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Development of a rapid LC-MS/MS method for direct urinalysis of designer drugs

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The current immunoassay screening methodologies used to detect sympathomimetic amines within the context of workplace drug testing may fail to detect a number of the emerging designer drugs, for example β-keto amphetamines and piperazine derivatives, commonly referred to as 'legal highs'. Therefore, a rapid multi-analyte qualitative screening method, using ultra-high-pressure liquid chromatography-tandem mass spectrometry (LC-MS/MS), was investigated for analysis of new designer drugs that have emerged from the former legal highs market.

Eight analytes were targeted as model compounds: 4-methylmethcathinone (mephedrone), 3,4-methylenedioxymethcathinone (bk-MDMA, 'methylone'), 2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one (bk-MBDB, 'butylone'), 4-methoxymethcathinone (bk-PMMA, 'methedrone'), 1-benzylpiperazine (BZP), 1-(3-trifluoromethyl phenyl)-piperazine (TFMPP), 1-(3-chloro phenyl)-piperazine (mCPP), and 3, 4-methylenedioxypyrovalerone (MDPV).

The LC-MS/MS method developed encompassed direct analysis following a 1 : 4 dilution of urine with mobile phase to reduce matrix effects. Although not all compounds were completely resolved chromatographically, two product ions conferred sufficient specificity to allow target analyte identification. Although all target analytes were readily detected at 500 ng/ml, a cut-off of 1000 ng/ml was chosen to mirror the amphetamine screening cut-off commonly used for routine analysis of workplace drug testing samples.

In conclusion, direct analysis using LC-MS/MS offers an attractive way forward for the development of a rapid routine screen for new psychoactive substances, particularly given the growing number of novel compounds. **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: direct analysis; LC-MS/MS; designer drugs

Introduction

The term 'designer drug' encompasses both synthetically altered, naturally occurring compounds and substances which have been entirely designed from molecular level upwards, all of which have psychoactive properties.[1,2]

Archetypal designer drugs include amphetamines, methamphetamine and 3, 4-methylenedioxymethamphetamine (MDMA). Legislation against such drugs has resulted in the synthesis of alternative analogues marketed as 'legal' or 'herbal highs' – substances not subject to control under the Misuse of Drugs Act (1971).[3] This is achieved by manipulation of functional groups on the structural backbone as an attempt to evade legislation.

These new designer drugs are predominantly phenylethylamine, piperazine, tryptamine, pyrrolidinophenone, and phenylcyclohexyl derivatives.[2] Such is their rapid emergence that minimal scientific information is available regarding their pharmacotoxicology, making detection and determination more complex. Reportedly all confer a similar mechanism of action to amphetamine and/or MDMA, i.e. sympathomimetic stimulation and/or empathogenic effects respectively dependent on their exact configuration.[4,5]

Control measures have come into effect in the UK targeting these new psychoactive substances and similar legislation is being considered in several other countries. Detection and determination of these new designer drugs (Figure 1a–1h) as part of a workplace drug testing program is increasingly expected because they are being administered as 'recreational replacements' for their more well-known counterparts (Figure 1i–k).

Immunoassay is commonly used for preliminary screening of abused drugs in urine and is an integral part of the approach used for testing samples collected as part of a workplace drug testing program.[6] Hybridoma production (including the period of immunization) seldom takes less than two months from start to finish, and it can take well over a year[7] combined with the further development to launch a consistent commercial immunoassay for drug screening purposes; the whole process is time consuming. Given the diversity of these new designer drugs combined with their ever changing popularity and availability, that the development of new immunoassays to specifically target these drugs is unlikely to be financially viable.[2] Currently there are no immunoassay available which specifically target new psychoactive substances, for example, β-keto amphetamines and piperazine derivatives.

The lack of a suitable immunoassay means that a rapid alternative technique is necessary to enable detection of such compounds.[10] Although common, gas chromatography mass spectrometry (GC-MS) is not an ideal alternative to immunoassay screening because of the protracted preparative requirements, including extraction often followed by derivatization of the

