in multiple reaction monitoring mode aswell as generating an EPI spectra under collision energy spread conditions. The method has been carried out using different batches of urine, different QC materials, by different analysts, using different batches of reagents, under varying environmental conditions and the method was shown to be robust. To demonstrate the applicability of the method incurred urine samples taken from subjects treated with MOR, COD and BENZOYL from the QC reference material were tested. These QC samples had values ranging from 0.25 to 0.37 μg mL^{-1} for MOR, 0.23–0.35 μg mL^{-1} for COD and 0.13–0.19 μg mL^{-1} for BENZOYL. The QC for MOR, COD and BENZOYL was found to be non-compliant as they contained levels above CCα and the calculated concentrations were within the specified range of the QC material. Furthermore the EPI spectra confirmed unambiguously the presence of MOR, COD and BENZOYL as spectra matched the corresponding spectra in the libary developed in-house. To further demonstrate the method applicability the method has been used to analyse a number of urine proficiency testing (PT) samples in which subjects were treated with MOR, BENZOYL, BUPREN, AMP, EDDP, METH and KET. The PT samples were analysed by the method developed in this study and were found to be non-compliant as they contained levels above the calculated CCα. The EPI spectra matched the corresponding spectra in the libary developed in-house in Analyst 1.5 software and satisfactory *Z*-scores of below 1.4 were obtained for PT samples. Furthermore a PT sample negative for cocaines, opioids and amphetamines was analysed by this analytical strategy and was reported as being negative thus further ensuring that an accurate analytical strategy was developed. The method was also stringently evaluated in-house by comparison with established GC methods ($3\times$ GC-ion trap methods for opiates, cocaines and amphetamines) and running all incoming samples simultaneously with old and new analytical methods. The results (unpublished data) were acceptable. The developed analytical strategy performs very well in terms of accuracy and within-laboratory reproducibility.

Table 4
Calculated CCα and CCβ values in urine for of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO.

	CCα (ng mL^{-1})	CCβ (ng mL^{-1})
MOR	0.05	0.09
COD	0.04	0.08
M-3-G	0.05	0.08
6-MAM	0.04	0.06
COC	0.04	0.07
BENZOYL	0.04	0.06
BUPREN	0.04	0.07
DHC	0.04	0.07
COCA	0.04	0.07
MDA	0.03	0.05
KET	0.03	0.04
MDMA	0.03	0.06
PSEUDOEPH	0.05	0.09
LIGNO	0.03	0.05
BZP	0.04	0.07
METHAMP	0.03	0.06
AMP	0.03	0.06
EDDP	0.05	0.08
METH	0.04	0.06

3.4. Case study

The described methodology has been applied in the laboratory since 2009 and positive drugs of abuse were identified in forensic criminal samples from crime offenders, abusers or victims using this method. The method has been used to analyse 233 samples from 01/03/2009 until 14/12/2009. The following substances were confirmed to be present in samples during this time period.

The presence of 6-MAM was confirmed in 110 samples. The presence of BENZOYL was confirmed in 91 samples, LIGNO was found in 78 samples, COC was found in 67 samples, METH was found in 65 samples, EDDP was found in 63 samples, AMP was found in 45 samples, COCA was found in 43 samples, MOR was found in 25 samples, BNZY was found in 21 samples, MDA was found in 17 samples, PSEUDOEPH and COD was found in 12 samples, MDMA was found in 7 samples, METHAMP was found in 6 samples and DHC was found in 3 samples during this time period. BENZOYL is the main metabolite of COC in urine and LIGNO is often used as an adulterant in COC.

It can be concluded that 6-MAM which is a marker for heroin and COC dominates the picture during this time period. The results obtained using the analytical strategy developed in this study were reported in forensic cases in the Republic of Ireland.

4. Conclusions

The developed strategy has been carried out using different batches of urine, different QC materials, by different analysts, using different batches of reagents, under varying environmental conditions. The developed method shows good agreement with

reference GC–MS methods (not shown). The advantage of a small sample size and the ability to confirm the identity of a wide variety of drugs in a single injection have important advantages for high sample throughput in a regulatory laboratory. Matrix effects studies were carried out and results have shown that utilizing a labelled internal standard, dilution of samples and a diverter valve minimised the effects. The accuracy of the method has been further certified as acceptable results were obtained by method comparison with PT samples and reference GC–MS methods. In conclusion the method shows that simple dilution of urine and analysis by hybrid LC–MS technology can present a rugged analytical strategy. There are no methods in the literature to the best of our knowledge that analyse the 19 drugs simultaneously in this study in urine representing opioids, cocaines or amphetamines by simple dilution and hybrid LC–MS using a hybrid linear ion trap-triple quadrupole mass spectrometer in MRM mode and product ion spectra in the linear ion trap mode. Therefore the aim of developing a fast, simple and reliable sample preparation and hybrid LC–MS strategy for opioids, cocaines and amphetamines in this study has been achieved successfully.

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