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A new mixed mode solid phase extraction strategy for opioids, cocaines, amphetamines and adulterants in human blood with hybrid liquid chromatography tandem mass spectrometry detection

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ABSTRACT

A rapid method has been developed to analyse morphine, codeine, 6-monoacetylmorphine, cocaine, benzoylecgonine, dihydrocodeine, cocaethylene, 3,4-methylenedioxyamphetamine, ketamine, 3,4-methylenedioxymethamphetamine, pseudoephedrine, lignocaine, benzylpiperazine, methamphetamine, amphetamine, methadone, phenethylamine and levamisole in human blood. Blood samples were cleaned up using mixed mode solid phase extraction using Evolute™ CX solid phase extraction cartridges and the sample aliquots were analysed by hybrid triple quadrupole linear ion trap (QTRAP) 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 blood, 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. Deuterated analogues of analytes were used as internal standards for quantitation where possible. The method proved to be simple and time efficient and was implemented as an analytical strategy for the illicit drug monitoring of opioids, cocaines, amphetamines and adulterants in forensic cases of crime offenders, abusers or victims in the Republic of Ireland.

1. Introduction

Acute intoxication of drugs either alone or in combination with other drugs is well documented. In 2009 the veterinary anthelmintic drug levamisole (LEV) has come into recent attention in the public health and medical communities as an alleged new cutting agent in adulterated cocaine. There have been a few cases first in Canada and then in the United States of patients with life-threatening cases of neutropenia/agranulocytosis as a result of LEV-adulterated cocaine [1,2]. Phenethylamine (PHEN) is a substance that can cause false positive results for amphetamines when immunoassay is used [3]. The molecular structures of these compounds are shown in Fig. 1. The analysis of these and drugs such as morphine (MOR), codeine (COD), 6-monoacetylmorphine (6-MAM), cocaine (COC), benzoylecgonine (BENZOYL), dihydrocodeine (DHC), cocaethylene (COCA), 3,4-methylenedioxyamphetamine (MDA), ketamine, (KET), 3,4-methylenedioxymethamphetamine (MDMA), pseudoephedrine (PSEUDOEPH), lignocaine (LIGNO), benzylpiperazine (BZP), methamphetamine (METHAMP), amphetamine (AMP) and methadone (METH) in blood is of vital importance in forensic toxicology. Blood is an important matrix as provides a sample screen of toxic substances present in the body at the time of collection. In our laboratory in the Republic of Ireland, the analysis of opioids, amphetamines and cocaines in blood was carried out using three separate sample preparation procedures and three different GC–MS instruments with additional derivatisation procedures. LEV was not monitored previously but the procedures were well established in blood but time consuming. The aim of this work was to develop a single fast, simple and reliable sample preparation procedure in blood to analyse the 18 drugs in this study. A study carried out by Juhascik and Jenkins [4] investigated whether switching from an established liquid/liquid partitioning (LLE) sample preparation procedure in blood to a solid phase extraction (SPE) sample preparation procedure was feasible for drugs of abuse. The study found that SPE had lower limits of detection for a wider range of drugs, was capable of detecting drugs that were previously not detectable by LLE and was shown to be a faster technique than LLE. Further benefits of the technique of SPE include reduction of matrix effects, the ability to automate the sample preparation procedure, dual reten-

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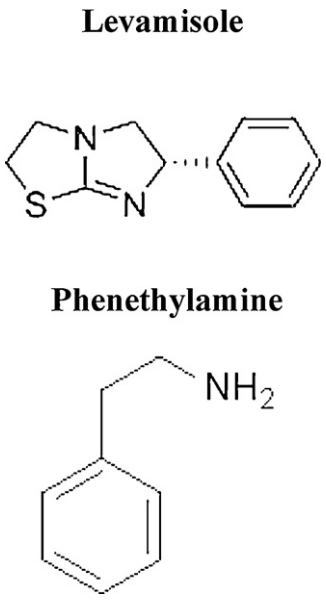

Fig. 1. Structures of levamisole and phenethylamine.