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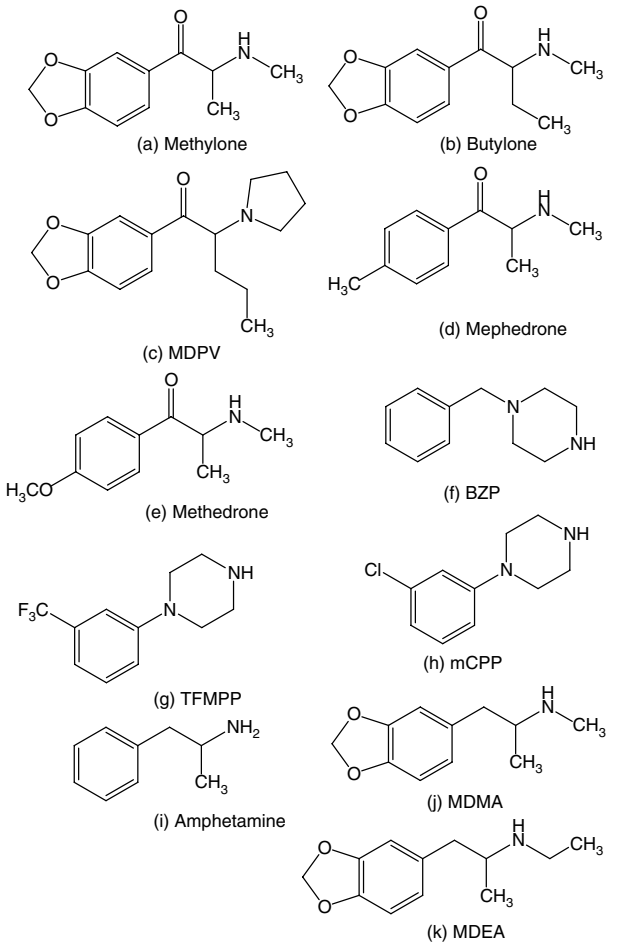

Figure 1. Examples of new (a–h) designer drugs recently legislated against and some of their traditional designer drug counterparts (i–k). Drugs a–e are beta-keto amphetamines (cathinone derivatives), and examples f–h are piperazine derivatives.

amine function of these fairly polar drugs.[8] LC-MS/MS has the potential to screen for multiple analytes within one assay but it is susceptible to matrix effects.[9,10,16] This phenomenon must be considered for each analyte of interest and several approaches exist to evaluate the extent of matrix effects on a particular assay.[11,13] Validated screening methodologies using LC-MS have been developed, although these have predominantly used liquid-liquid extraction (LLE) or solid-phase extraction (SPE) thus increasing sample analysis time.[1,2] A direct analysis approach has been used in several bioanalytical areas for many years, such as metabolic fingerprinting, and is now being increasingly applied for the determination of drugs in urine, for example having been demonstrated to be robust and reliable for the confirmatory analysis of the amphetamines.[13] Moreover, direct injection has been routinely used for the screening of some of the more common drugs of abuse within the Concateno laboratories for the last eight years. There is, however, minimal information regarding the use of 'dilute and shoot' for direct analysis of designer drugs such as those in this report.

The aim of this study was to develop a rapid, multi-analyte qualitative screening method using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to specifically identify the most recently legislated against designer drugs in urine (Figure 1a–h). The use of direct analysis, rapid chromatography and short analysis times could make it attractive as a viable addition to the immunochemical screening panel used for workplace drug testing.

In addition, the cross reactivity of some of the new designer drugs (Figure 1a–h) in the CEDIA® amphetamine/ecstasy assay was investigated.

Experimental

Chemicals and reagents

The following drug standards were obtained from Sigma Aldrich (Poole, Dorset, UK); 4-methylmethcathinone (mephedrone), 3,4-methylenedioxymethcathinone (bk-MDMA, 'methylone'), 2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one (bk-MBDB, 'butylone'), 4-methoxymethcathinone (bk-PMMA, 'methedrone'), 1-benzylpiperazine (BZP), 1-(3-trifluoromethyl phenyl)-piperazine (TFMPP) and 1-(3-chloro phenyl)-piperazine (mCPP). A sample of 3,4-methylenedioxypyrovalerone (MDPV) was kindly provided by John Ramsey, TICTAC Communications, St Georges University of London. The deuterated internal standards; amphetamine-d5, 3,4-methylenedioxymethamphetamine-d5 (MDMA-d5), methylenedioxyamphetamine-d5 (MDA-d5) and methylenedioxyethylamphetamine-d5 (MDEA-d5) were purchased from LGC Standards (Teddington, UK). HPLC grade methanol, ammonium acetate, acetonitrile, and analytical grade formic acid were purchased from Fisher Scientific (Loughborough, UK).

Stock standard solutions (1 mg/ml) of each analyte were prepared in methanol and used to prepare methanolic working solutions of the individual analytes at concentrations of 100 μg/ml and 10 μg/ml respectively. A working internal standard solution containing amphetamine-d5, MDMA-d5, MDA-d5 and MDEA-d5 was prepared at a concentration of 10 μg/ml.

Preparation of calibrators and quality control (QC)

To assess the detection capabilities of the LC-MS/MS screening procedure, calibrators containing mephedrone, methylone, butylone, methedrone, BZP, TFMPP, mCPP and MDPV were prepared daily in drug-free urine prior to analysis at concentrations of 500 ng/ml, 1000 ng/ml and 2000 ng/ml. A limited 3-point calibration curve was considered appropriate because the method would be applied to the qualitative analysis of samples as a preliminary screening procedure.

