Journal of Analytical Toxicology 2012;36:381–389
doi:10.1093/jat/bks049 Advance Access publication May 16, 2012

Article

Detection and Quantification of New Designer Drugs in Human Blood: Part 2 – Designer Cathinones

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In recent years, derivatives of cathinone, a naturally occurring beta-keto phenylethylamine, have entered the illicit drug market. These compounds have been marketed over the internet or in so-called head shops as "legal highs" and have gained popularity among drug users. Numerous fatalities due to the abuse of these drugs in recent years have increased the need for their detection in human blood samples.

For detection and determination of 25 designer cathinones and their related ephedrines in blood samples, a liquid chromatography–tandem mass spectrometry (LC–MS-MS) method was developed using only 100 μL of blood. The blood was extracted using liquid–liquid extraction with 1 mL of 1-chlorobutane containing 10% of isopropanol. The final extract was analyzed using a Shimadzu 8030 LC–MS-MS system operated in electrospray positive ionization multiple reaction monitoring mode.

The method has been validated according to international guidelines and was found to be selective for all tested compounds. Calibration for all 25 studied analytes was satisfactory from 10–1,000 ng/mL. Accuracy data were within the acceptance interval of ±15% [±20% at the lower limit of quantification (LLOQ)] of the nominal values for all drugs. Within-day (repeatability) and intermediate precision data were within the required limits of 15% relative standard deviation (RSD) (20% RSD at LLOQ).

Introduction

Cathinone, a beta-keto phenylethylamine, is one of the active constituents of the khat plant and was first identified in the plant in the 1970s (1). The pharmacological effects of cathinone are considered to be the same as amphetamine; however, the potency of cathinone is about one half of that of amphetamine (1–5). Khat users reported the associated effects to be increased energy, confidence, alertness, concentration, friendliness and flow of ideas, as well as decreased hunger and fatigue (2, 3, 5). Methcathinone was the first cathinone derivative, which was synthesised in 1928 as an intermediate of the synthesis of d,l-ephedrine (6). Shortly after, mephedrone (4-methylmethcathinone, 4-MMC), was synthesized by a French chemist named Saem de Burnaga Sanchez in 1929 (7); the compound was later considered to be an obscure chemical product.

Before the widespread abuse of cathinone derivatives began, some substances, namely bupropion, amfepramone (diethylcathinone) and metamfepramone, were marketed as appetite suppressants, and in case of bupropion, as a drug for smoking cessation (8, 9). To date, these substances are still prescribed and also abused as anorectic drugs (10).

The class of pyrrolidinopropiophenones consist of a pyrrolidino ring system as the amine function of the beta-keto phenylethylamine, and the group can therefore also be classified as cathinones. Amongst the pyrrolidinopropiophenones, pyrovalerone was first synthesized in 1964 and marketed in the treatment of chronic fatigue and as an appetite suppressor (11).

In recent years, the so-called designer cathinones have entered the illicit drug market (6, 12–16). Although most cathinones are now banned in numerous countries, many compounds are still available over the internet or in so-called head shops as "legal highs" in research chemicals, bath salts, herbal bath salts or plant food, and are occasionally marked with "not for human consumption" (17, 18).

The abuse of cathinone-derived designer drugs has been associated with acute toxicity and numerous fatalities have been reported (19–21). Commonly used laboratory and roadside drug tests are not able to detect the wide range of designer cathinones; therefore, there is a need for multi-analyte procedures for the detection of a wide range of designer cathinones. To date, only limited studies have been published for the detection of some designer cathinones in plasma or blood (20–27). Therefore, the aim of this study was the detection and quantification of 25 commonly reported designer cathinones in Australia in human whole blood using liquid chromatography–tandem mass spectrometry (LC–MS-MS).