tion mechanisms, improved sorbent chemistries for rigorous wash and elution protocols, improved sensitivity and decreases exposure and costs due to hazardous solvents. The main challenge of SPE is the large number of parameters that need to be adjusted to optimise the sorbent chemistry performance. To date sample preparation procedures in whole blood in the literature using LC coupled to various detection systems for the determination of drugs included in this study were achieved by solvent extraction [4,5], liquid/liquid extraction [4,6–9] and SPE [7,10–15]. SPE sorbent chemistries utilised were reverse phase [11,13,15], cation exchange [13,10,12], strong/weak mixed mode cation exchange sorbents [12–14] and polymeric sorbents utilising Oasis HLB [13]. A new mixed mode SPE sorbent chemistry technology was developed by Biotage™ with an optimised pore size and was evaluated as a single purification strategy for the 18 drugs in blood. To date drugs in our study have been analysed using LC coupled to different detectors including LC–diode array and fluorescence detectors [12], LC–electrochemical detector [9,14,15], LC–MS [6,11], LC–MS/MS [5,8,10,13,16] and hybrid LC–MS [7]. The benefits of the technique of LC–MS are that it does not require derivatisation, can analyse both free and conjugated drugs simultaneously, can analyse thermolabile drugs directly, short chromatographic runtimes, easy online coupling to SPE and sample preparation procedures prior to analysis by LC–MS are generally more simplified. The disadvantage is the possibility of matrix effects. Evaluation of the literature showed the majority of studies in blood analysed by tandem LC–MS with chromatographic runtimes varying from 10 to 30 min. There are limited studies using hybrid LC–MS technology such as the 4000 QTRAP LC–MS system. The hybrid LC–MS method in this study was based on work undertaken at our laboratory for drugs of abuse in urine [17]. The second aim of this study was to extend the method to include LEV and PHEN. In the 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer, Q3 can be operated as a quadrupole or as a linear ion trap with axial ion injection [18]. The instrument also has the capability to perform a large number of survey scans because it has a linear acceleration collision cell (LINAC) [19] that enables ions to be transported through the system rapidly. Further information relating to this type of LC–MS is previously described [17]. A method developed by Mueller et al. [7] analyses 301 drugs qualitatively in blood and urine by 3200 QTRAP hybrid LC–MS with a chromatographic runtime of 30 min. Blood was mentioned in the manuscript but no validation results were given and results were qualitative only. A disadvantage was that only one MRM transition is monitored and if a situation arises where the EPI scan does not trigger re-injection of samples would be necessary. Three EPI scans in addition are utilised at three separate collision energies (CE) which increase the duty cycle in the study and substances such as COCA, BNZY, LEV and PHEN were not analysed in blood. This study describes a new single solid phase extraction sample preparation procedure using Evolute™ ABN CX in blood for the analysis of COCA, BNZY, LEV and PHEN and the 14 other drugs with detection by hybrid LC–MS running two MRMs and a single CES at ±15 in positive mode. Validation was based on Commission Decision 2002/657/EC [20] and in-house procedure and drug identification was achieved by library searching based on EPI spectra at a single CES of 35 ± 15 in positive mode. The method is being used as an analytical strategy in the Republic of Ireland in forensic cases.

2. Experimental

2.1. Materials and reagents

LC–MS grade water, ethyl acetate, methanol, propan-2-ol (HPLC) were obtained from Reagecon and formic acid was obtained from BDH (Merck, UK). Ammonium acetate and ammonium hydroxide were obtained from Sigma–Aldrich. Evolute™ ABN CX solid phase extraction cartridges were obtained from Biotage (Biotage, UK).

MOR, COCA, COD, DHC, 6-MAM, METH, BUPREN, EDDP, COC, BENZOYL, LIGNO, LEV, PHEN, MOR-d_6, COD-d_6, DHC-d_6, METH-d_9, M-3-G-d_3, BENZOYL-d_8, COC-d_3, KET-d_4, PSEUDOEPH-d_3, 6-MAM-d_6, EDDP-d_3, BNZY-d_8, METHAMP, KET, MDA, MDMA, PSEUDOEPH, BZP, AMP, METHAMP-d_{14}, MDA-d_5, MDMA-d_5, 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 μg ml^{-1} except for BZP. A stock solution of BZP standard was prepared in methanol at a concentration of 1000 μg 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, KET-d_4, PSEUDOEPH-d_3, 6-MAM-d_6, EDDP-d_3, BNZY-d_8, METHAMP-d_{14}, MDA-d_5, MDMA-d_5, AMP-d_{11} was prepared at a concentration of 2 μg ml^{-1} (stable for 6 months). An intermediate standard solution (stable for 6 months) of MOR, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP, LIGNO, LEV and PHEN was prepared at a concentration of 10 μg ml^{-1} (stable for 6 months). Standard fortification solutions (stable for 6 months) were prepared in methanol at a concentration of 1.25 μg ml^{-1} and 5 μg ml^{-1} from the 10 μg ml^{-1} intermediate stock solution. All standards were stored at 4 °C in the dark. Injection solvent was water:methanol (50:50, v/v). 50 mM ammonium acetate, 2% formic acid and 100% methanol were used as solid phase extraction wash solvents. 5% ammonium hydroxide in ethyl acetate (70:30, v/v) was used as the solid phase extraction elution solvent. Injection solvent was water:methanol (50:50, v/v).

Generic manufacturer's SPE procedure (Procedure A) involved dilution of blood 1:5 with 50 mM ammonium acetate buffer at pH 6.0, sonication of the samples (10 min), centrifugation (3000 rpm, 10 min) and passing the supernatant under gravity through an SPE cartridge preconditioned with methanol (3 ml) and ammonium acetate buffer (3 ml). The cartridges were washed with ammonium acetate buffer (2 ml), methanol (2 ml) and eluted with 5% ammonium hydroxide:methanol (3 ml). *Modified generic manufacturer's SPE procedure* (Procedure B) consisted of an acid wash step using 2% formic acid being introduced prior to the methanol wash step to ensure that all drugs were ionised and

retained on the cartridge prior to the high organic wash step. Wash solvent volumes were increased from 2 to 3 ml. A drying step of 20 min was introduced also. *Elution solvent study* (Procedure C) involved using Procedure B but elution with ethyl acetate:methanol:ammonium hydroxide (68:25:2, v/v/v) or (70:25:5, v/v/v) ethyl acetate:ammonium hydroxide (98:2, v/v) or (95:5, v/v) methylene chloride:isopropanol:ammonium hydroxide (78:15:2, v/v/v) or (80:15;5, v/v/v). Elution volumes of 3 × 2 ml were used.