Combined quality control (QC) samples containing the analytes of interest were prepared independently from the calibrators in drug-free urine, i.e. using a different blank matrix to that used for the preparation of calibrators, at a concentration of 1000 ng/ml. These were stored in 5-ml aliquots at −20 °C prior to analysis.

The calibrators, together with the QC samples, were analyzed alongside each run and used to assess the method performance (reproducibility, %CV) as well as the stability of samples under storage conditions.

A 'carry over' standard was prepared at a concentration of 50 000 ng/ml by spiking all of the analytes of interest into drug-free urine. The carry over standard was run at the beginning and the end of each analytical run and was immediately followed by a 'blank' sample to ensure that any analyte carry over would be detected.

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Table 1. List of analytes including relevant deuterated (d) internal standards, together with their respective mass spectrometry parameters

Drug name	Retention time ($t_R$min)	Q1 mass (amu)	Q3 mass (amu)	Cone (V)	Collision energy (kV)
BZP	1.27	176.1	90.7	25.0	27.0
	1.27		84.7	25.0	25.0
Methylone	1.79	208.0	159.9	20.0	20.0
	1.79		131.9	20.0	27.0
Amphetamine-d5	1.86	141.0	124.0	12.0	12.0
MDA-d5	1.96	185.0	168.0	17.0	15.0
Methedrone	2.02	193.9	175.9	22.0	15.0
	2.02		160.9	22.0	25.0
MDMA-d5	2.09	199.0	165.0	20.0	15.0
Butylone	2.17	222.0	203.9	20.0	20.0
	2.17		173.9	20.0	22.0
MDEA-d5	2.28	213.0	163.0	25.0	12.0
Mephedrone	2.28	177.9	159.9	22.0	20.0
	2.28		144.8	22.0	25.0
mCPP	2.75	196.0	153.8	37.0	25.0
	2.75		118.8	37.0	32.0
MDPV	2.89	276.1	174.9	35.0	35.0
	2.90		148.9	35.0	35.0
TFMPP	3.24	231.0	188.0	35.0	32.0
	3.24		44.0	35.0	35.0

Immunoassay screening

To establish the immunoreactivity of the target analytes with the CEDIA® Amphetamines/Ecstasy immunoassay, individual drug standards at concentrations of 1000 ng/ml, 5000 ng/ml, and 10 000 ng/ml were prepared in drug-free blank urine.

The standards were analyzed according to the manufacturer's instructions using an Olympus AU2700 (High Wycombe, Beckman Coulter, UK) in conjunction with the CEDIA® Amphetamines/Ecstasy assay (Microgenics, Freemont, CA, USA) using a 1000 ng/ml d-methamphetamine standard as the cut-off calibrator.

QC samples were prepared from commercially available material at ±20% of the assay cut-off and run alongside the standards to ensure acceptable assay performance.

LC-MS sample preparation

A 1-ml aliquot of sample check/sample/QC/calibrator was prepared for analysis by centrifuging at 15 830 g for 5 min. Following the addition of 50 µl of internal standard solution (10 µg/ml) samples were vortex mixed for 10 s and then diluted 1 : 4 (*v/v*) with 98% 20 mM ammonium acetate, adjusted to pH using 0.1% formic acid 3 : 2% acetonitrile. The samples were then directly injected into the LC-MS system.

Instrumentation

Chromatography

Analysis was performed using a Waters Acquity ultra performance liquid chromatography (UPLC) system (Waters, Elstree, UK). Chromatographic separation was achieved using an Acquity UPLC BEH C18 2.1 × 50 mm column with 1.7 µm particle size fitted with a 0.2 µm stainless steel Acquity column in line filter unit (Waters, Elstree, UK). The mobile phase consisted of solvent A (20 mM ammonium formate/0.1% formic acid, pH 3) and solvent B (acetonitrile/0.1% formic acid). A gradient method was used starting with 2% B, ramping to 25% B by 2.5 min. Following a 1-min hold period, it was then subsequently increased to 40% B by 4.2 min and then rapidly returned to starting conditions by 4.3 min. The total run time was 5 min. The flow rate was set at 0.400 ml/min with an injection volume of 15 µl. The auto-sampler was kept at 20 °C and column temperature set to 35 °C.

Mass spectrometry

Analyses were performed using a Quattro Premier XE triple quadrupole mass spectrometer (Waters, Elstree, UK) using electrospray ionisation in the positive mode. The following parameters were optimised and used during analysis; capillary voltage 1.00 kV; source temperature 120 °C; desolvation temperature 400 °C; desolvation gas flow rate 887 l/hr. Direct infusion of the individual analytes under investigation was used to identify the molecular ion ($M + H^+$) followed by a product ion scan to identify the most prominent fragments following collision energy optimisation. This information was used to determine the appropriate selected reaction monitoring (SRM) transitions which were used during analysis (Table 1). Due to the lack of availability of deuterated internal standards for the compounds studied, deuterated amphetamine, MDA, MDMA and MDEA were added as chromatographic markers for the following:- Amphetamine-d5: BZP and Methylone; MDA-d5: Methedrone; MDMA-d5: Butylone; MDEA-d5: Mephedrone, mCPP, MDPV and TFMPP. To ensure continued sensitivity of the method, the cone was cleaned daily prior to use.