Materials and Methods

Chemicals and reagents

Standards of ethcathinone (ethylpropion), *N*, *N*-dimethylcathinone (*N*, *N*-DMMC), 3,4-dimethylmethcathinone (dimephedrone), methylenedioxyethcathinone (ethylone), methylene dioxy-α-pyrrolidionopropiophenone (MDPPP) and α-pyrrolidionopropiophenone (PPP) were purchased from the National Measurement Institute, Australian Government (Pymble, Australia); 4-methylethcathinone, butylone (bk-MBDB), 4-fluoromethcathinone (flephedrone), 4-methylmethcathinone (mephe drone), 4-methylmethcathinone-d3 (4-MMC-d3), pyrovalerone, norpseudoephedrine, pseudoephedrine, diethylcathinone-d10, para-methoxymethcathinone (methedrone) and methcathinone (ephedrone) were obtained from PM Separations (Capalaba, Australia); butylone-d3 (bk-MBDB-d3), diethylcathinone (amfepramone), methylenedioxymethcathinone (methylone), methyl enedioxymethcathinone-d3, methylenedioxypyrovalerone (MDPV), naphtylpyrovalerone (naphyrone), phenylephrine, 3-fluormethcathinone, cathinone, norephedrine-d3, norephedrine, pseudoephedrine-d3 and methylephedrine were obtained from Kinesis (Redland Bay, Australia); and pentylone (bk-MBDP) was obtained from Sapphire Bioscience (Waterloo, Australia).

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Methanol, isopropanol, 1-chlorobutane and trizma base were obtained from Merck (Darmstadt, Germany). Ammonium formate, acetonitrile and hydrochloric acid were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

Trizma buffer was prepared by dissolving 242 g trizma base in 1 L deionized water and pH adjusted to 9.2 with hydrochloric acid.

Specimens

Preserved blank blood samples (containing 1% sodium fluoride–potassium oxalate) for calibration purposes and validation experiments were obtained from a local blood bank. All blood samples were stored at −20°C before analysis.

Apparatus

The LC–MS-MS system consisted of a Shimadzu LCMS-8030 quadrupole mass spectrometer (Melbourne, VIC, Australia) operated in the electrospray ionization (ESI) in positive mode, and a Shimadzu Nexera high-performance liquid chromatography (HPLC) system (Melbourne, VIC, Australia) that consisted of a degasser, two eluent pumps, a column oven and an autosampler.

HPLC conditions

Gradient elution was performed on an Agilent Eclipse XDB C-18 (4.6 × 150 mm, 5 µm) column coupled with an Eclipse XDB C-18 (4.6 × 12.5 mm, 5 µm) guard column. The mobile phase consisted of 50 mmol/L aqueous ammonium formate pH 3.5 (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). During use, the mobile phase was degassed by the integrated Shimadzu Nexera degasser. Before starting the batch analysis, the HPLC system was equilibrated for 10 min with a mixture of 90% eluent A and 10% eluent B before the injection of the first sample in the batch. The gradient and flow rate were programmed as follows: 0.01–2.00 min, 10% eluent B, flow rate 0.6 mL/min; 2.01–17.00 min, eluent B increasing to 100%, flow rate increasing to 0.8 mL/min; 17.01–20.00 min, 100% eluent B, flow rate 0.8 mL/min; 20.01–24.00 min, starting conditions (10% eluent B, flow rate 0.6 mL/min) to re-equilibrate the column.

The column oven was set at 60°C. The autosampler was operated at 4°C and the autosampler needle was rinsed before and after aspiration of the sample using methanol.

MS-MS conditions

The mass spectral data were acquired with the following ESI inlet conditions: nebulizing gas and drying gas were nitrogen at a flow rate of 3.0 and 15.0 L/min, respectively; the interface voltage was set to 4.5 kV; desolvation line (DL) temperature was 280°C and the heat block temperature was 450°C. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) with argon as the collision induced dissociation gas (CID) at a pressure of 230 kPa; the detector voltage was set to 1.72 kV. All other settings were analyte-specific and were auto-optimized by flow injection of 1 µL of a 1 mg/mL solution in methanol containing one analyte. The results of the auto-optimizations are summarized in Table I. The chemical structures of all included analytes are summarized in Figure 1.