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) 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 TurboIonSpray source from Applied Biosystems (Applied

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

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).

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

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	80	10
COD	300.0 → 151.9	101	95	10
	300.0 → 115.2	101	103	6
DHC	302.0 → 199.07	96	28	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	51	8	22
	310.0 → 105.2	51	43	6
COC	304.2 → 182.1	36	14	12
	304.2 → 77.0	36	89	12
BENZOYL	290.2 → 167.9	46	14	10
	290.2 → 77.3	46	79	4
COCA	317.0 → 82.0	80	30	5
	317.9 → 196.3	80	14	28
BZP	177.0 → 91.0	30	15	15
	177.0 → 65.0	30	45	20
METHAMP	150.0 → 91.0	60	10	4
	150.0 → 65.0	60	35	4
LIGNO	235.1 → 86.2	71	10	14
	235.1 → 58.0	71	38	10
PSEUDOEPH	166.0 → 148.0	60	8	4
	166.0 → 91.0	60	35	4
AMP	136.0 → 91.0	60	10	4
	136.0 → 65.0	60	40	4
KET	238.0 → 125.0	60	20	4
	238.0 → 220.0	60	10	4
MDA	180.1 → 103.0	60	20	4
	180.1 → 133.0	60	20	4
MDMA	194.1→ 163.0	31	5	2
	194.1 → 105.2	31	18	2
LEV	204.6 → 178	90	20	13
	204.6 → 123	90	40	10
PHEN	122.2 → 105	43	17	10
	122.2 → 77	43	45	11
MOR-d6	292.06 → 152.0	106	83	10
COD-d6	306.0 → 152.1	101	95	10
DHC-d6	308.0 → 202.0	96	28	16
COC-d3	307.2 → 185.0	36	14	12
BENZOYL-d8	298.2 → 171.0	46	14	10
METH-d9	319.0 → 268.1	51	8	22
METHAMP-d14	164.1 → 130.0	60	20	4
AMP-d11	147.0 → 130.0	60	10	22
MDA-d5	185.0 → 110.0	60	31	4
MDMA-d5	199.1 → 165.1	31	5	2
KET-d4	242.1 → 129	60	8	4
PSEUDOEPH-d3	169.1 → 150.9	60	20	4
6-MAM-d6	334.2 → 105	121	55	12
BNZY-d8	184.8 → 90.9	30	20	15

Note: Matrix matched curves were used for quantification of all compounds.