Data acquisition, data review and instrument controls were performed using MassLynx and Targetlynx software (Waters, Elstree, UK).

Limit of detection, limit of quantitation, matrix effects, and analyte recovery

There are several approaches which can be used to calculate the limit of detection (LOD) and limit of quantitation (LOQ) of an

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Table 2. Results generated in the CEDIA amphetamine/ecstasy assay following the analysis of drug-free urine spiked with β-keto amphetamines and piperazine derivatives

Sample type	Apparent concentration (ng/ml). 10 000 ng/ml solution	Apparent concentration (ng/ml) 5000 ng/ml solution	Apparent concentration (ng/ml) 1000 ng/ml solution
Butylone	1592	646	239
Methylone	369	176	37
Methedrone	244	141	53
Mephedrone	211	88	67
BZP	435	254	60
TFMPP	850	459	101
mCPP	847	463	64
MDPV	169	94	10

analytical method. Since the method described is qualitative and incorporates the use of a relatively high analytical cut-off (1000 ng/ml) to reflect the cut-off commonly applied when screening using an amphetamine immunoassay, the LOD and LOQ were estimated from the signal to noise (S/N) of the lowest calibrator (500 ng/ml). A S/N ratio of 3 is recognized as acceptable for estimating LOD with an S/N of 10 being acceptable for the determination of the LOQ. These criteria were applied to the data generated as part of this study.

Matrix effects and recovery were assessed using the direct comparison method as described by Matuszewski *et al*.[12] Sets of samples containing 500 ng/ml of each working standard and internal standards were prepared in matrix free solvent (0.1% formic acid) and drug-free urine samples from 5 different sources (pH 6–9).

Ion suppression and enhancement was assessed by comparing the peak area of standards in 0.1% formic acid (A) and standards spiked into the urine samples post-dilution (B) and pre-dilution (C):

$$\text{Matrix effect (\%)} = \frac{\text{area ratio of B}}{\text{area ratio of A}} \times 100$$

$$\text{Recovery (\%)} = \frac{\text{area ratio of C}}{\text{area ratio of B}} \times 100$$

Case sample analysis

During the course of the study two samples were submitted to the laboratory specifically for mephedrone analysis. Sample 1 was collected from a known mephedrone user. The second sample was collected from a known associate of the donor of sample 1. The method described was applied to the analysis of these samples.

Results and discussion

Immunoassay screening

Currently neither β-keto amphetamines or piperazine derivatives whether in their parent drug form or their metabolites (where elucidated) are listed as cross-reactants in amphetamine and/or methamphetamine immunoassays.[2,14] The data presented in this report support this assumption with the results for all target analytes falling well below the 1000 ng/ml cut-off of the immunoassay, with the exception of butylone, where a 10 000 ng/ml concentration produced a positive screening result (Table 2), with an apparent amphetamines concentration of 1592 ng/ml, 16% of the true concentration.

It is not clear why butylone has a higher immunoreactivity than the other analytes studied. According to Loor *et al*.,[15] when compared to their non-keto equivalents using the Microgenics multiplex CEDIA Amphetamine/Ecstasy assay, MDMA (the non-keto equivalent of methylone) had the highest cross reactivity (199% when a 250 ng/ml concentration was tested) and not MBDB, (the non-keto equivalent of butylone) which had a 123% cross reactivity when 500 ng/ml was tested.

The piperazine derivatives (Table 2) exhibited a weak cross-reactivity with the CEDIA Amphetamine/Ecstasy assay as demonstrated by the analysis of samples spiked at 5 times and 10 times the assay cut-off. Most immunochemical research appears to have been performed on piperazine derivatives particularly BZP and TFMPP.[16,17] Using the CEDIA DAU Amphetamine/Ecstasy assay Button and Kenyon[18] found that BZP and TFMPP did not test positive above the 1000 ng/ml cut-off until concentrations of 150 000 ng/ml and 25 000 ng/ml, respectively were used. This supports earlier research by de Boer *et al*.[19] using the AxSYM Amphetamine/Methamphetamine II FPIA assay which did not detect urine spiked with 100 000 ng/ml BZP using a 300 ng/ml cut-off. However, some degree of cross-reactivity was observed with the Dade Behring EMIT d.a.u. Amphetamine assay using a cut-off of 300 ng/ml.