Preparation of stock solutions, calibration standards and control samples

Stock solutions of each analyte were additionally purchased as commercially available calibrated liquid reference standards at a concentration of 1 or 0.1 mg/mL using methanol or acetonitrile as solvent. Working solutions of each analyte were prepared using methanol by independent dilution of each stock solution at the following concentrations: 0.1, 0.01 and 0.001 mg/mL. All solutions were stored at −20°C for a maximum time frame of three months.

The calibration standards were prepared using pooled blank blood and spiking solutions were prepared from the working solutions as mixtures of the 25 designer cathinones and related ephedrines at concentrations 10 times higher than the corresponding calibration standards. The quality control samples were prepared using pooled blank blood and independently prepared mixtures of the 25 designer cathinones and related ephedrines at concentrations 100 times higher than the concentrations of the corresponding quality control samples.

The final blood concentrations of calibration standards and quality control samples were as follows:

Calibration standards were 10, 20, 200, 400, 600, 800 and 1,000 ng/mL; respective quality control concentrations were 30 ng/mL (low), 500 ng/mL (med) and 900 ng/mL (high). All samples were stored at −20°C before analysis.

Extraction procedure

In a 2-mL Eppendorf tube (Eppendorf Australia, North Ryde, NSW), 0.1 mL blood was mixed with 0.01 mL of the internal standard (IS; 1 µg/mL of 4-MMC-d3, bk-MBDB-d3, diethylcathinone-d10, pseudoephedrine-d3 and norephedrine-d3). To the blood, 0.2 mL of trizma buffer and 1 mL of 1-chlorobutane containing 10% of isopropanol were added and mixed thoroughly. The sample was extracted for 5 min on a VXR basic IKA Vibrax shaker at 1,500 rpm. After a brief centrifugation to separate layers, the solvent layer was transferred to an autosampler vial and evaporated to dryness using a Ratek dry block heater DBH10 operated at room temperature.

The residue was reconstituted in 0.05 mL of eluent B and diluted with 0.45 mL of eluent A. The final extract (0.04 mL) was injected into the LC–MS-MS system.

Validation Experiments

Selectivity

Selectivity experiments were carried using postmortem and antemortem blood samples sent to the authors' laboratory for toxicological analysis. Ten postmortem and 10 antemortem samples were extracted as described previously without the

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Table I

Analyte, retention times (RT) [min], multiple reaction monitoring (MRM) transitions, Dwell times [msec], Q1 Pre-bias [V], collision cell energy (CE)[V], and Q3 Pre-bias [V] used in LC-ESI -MS-MS