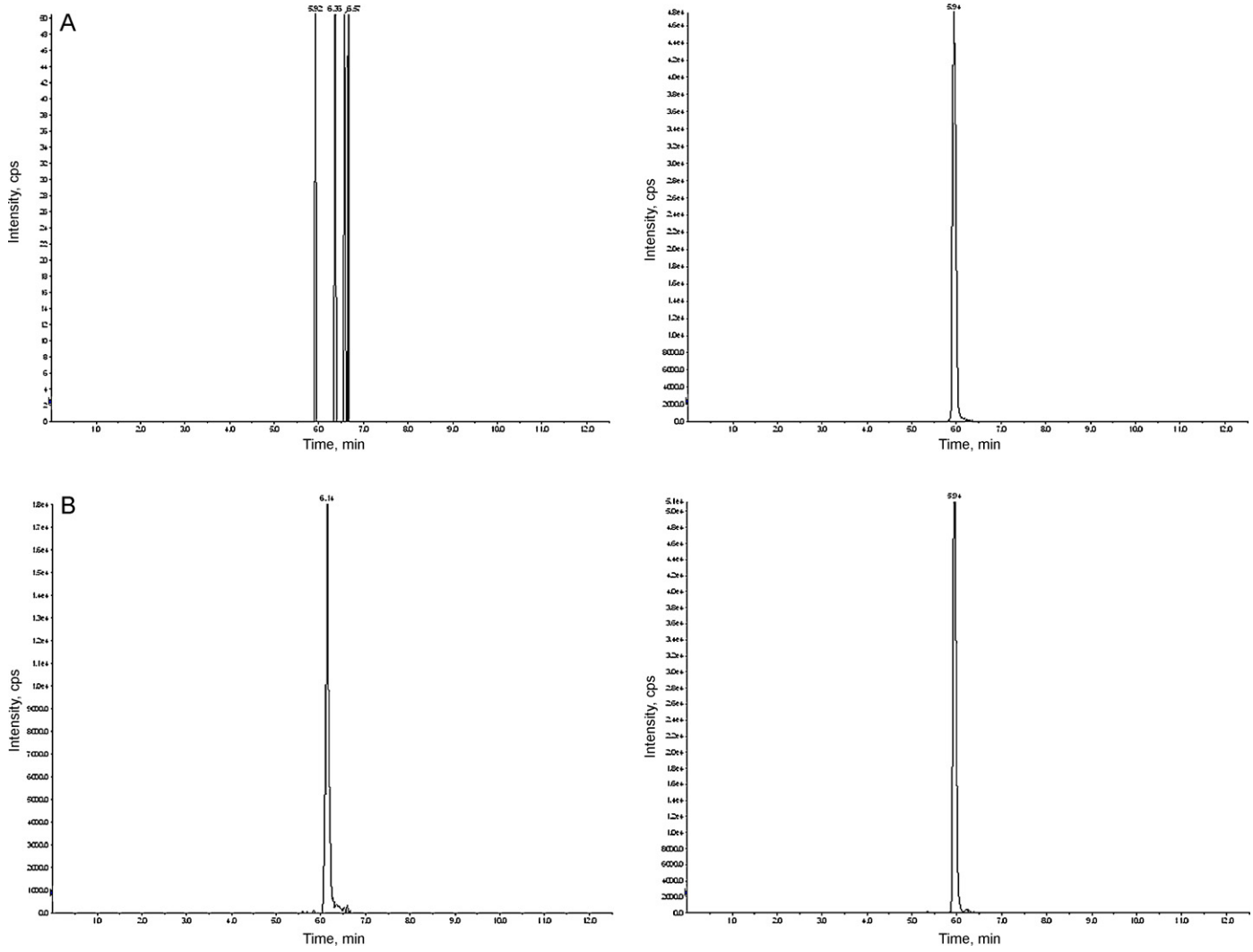

Fig. 2. Chromatogram of negative control blood (A) fortified at 0.2 μg ml^{-1} with internal standard d_6-COD and fortified with 0.025 μg ml^{-1} of LEV in blood (B) and 0.2 μg ml^{-1} with internal standard d_6-COD.

Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software.

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 library. 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 library. The set up of the library 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}.

2.4. Blood samples

Blood obtained for use as negative controls was separated into 50 ml aliquots and stored at −20 °C. The blood was analysed by the methodology described in this paper to ensure it was negative before being used in validation studies.

2.5. Sample preparation

Blood samples (500 μl) were aliquoted into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 0.2 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, KET-d_4, PSEUDOEPH-d_3, 6-MAM-d_6, EDDP-d_3, METHAMP-d_{14}, MDA-d_5, MDMA-d_5 and AMP-d_{11}. Samples were fortified at levels corresponding to 0.05, 0.075 and 0.1 μg ml^{-1} by adding 20, 30 and 40 μl of a 1.25 μg ml^{-1} fortification solution. After fortification, samples were held for 15 min prior to extraction. Ammonium acetate (50 mM, 5 ml) buffer pH 6 (adjusted with concentrated formic acid) was added and the samples were sonicated (10 min). The samples were centrifuged (3000 rpm, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The sample extracts were further purified by mixed mode cation

exchange solid phase extraction using Evolute™ CX SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with methanol (3 ml)) and ammonium acetate buffer (50 mM, 3 ml). The cartridges were washed with ammonium acetate buffer (3 ml), 2% formic acid (3 ml), methanol (3 ml) and then dried using a vacuum pump (20 min). The cartridges were eluted with 5% ammonium hydroxide in ethyl acetate:methanol (70:25, v/v) (3× 2 ml). The eluates were reduced to dryness under nitrogen at 40 °C before re-dissolving in 500 μl of methanol:water (50:50, v/v). An aliquot (20 μl) was injected onto the LC column.

2.6. Matrix-matched calibration

Matrix matched calibration curves were prepared and used for quantification. Control blood previously tested and shown to contain no residues was prepared as above (Section 2.4). Control blood samples (500 μl) were weighed into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 0.2 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, KET-d_4, PSEUDOEPH-d_3, 6-MAM-d_6, EDDP-d_3, METHAMP-d_{14}, MDA-d_5, MDMA-d_5 and AMP-d_{11}.

Samples were fortified at levels corresponding to 0, 0.025, 0.05, 0.1 and 0.0.25 μg ml^{-1} by adding 0, 10, 20, 40 and 100 μl portions of a 1.25 μg ml^{-1} standard solution. Samples were fortified at the 0.5, 1.0 and 2.0 μg ml^{-1} calibration levels by adding 50, 100 and 200 μl portions of a 5 μg ml^{-1} standard solution. After fortification, samples were held for 15 min prior to extraction procedure as above (2.5). The concentrations of the drugs (μg ml^{-1}) were determined from the matrix matched calibration curves.

2.7. Method validation

For estimation of accuracy, blank blood samples were fortified with MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP, LIGNO, LEV and PHEN at 0.05, 0.075 and 0.1 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 was 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 6 replicates at 3 levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα. 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) only and subsequently with blank matrix diluted in methanol:water (1:1, v/v) onto the analytical column. The specific ion transitions of the analytes 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. Development/optimisation experiments