Although all the target analytes discussed in this study are thought to act to varying degrees on the same receptors as (*S*)-amphetamine, the results support previous research which suggests that binding to the same receptors does not ensure that they will also interact with the same assay antibodies as amphetamine.[8,19]

Chromatographic analysis

Thorough sample preparation is integral before using hyphenated techniques as part of bioanalysis, particularly with the advent of more potent drugs which may be present at lower levels than previously encountered.[20,21] Yet it is sample preparation, especially if performed manually, that is believed to be a fundamental rate-limiting step to high throughput analysis.

In this study, centrifugation was used in an attempt to remove any particulates as quickly as possible prior to the addition of the internal standard and sample dilution. A dilution factor of 1 : 4 (*v*/*v*) was chosen based on previous in-house (unpublished) research into β-keto amphetamine analysis. The dilution factor chosen is integral because of potential column damage and loss of MS sensitivity.[22]

The combination of reduced sample preparation time by the use of a simple sample dilution approach together with the use of UPLC chromatographic conditions allowed the development of a fast and accurate analytical method with increased chromatographic

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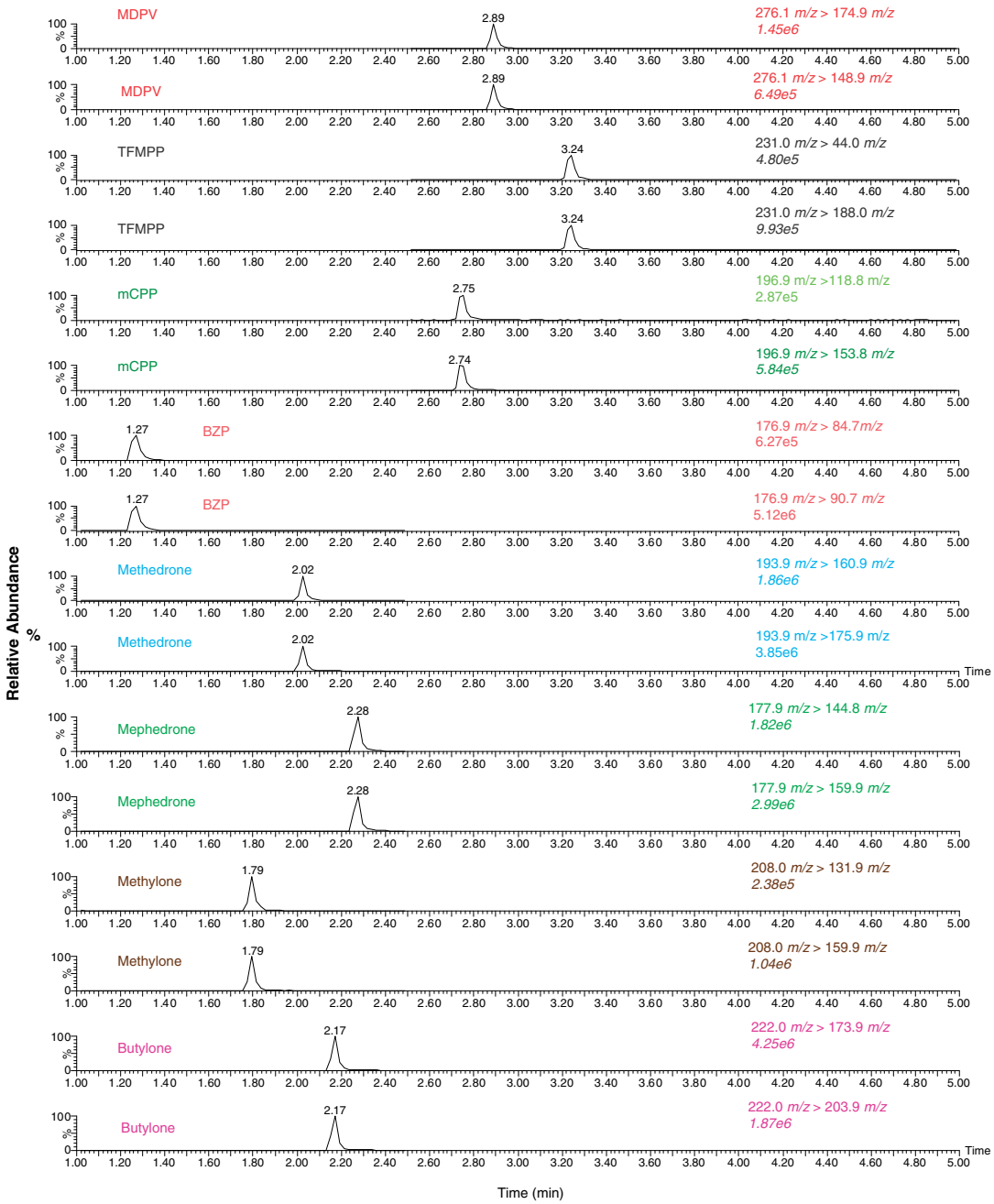