Drug	Retention Time [min]	Precursor Ion [m/z]	Product Ion [m/z]	Dwell time [ms]	Q1 Pre-bias [V]	LC-MS-MS	Q3 Pre-bias [V]
Phenylephrine	3.06	168	150.1	50	−8	−16	−50
			109.1		−8	−22	−20
			91.1		−8	−24	−34
Norephedrine	5.26	152	134.1	50	−16	−16	−8
			115.1		−16	−20	−46
			91.1		−16	−36	−34
Norpseudoephedrine	5.43	152	134.1	50	−16	−16	−8
			115.1		−16	−20	−46
			91.1		−16	−36	−34
Norephedrine-*d₃*	5.29	155.1	137.1	50	−6	−16	−14
			119.1		−6	−22	−22
			91.1		−6	−14	−40
Cathinone	5.68	150	132.1	50	−8	−18	−34
			117.2		−4	−22	−10
			105		−16	−24	−10
Pseudoephedrine	6.19	166.1	148.2	15	−12	−16	−6
			115		−14	−28	−40
			91.1		−12	−34	−34
Pseudoephedrine-*d₃*	6.21	169.1	151	15	−12	−16	−46
			115		−8	−28	−44
			91.1		−8	−40	−32
Methcathinone	6.21	164.2	146.1	10	−16	−16	−14
			131		−16	−22	−26
			130.1		−14	−34	−26
Methylenedioxymethcathinone	6.45	208	159.9	10	−8	−18	−16
			190.2		−8	−14	−20
			132.1		−8	−30	−48
Methylenedioxymethcathinone-*d₃*	6.44	211	163	10	−20	−20	−10
			192.8		−20	−14	−20
			135		−8	−30	−44
N,N-dimethylcathinone	6.48	178.1	105.1	10	−8	−22	−10
			77.1		−8	−44	−28
			72.1		−8	−26	−12
Methylephedrine	6.53	180.1	162	10	−8	−18	−10
			117		−8	−22	−40
			91.1		−8	−34	−32
3-fluoromethcathinone	6.71	182.2	164	10	−16	−16	−16
			149		−16	−24	−48
			148		−18	−32	−50
4-fluoromethcathinone	6.78	182.2	164	10	−16	−16	−16
			149		−16	−24	−48
			148		−18	−32	−50
Ethcathinone	6.80	178.1	160	10	−8	−16	−10
			132.1		−8	−20	−28
			130		−8	−34	−24
Methylenedioxyethcathinone	6.99	222	174	10	−10	−20	−18
			204.3		−10	−14	−22
			146.1		−10	−30	−48
para-Methoxymethcathinone	7.03	194.1	175.9	10	−8	−14	−18
			160.9		−8	−22	−16
			146		−8	−32	−48
α-pyrrolidionopropiophenone	7.17	204.1	105.1	10	−6	−26	−10
			132.9		−6	−20	−26
			98.2		−6	−26	−36
Methylenedioxy-α-pyrrolidionopropiophenone	7.33	248.1	98.1	10	−12	−26	−18
			147		−6	−26	−48
			91		−12	−46	−30
Butylone	7.34	222	174	10	−16	−18	−18
			204.3		−10	−14	−14
			146		−6	−28	−30
Butylone-*d₃*	7.33	225.1	177.1	10	−10	−18	−18
			207.1		−10	−14	−22
			134.1		−10	−40	−48
Amfepramone	7.53	206.1	105	10	−6	−24	−10
			100.1		−10	−24	−18
			77.1		−10	−50	−30
Amfepramone-*d₁₀*	7.49	216.1	105.1	10	−10	−24	−10
			110.1		−10	−26	−40
			77		−22	−52	−26
4-methylmethcathinone	7.60	178.2	160.2	10	−8	−12	−10
			145		−8	−22	−50
			143.9		−16	−36	−48
4-methylmethcathinone-*d₃*	7.60	181.05	163.1	10	−32	−16	−10
			147.9		−12	−24	−30
			147		−12	−34	−48

(continued)

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Table I Continued

Drug	Retention Time [min]	Precursor Ion [m/z]	Product Ion [m/z]	Dwell time [ms]	Q1 Pre-bias [V]	LC-MS-MS	Q3 Pre-bias [V]
4-Methylethcathinone	8.03	192.1	174.3	15	−8	−16	−18
			144.1		−18	−32	−48
			91.1		−8	−34	−32
Pentylone	8.22	236.1	188	15	−6	−18	−12
			218		−6	−14	−14
			175		−10	−22	−32
3,4-dimethylmethcathinone	8.48	192.05	174	15	−36	−16	−18
			159		−14	−24	−50
			158		−14	−34	−50
Methylenedioxypyrovalerone	8.83	276.1	126.1	15	−8	−32	−44
			175		−8	−24	−18
			135		−8	−30	−46
Pyrovalerone	9.68	246.1	105.1	15	−26	−24	−10
			174.9		−6	−18	−18
			91.1		−12	−48	−32
Naphtylpyrovalerone	10.54	282.1	141.1	15	−14	−26	−14
			211		−8	−20	−22
			127.1		−8	−56	−46