In this study a methodology was developed as a tool for the analysis of drugs in forensic cases in the Republic of Ireland using a new solid phase extraction sorbent technology and the drugs were detected by hybrid LC–MS. The hybrid LC–MS/MS method was based on a previous method developed in the author's laboratory for urine analysis [17] using MRM mode and product ion spectra in the linear ion trap mode (Q3) however in addition LEV (Fig. 2) and PHEN (Fig. 3) were monitored allowing the analysis of 18 drugs simultaneously in a single injection. The ionisation of the drugs was studied in positive mode. The optimum conditions (declustering potential, collision energy, collision cell exit potential) were determined and the best diagnostic ions for MS/MS analysis were obtained (Table 2). For a method to be deemed confirmatory 4 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) in accordance with 2002/657/EC [20]. Precursor and product ions were determined by direct infusion of single analyte solutions (1 μg ml^{-1} in methanol:water (50:50, v/v)). Chromatography conditions were described in Section 2.2. An 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 dependent scan was an EPI scan. One of the drawbacks of the Analyst 1.5 software in data dependent mode was that the software only allows a single preselected CE or CES for all analytes in an EPI experiment. It would be more advantageous if individual DP and CE settings per compound could be set. The dependent scan was an EPI scan and experiments to evaluate the optimum CES conditions for each analyte showed that using simultaneously different settings improved fragmentation patterns. The optimum CES

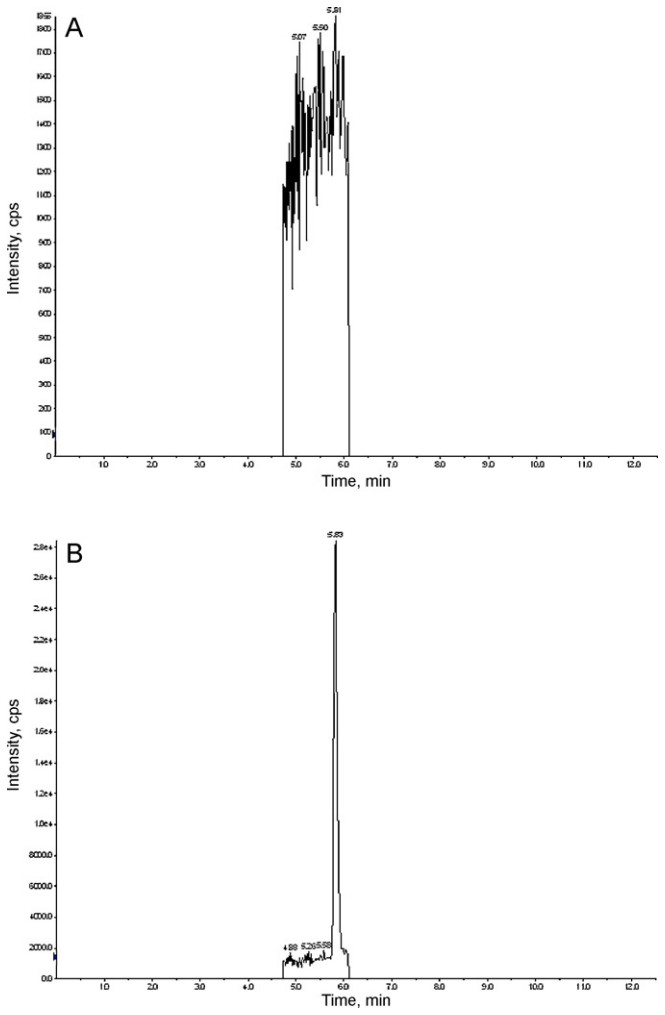

Fig. 3. Chromatogram of negative control blood (A) and negative control blood fortified with 0.025 μg ml^{-1} of PHEN in blood (B).