Figure 2. SRM mass chromatograms obtained following the analysis of 500 ng/ml standards prepared in 0.1% formic acid.

resolution and sensitivity.[10] Not all compounds were completely resolved from each other (Figures 2 and 3) in the chromatographic run adopted (5 min), but two product ions conferred sufficient specificity to allow identification of all eight of the drugs targeted. With the growing number of new psychoactive substances, positional isomers may give rise to identical transitions and without adequate chromatography, these may not be distinguished. Even so, with the 'dilute and shoot' approach, this is not an issue, the emphasis being on the rapid screening of drugs, presumptive positive samples then being subjected to analytical procedures incorporating adequate chromatographic resolution and relative ion intensities for identification purposes. The inter-assay QC data obtained using this method demonstrated acceptable between run reproducibility, with a CV of 12%. No analyte carry over was detected throughout the study.

It should be noted that retention times for each analyte were reproducible (+/− 2%) despite the lack of a deuterated internal standard. Ideally the method should include a deuterated internal standard for each compound targeted, particularly for the early eluting analytes, as a simple correction technique. For screening procedures, however, using a different internal standard for every analyte is impractical because it starts to reduce SRM sensitivity by introducing too many transitions.

SRM was chosen because of its recognized specificity. Such an approach was ideal for this study because the method was to be developed as a targeted screening approach, i.e. tested against

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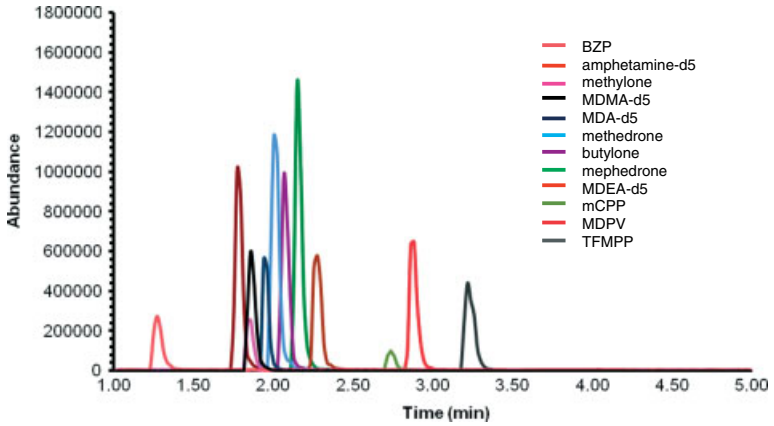

Figure 3. Smoothed chromatogram of the primary transitions (see Table 1) obtained following the analysis of a 500 ng/ml standard prepared in drug-free urine.

Table 3. Limit of detection and limit of quantitation calculated as three times the S/N (LOD) and ten times the S/N (LOQ) following the analysis of the 500 ng/ml standard prepared in drug-free blank urine

Analyte	Estimated Limit of detection (ng/ml) (S/N 3 : 1)	Estimated Limit of quantitation (ng/ml) (S/N 10 : 1)
Butylone	2.0	6.5
Methylone	2.8	9.3
Methedrone	2.4	8.0
Mephedrone	2.0	6.8
BZP	8.3	27.3
TFMPP	6.5	21.5
mCPP	29.0	96.0
MDPV	3.4	11.3

Table 4. Matrix effects (mean % +/− SD) and analyte recovery (mean % +/− SD) calculated following the analysis of a 500 ng/ml standard prepared in drug-free blank urine (n = 5)

Analyte	Matrix effects (Mean % ± SD)	Recovery (Mean % ± SD)
Butylone	100.1 (+/− 3.09)	93.0 (+/− 7.10)
Methylone	99.9 (+/− 10.7)	97.8 (+/− 9.33)
Methedrone	92.8 (+/− 4.1)	105.3 (+/2.40)
Mephedrone	106.8 (+/− 3.7)	96.4 (+/− 3.33)
BZP	75.4 (+/− 1.4)	85.8 (+/− 5.61)
TFMPP	104.9 (+/− 7.7)	79.5 (+/− 5.12)
mCPP	92.6 (+/− 7.7)	88.2 (+/− 3.10)
MDPV	108.0 (+/− 0.9)	77.2 (+/− 3.25)

a panel of selected compounds. This approach is not suitable for analysis of complete unknowns.[23]

Numerous reports[24–26] state that three ions or at least two SRM transitions should be chosen, preferably including the molecular ion, as was performed in this study to increase selectivity of the technique. This highlights that not only are the specific transitions important for selective and sensitive detection but also the analyte specific instrument settings.[24–26] Nordgren *et al*.[25] reported that when using only one SRM transition for screening urine samples, approximately one-third of their results yielded false positives. This is because either natural and/or synthetic interferents or metabolites found in biofluids can produce both precursor and product ions with *m/z* values exactly the same as those of the substances being analyzed.[26] For example, cotinine, a metabolite of nicotine which is often detected in urine, has an *m/z* of 176, the same *m/z* ratio as BZP. Although they have the same *m/z* ratio, cotinine does not have an equivalent product ion at *m/z* 91 which is important in terms of possible mistaken identification of BZP use.[5]

Limit of detection, limit of quantitation, matrix effects, and analyte recovery

The LOD and LOQ were estimated from the lowest calibrator (500 ng/ml) as 3 times the noise value and 10 times the noise value, respectively. The calculated LOQ values were below 30 ng/ml for all analytes, with the exception of mCPP where the LOQ was estimated at 96 ng/ml (Table 3).