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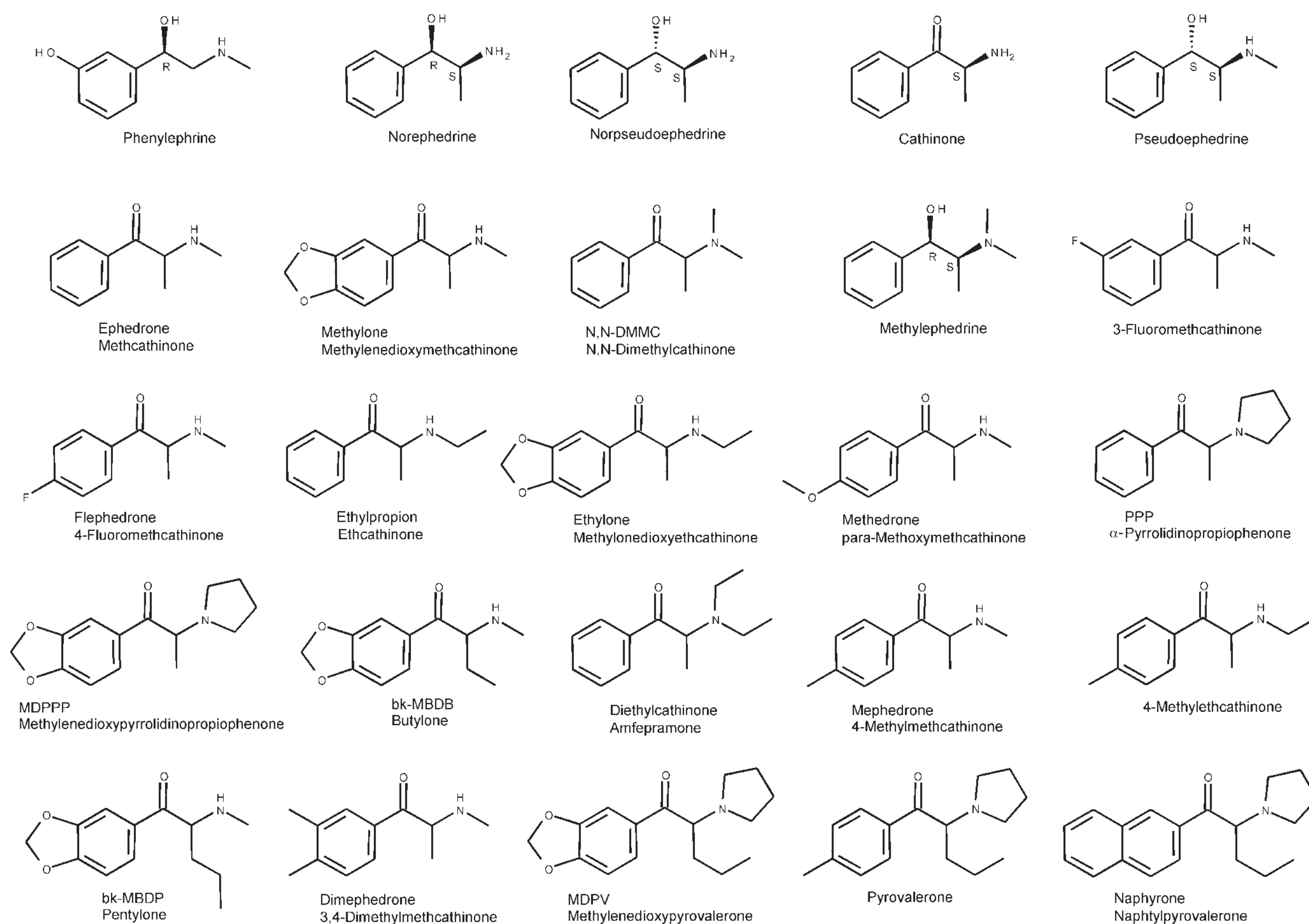

Figure 1. Chemical structures of the designer cathines and related ephedrines included in the method.

addition of IS. The samples were analyzed to exclude any interference with endogenous peaks. Additionally, two zero samples (blank sample + IS) were analyzed to check for the absence of analyte ions in the respective peaks of the IS.

Linearity

Aliquots of blank blood samples were spiked and extracted at concentrations described previously to obtain calibration standards.