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

Analyte	Fortification level ($\mu g\,ml^{-1}$)	Accuracy (%)	Within run CV (%)	Between run CV (%)	Total CV (%)
MOR	0.05	99	6.025	12.033	13.446
	0.075	94	10.356	11.367	15.377
	0.1	93	4.575	10.622	11.565
Combined variance	0.05, 0.075, 0.1				13.56
COD	0.05	92	6.335	9.403	11.337
	0.075	90	5.590	10.767	12.132
	0.1	87	8.611	6.031	10.513
Combined variance	0.05, 0.075, 0.1				11.35
DHC	0.05	94	8.117	9.169	12.246
	0.075	90	13.069	0.000	13.069
	0.1	94	9.727	6.910	11.932
Combined variance	0.05, 0.075, 0.1				12.42
6-MAM	0.05	87	7.969	11.157	13.711
	0.075	91	7.103	0.000	7.103
	0.1	94	8.639	0.000	8.639
Combined variance	0.05, 0.075, 0.1				10.22
METH	0.05	83	3.231	9.350	9.992
	0.075	91	3.477	1.131	3.656
	0.1	97	3.661	2.300	4.324
Combined variance	0.05, 0.075, 0.1				6.58
COC	0.05	89	4.415	14.280	14.947
	0.075	89	3.912	5.281	6.572
	0.1	92	5.059	0.000	5.059
Combined variance	0.05, 0.075, 0.1				9.87
BENZOYL	0.05	80	3.413	6.424	7.275
	0.075	87	6.394	9.331	11.312
	0.1	84	3.588	6.223	7.183
Combined variance	0.05, 0.075, 0.1				8.80
COCA	0.05	102	4.222	8.051	9.090
	0.075	101	3.182	2.126	3.827
	0.1	103	2.781	2.206	3.549
Combined variance	0.05, 0.075,0.1				6.05
BZP	0.05	96	3.852	2.324	4.499
	0.075	98	4.061	6.907	8.013
	0.1	97	5.641	6.200	8.382
Combined variance	0.05, 0.075, 0.1				7.18
METHAMP	0.05	92	5.105	6.379	8.170
	0.075	101	4.242	10.125	10.978
	0.1	99	4.551	2.122	5.022
Combined variance	0.05, 0.075, 0.1				8.42
LIGNO	0.05	88	4.862	19.195	19.801
	0.075	92	4.242	4.855	6.448
	0.1	100	4.551	5.729	7.317
Combined variance	0.05, 0.075, 0.1				12.74
PSEUDOEPH	0.05	93	3.403	8.670	9.314
	0.075	97	5.901	2.995	6.617
	0.1	99	6.400	0.0760	6.445
Combined variance	0.05, 0.075, 0.1				7.57
AMP	0.05	84	6.892	21.327	22.413
	0.075	87	4.662	10.886	11.843
	0.1	89	5.743	6.945	9.012
Combined variance	0.05, 0.075, 0.1				15.53
KET	0.05	83	3.138	8.756	9.301
	0.075	90	4.253	6.901	8.106
	0.1	97	3.627	4.895	6.093
Combined variance	0.05, 0.075, 0.1				7.94
MDA	0.05	96	6.035	8.708	10.595
	0.075	95	6.847	4.458	8.170
	0.1	97	4.093	2.172	4.634
Combined variance	0.05, 0.075, 0.1				8.17
MDMA	0.05	86	3.714	7.873	8.705
	0.075	89	4.527	2.810	5.328
	0.1	90	3.318	2.079	3.916
Combined variance	0.05, 0.075, 0.1				6.31
LEV	0.05	98	4.353	0.415	4.732
	0.075	100	6.796	0.000	6.796
	0.1	97	6.993	0.000	6.993
Combined variance	0.05, 0.075, 0.1				6.17
PHEN	0.05	103	4.002	3.019	5.013
	0.075	101	9.127	6.160	11.012
	0.1	100	7.848	7.891	11.129
Combined variance	0.05, 0.075, 0.1				9.49

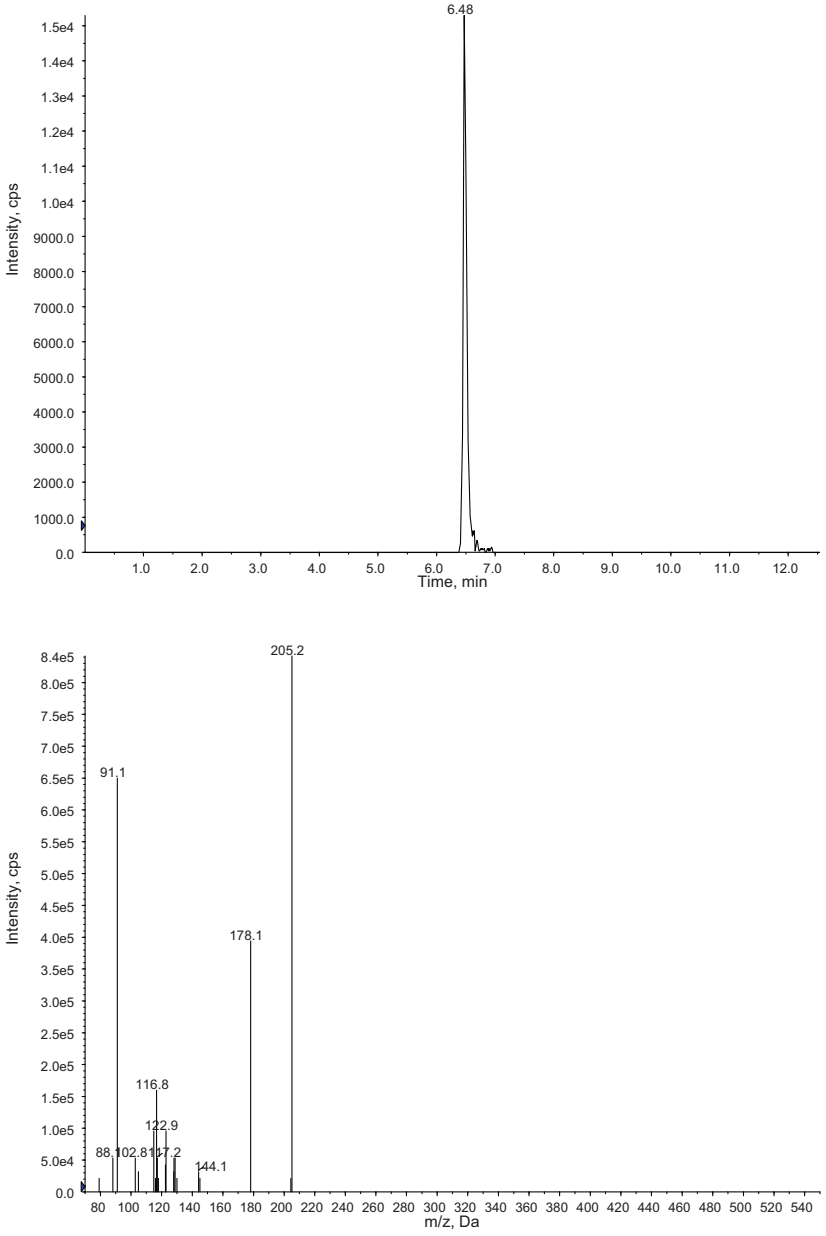

Fig. 4. Chromatogram of LEV positive and EPI spectra.