Matrix effects were evaluated following the method described by Matuszewski *et al*.[12] Samples of the individual analytes were prepared at a concentration of 500 ng/ml in drug-free urine collected from five different sources. The use of more than one source of drug-free matrix is important when investigating the effect of the matrix. Analyzing only one matrix source means that the question of different recoveries in samples from different sources and the potential of matrix effects on analyte quantitation are not dealt with. Analysis of drug-free urine from a number of different sources means that differences are highly likely to appear between samples and an indication of whether sample matrix or differences in sample recovery are likely to affect the basic accuracy and precision of the method.[12]

Unsurprisingly every analyte suffered from ion suppression (results <100%) or enhancement (results >100%), in the five different sample matrices studied. This is to be expected because the non-selectivity of direct injection means that the appearance of interfering substances, for example, salts, fatty acids, organic bases compete for ionization.[27] The differences in responses of all the analytes in the five different matrices studied was found to be within +/− 15% (92.6–108%) of the nominal values with the exception of BZP where it was determined to be 75.4% +/− 1.4% (Table 4). It should be noted that the impact of matrix effects on assay performance were evaluated at 500 ng/ml, 50% below the proposed 1000 ng/ml assay cut-off.

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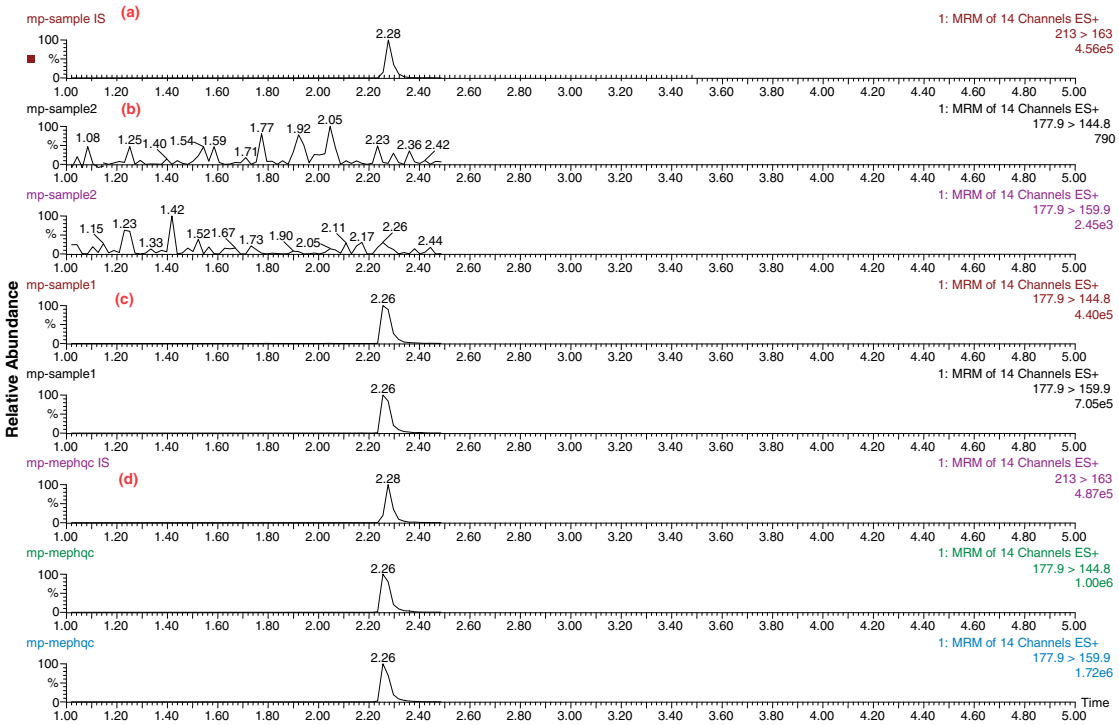

Figure 4. Chromatograms for (A) MDEA-d5 (B) Case sample 2 (C) Case sample 1 (D) QC sample prepared in drug-free urine at a concentration of 1000 ng/ml.