conditions for amphetamine, cocaine classes and adulterants was 35 ± 15 however for certain opioids a 50 CES $\pm$ 15 gave improved fragmentation. Due to the ability of the software to only set one CES value. A value of 35 ± 15 was chosen and the spectra generated under these conditions were utilisable for opioids. Evaluation of EPI spectra of a sample peak and spectra obtained from analysis of reference standards in a mass spectral library was subsequently carried out.

A new mixed mode solid phase extraction technology was launched by Biotage called Evolute™ ABN CX. The cartridge is a mixed mode resin based cation exchange SPE sorbent with an optimised pore size that minimises retention of high molecular weight matrix components. In this study a sample size of 500 μl was chosen based on the sensitivity required. Preliminary studies were carried out using the generic solid phase extraction protocol (Procedure A) obtained from the sorbent manufacturer using ultra pure water spiked with target compounds at a concentration of 0.25 $\mu g\,ml^{-1}$. The results showed that poor recoveries were obtained for KET, BENZOYL and LIDO. Collection of wash solvents in the generic procedure and analysis showed that KET, BENZOYL and LIDO were recovered at a high percentage in methanol. The generic manufacturer's procedure was modified as described (Procedure B). Studies showed that the methanol wash solvent contained no analytes upon addition of acid wash step. The addition of the drying procedure is important when elution solvents are non-polar to maintain a good recovery. Subsequent work was carried out to evaluate the best elution solvent (Procedure C) for the range of drugs tested. The results of the elution study showed that the best recoveries were obtained when a solution of ethyl acetate:methanol:ammonium hydroxide (70:25:5, v/v/v) was used.

The blood purification procedure developed as described in Section 2 has the following advantages:

- It reduces the workload in monitoring for these substances at our laboratory replacing three separate sample preparation methodologies.
- The ability to automate the SPE procedure.

- The option to collect the methanol wash fraction for the analysis of neutral and acidic compounds.
- The ability to fractionate using using different types of elution solvents thus widening the potential number of analytes that can be detected in a single injection.
- It is a very stringent purification procedure and can be adopted for additional matrices such as vitreous humor, muscle and urine (unpublished data).
- The strategy can be used with other detection techniques.

The new purification strategy produces extremely clean extracts and the optimised pore size of the cartridges ensures the whole blood samples passed unhindered through the cartridges making the method very fast. Furthermore the matrix of blood alone is a very complicated matrix so a thorough sample purification procedure is essential and a 100% methanol wash step ensures clean sample extracts.

A hybrid LC-MS detection method developed as described in Section 2 has the following advantages:

- It replaces three separate detection technologies for opioid, cocaine and amphetamine classes in blood using GC–MS.
- Fast run-time of 12.5 min per injection and the ability to analyse LEV and PHEN and 16 important drugs simultaneously in blood.
- High and low concentrations of drugs in blood samples can be identified, quantified and confirmed simultaneously in a single injection with no need for re-injection as EPI spectra can be used to unambiguously confirm overdose cases in a straightforward manner.
- Analysis of PHEN and the amphetamine class simultaneously allows identification of false positive results for amphetamines reducing significant time spent on sample re-analysis to identify this and the elimination of derivatisation steps.

The disadvantage of using MRM ratios only instead of MRM-to-EPI experiments is that the sample will require dilution as a result of detector saturation and re-injection. The method developed has been used in 2010 for detection of opioid, cocaine, amphetamine and adulterant drugs in blood in forensic cases in the Republic of Ireland. 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 blood forensic toxicology samples at our laboratory.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [20] 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, blood samples were fortified with the above drugs and also non-fortified samples were analysed. No interfering peaks were observed at the retention time of some of the analytes in the chromatograms of the non-fortified samples.

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 0–2.0 $\mu g\,ml^{-1}$. The regression coefficients (r^2) for all the calibration curves used in this study were $\geq$0.99.

Table 4
Calculated CCα and CCβ values in blood for MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN.

	CCα ($\mu g\,ml^{-1}$)	CCβ ($\mu g\,ml^{-1}$)
MOR	0.014	0.024
COD	0.015	0.025
DHC	0.019	0.033
6-MAM	0.013	0.022
METH	0.005	0.009
COC	0.008	0.013
BENZOYL	0.010	0.017
COCA	0.006	0.010
BZP	0.009	0.016
METHAMP	0.011	0.019
LIGNO	0.009	0.015
PSEUDOEPH	0.011	0.018
AMP	0.010	0.018
KET	0.008	0.013
MDA	0.010	0.016
MDMA	0.007	0.013
LEV	0.012	0.020
PHEN	0.013	0.023

3.2.3. Accuracy

The accuracy (n = 18) of the method was determined using human blood samples fortified at 0.05, 0.075 and 0.10 $\mu g\,ml^{-1}$ in three separate assays was 80–103%.