The value of +/− 15% was proposed as an acceptable limit for matrix effects variability as part of the 2007 American Association of Pharmaceutical Scientists (AAPS)/Food and Drug Administration (FDA)conference report: *Quantitative Bioanalytical Methods Validation and implementation: Best Practices for Chromatographic and Ligand Binding Assays*.[28] The use of these criteria would suggest that an awareness and quantification of matrix effects are more important than automatically trying to eliminate them entirely.[29] However, when variability is observed outside of the +/− 15% cut-off value, adjustments should be made to the method where possible in order to reduce the matrix's influence.[29]

McCauley-Myers *et al*.[22] state that the success of direct injection is dependent on the combination of analyte, dilution factor, diluents, organic content of the mobile phase, and injection volume. It would also appear that a balance must be found between the dilution factor and the ion suppression or enhancement likely to be encountered. One option which could be investigated to minimize matrix effects is the use of alternative dilution factors. In theory, the more dilute the matrix, the less interferents and impact on the column and/or source. However, it is possible over-dilution may reduce the detection of some analytes due to the LOD of the assay.[27] The results of this study (Table 3) show that using a 1: 4 (*v*/*v*) dilution in conjunction with the UPLC-MS/MS parameters described, all compounds could be detected at 500 ng/ml, half that of the chosen assay cut-off, providing a reassuring degree of latitude if instrumental sensitivity decreases prior to routine maintenance.

Analyte recovery appears to be consistently better for the cathinone derivatives (~93.0% – ~105.3%) compared to the piperazine derivatives (~77.2% – ~88.2%), which might suggest they are less stable in the matrix. The fact that BZP, as the earliest eluting substance, has the greatest amount of ion suppression (75.4% +/− 1.4%) is not surprising. Ion suppression particularly affects early eluting analytes because when using reverse phase chromatography the most polar analytes elute most quickly.[30,31] There is little distinction between the unwanted polar interferents and the polar analyte of interest.

Research shows that using alternative LC-MS methods to those used in this study, BZP elutes significantly earlier than other piperazine derivatives and consequently undergoes the most ion suppression.[1]

Case samples analysis

During the course of this study, two samples were received by the laboratory for mephedrone analysis. Sample one was collected from a known mephedrone user.

The second sample was collected from an individual who was a known associate of the donor of the first sample. Both these samples were analyzed using the method described.

The calibration standards (500 ng/ml, 1000 ng/ml, and 2000 ng/ml) showed good linearity for mephedrone ($r^2 = 0.999$).

Analysis of sample 1 identified the presence of mephedrone. However, this result was reported as negative as the signal intensities of both the target transition (m/z 177.9 > 144.8) and the qualifier transition (m/z 177.9 > 159.9) were below that of the 1000 ng/ml cut-off (Figure 4). None of the target analytes were identified in sample 2.

Conclusions and future direction

The results of this study support previously published research which suggests that drug-testing laboratories will not detect the presence of the emerging designer drugs, for example, β-keto amphetamines and piperazine derivatives using routine

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immunochemical screening techniques.[2,10,14,18,19] Screening by hyphenated mass spectrometry is an obvious way forward with the rapid increase in availability of new psychoactive substances, as the retention time and the *m/z* of ions for each new drug can be quickly incorporated into existing runs. The incorporation of UPLC resulted in an overall analysis time after sample dilution within 5 min/sample. This short analytical run-time, combined with a simple sample preparation procedure allows the method to be adapted for routine workplace drug testing within high sample throughput laboratories.

The primary focus for a workplace drug testing programme, where the main aim is to deter drug misuse amongst the workforce, is the detection of recent drug administration which can be achieved by the detection of parent compounds in urine. For this reason, a relatively high reporting threshold of 1000 ng/ml was chosen. Even though this threshold is considerably higher than the LOD for drugs targeted, it correlates to the amphetamine immunoassay screening threshold currently employed. This threshold contrasts with that of forensic toxicology, where emphasis is placed on much lower reporting thresholds, including the analysis for late eliminating metabolites, often necessitating preliminary sample extraction. The metabolism of designer drugs has been reviewed[34,35] and, moreover, recent data has been published regarding the metabolism of mephedrone, butylone, methylone, and MDPV.[36,37] Whether any designer drugs are excreted to such a small extent that they are unlikely to exceed the reporting threshold chosen for workplace testing remains to be evaluated, but the targeting of the major metabolites will then be a logical alternative. In the interim, it seems sensible to add such major metabolites to drug screens to aid such comparison, as and when reference standards for metabolites become available.

It may be possible to develop this method further to achieve greater analytical sensitivity and therefore support its application to selected areas of clinical and forensic investigation. Of particular interest would be to investigate the combination of 'dilute and shoot' in conjunction with high resolution mass spectrometry to aid discrimination of drugs from matrix interferents. Moreover, full-scan accurate MS can give broad coverage of known compounds and the ability to rapidly locate new compounds, as exemplified recently regarding the analysis of cannabinomimetics.[38]

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