3.2.4. Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (0.05, 0.075 and 0.10 $\mu g\,ml^{-1}$) was less than 16% (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-β, were β = 5%. CCα and CCβ values obtained are shown in Table 4 and calculated using the intercept (value of the signal, y, where the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (0.05, 0.075 and 0.1 $\mu g\,ml^{-1}$). 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).

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 [21]. 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 blood sourced from different humans it was decided to use a safety factor of 3.0 instead of 2.33. The values are shown in Table 5 and were determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

Table 5
Calculated measurement uncertainty values in blood for MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN.

	Measurement uncertainty
MOR	41
COD	34
DHC	37
6-MAM	31
METH	20
COC	30
BENZOYL	26
COCA	18
BZP	22
METHAMP	27
LIGNO	38
PSEUDOEPH	23
AMP	47
KET	24
MDA	25
MDMA	19
LEV	19
PHEN	28

3.3. Evaluation

The analytical strategy in this study has been used to evaluate the presence of MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN in human blood in the Republic of Ireland in 2010. In monitoring for these substances at our laboratory drug identification was carried out by library search with a developed in-house MS/MS library based on EPI spectra at a collision energy spread (CES) of 35 ± 15. Additionally, it was routinely possible to detect the precursor ion and two daughter ions (within a single injection) in multiple reaction monitoring mode as well as generating an EPI spectra under collision energy spread conditions. The method has been carried out using different batches of blood, different QC materials, 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 blood samples taken from subjects treated with AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC from the QC Reference Material were tested. These QC values are shown in Table 6. The QC for AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC were found to be positive 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 AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC as spectra matched the corresponding spectra in the library developed in-house. To further demonstrate the method applicability the method has been used to analyse a number of PT schemes. In addition 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 established methods and this new analytical strategy. The developed analytical strategy performs very well in terms of accuracy and within-laboratory reproducibility.

Table 6
Theoretical values of incurred blood reference material containing AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC.

	Concentration ($\mu g\,ml^{-1}$)
AMP	0.0760–0.1208
MDA	0.0587–0.0951
MDMA	0.0427–0.0707
METHAMP	0.0991–0.1547
COC	0.0389–0.0642
BENZOYL	0.1140–0.1770
MOR	0.0247–0.0427
COD	0.1170–0.1808
DHC	0.1328–0.2032

3.4. Case studies

The described methodology has been applied in the laboratory since 2010 and positive drugs of abuse were identified in forensic cases from drug overdoses, suicidal or accidental poisonings using this method. The method has been used to analyse 40 blood samples received for toxicological analysis. The following substances were confirmed to be present in these samples. The presence of METH and MOR was confirmed in 9 samples. The presence of BENZOYL and LIGNO was confirmed in 6 samples, COD was found in 5 samples, PHEN was found in 4 samples, COC was found in 3 samples, 6-MAM, DHC, MDMA and LEV were found in 2 samples and COCA, PSEUDOEPH and MDA were each found once. It can be noted during the evaluation period that the adulterants LEV and LIGNO were identified. In 2009 the veterinary drug LEV was being used as an alleged new cutting agent in adulterated cocaine. Fig. 4 shows the EPI library spectrum of the veterinary drug and adulterant LEV which was found in a positive sample also with cocaine in Ireland. Information on fit, reverse fit and purity is previously described [17]. It can be concluded that METH and MOR were the most prevalent substances in the 40 samples studied.

4. Conclusions

The present investigation confirms that the sample preparation procedure using Evolute^{TM} ABN CX solid phase extraction cartridges and detection using hybrid triple quadrupole/linear ion trap mass spectrometer in blood can be used for the confirmation of opioids, cocaines, amphetamines and adulterants simultaneously.

There are no quantitative confirmatory methods in the literature to the best of our knowledge that analyse 18 drugs simultaneously in this study using this sample preparation procedure and hybrid LC–MS using 4000 QTRAP in MRM mode and product ion spectra in the linear ion trap mode and this study is the first. The sample preparation procedure produces extremely clean extracts and the volume of sample required is only 500 μl. The advantage of the analytical strategy at our laboratory is that it replaces 3 separate sample preparation procedures utilising 3 different GC instruments and that the sample preparation is dramatically simplified omitting extraction, hydrolysis, derivitisation steps and sample analysis time is reduced. The method has been carried out using different batches of blood, different QC materials using different batches of reagents, under varying environmental conditions and the method was shown to be rugged. The developed method shows good agreement with well established reference GC–MS methods (not shown) at our laboratory. The advantage of a small sample size and the ability to confirm the identity of a wide variety of drugs in a single injection has important advantages for high sample throughput in a regulatory laboratory. Matrix effects studies were carried out and no suppression effects were evident. The accuracy of the method has been further certified as the quantitative and qualitative results were obtained by method comparison with PT samples and reference GC–MS methods. The primary advantage of the developed analytical methodology is the quantitation and confirmation of a wide range of forensically important drugs can be carried out using a single analytical strategy and a single analyst with a short analysis time.

Therefore a reliable and fast sample preparation and detection strategy for opioids, cocaines, amphetamines and adulterants has been developed.

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