Replicates ($n = 6$) at each of the seven concentration levels were analyzed. Daily calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

Accuracy and precision

Quality control (QC) samples, QC low, QC med and QC high, were prepared at the previously described concentrations. Two samples of each QC concentration were measured over a period of eight consecutive days. Daily calibration curves were used to calculate the concentration of the QCs. Accuracy was calculated for each analyte as bias determined by calculating the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Precision data (given as relative standard deviations; RSD) for within-day (repeatability), and time-different intermediate precision (combination of within-day and between-day effects) of the method were calculated according to Beyer *et al.* (22, 28) using one-way analysis of variance (ANOVA) with the grouping-variable "day." The acceptance intervals of within-day (repeatability) and intermediate precision were $\leq$15% RSD at QC med and QC high ($\leq$20% RSD at QC low) and $\pm$15% for bias QC med and QC high ($\pm$20% at QC low) of the nominal values (29).

Processed sample stability

For estimation of stability of the processed samples under the conditions of LC–MS-MS analysis, QC low and QC high samples ($n = 8$ each) were extracted as described previously. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected into the LC–MS-MS system and analyzed under conditions given previously. The time intervals between the analyses of the QC samples were extended to two hours by the injection of five blank samples. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. The instability of the processed samples was indicated by a negative slope, significantly different from zero ($P \leq 0.05$) (30).

Freeze/thaw stability and bench-top stability

Combined freeze/thaw and bench-top stability were evaluated by analysis of QC samples (six replicates at each concentration) before (control samples) and after eight freeze/thaw cycles (stability samples). For each cycle, the samples were kept at 20°C for 21 hours. The thawed samples were kept at room temperature for three hours before the next freeze cycle to incorporate bench-top stability. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples versus control samples) and an acceptance interval of 80–120% from the control samples' mean for the 90% confidence interval (CI) of stability samples (30).

Long-term stability

Experimental design for the study of long-term stability was similar to the freeze/thaw stability. Analyte stability for long-term storage was evaluated by analysis of QC samples ($n = 6$ at each concentration) before (control samples) and after storage for six weeks at −20°C (stability samples). Stability was measured against an acceptance interval of 90–110% for the ratio of the means (stability samples versus control samples) and an acceptance interval of 80–120% from the control samples' mean for the 90% CI of stability samples (30).

Lower limits of quantification and detection

The lower limits of quantification (LLOQ) were defined as the lowest point of the calibration curve, as mentioned previously, and fulfilled the requirement of LLOQ signal-to-noise ratio of 10:1 (30, 31). The limit of detection (LOD) was not systematically evaluated.

Extraction efficiencies, matrix effects and process efficiencies

According to the approach of Matuszewski *et al.* (32), the extraction efficiencies, matrix effects and process efficiencies were estimated with a set of three different samples at two concentrations. Set A was a batch of neat standards. The neat samples were prepared with 0.01 mL IS, 0.01 mL of the respective spiking solution for the QC low (five samples) and high (five samples), 0.05 mL eluent B and 0.45 mL of eluent A. For the samples of set B, five different blank bloods (0.1 mL) were extracted as described previously and the residue of the

Table II

Matrix effects and recoveries in % [range] of all targets and IS. Datasets with variations (minimum and maximum value in %) greater than 20% difference of the mean value (not acceptable) are marked in bold type

Name	Matrix Effects		Extraction Efficiency	
	30 ng/mL	900 ng/mL	30 ng/mL	900 ng/mL
Phenylephrine	82 [80-84]	94 [91-98]	3 [3-4]	3 [3-3]
Norephedrine	88 [84-92]	102 [98-107]	28 [25-30]	31 [28-34]
Norpseudoephedrine	79 [76-84]	101 [96-108]	35 [29-43]	35 [29-39]
Cathinone	86 [76-93]	100 [95-106]	29 [16-43]	50 [43-60]
Pseudoephedrine	90 [87-93]	100 [97-105]	41 [34-47]	55 [52-59]
Methcathinone	96 [94-98]	101 [98-106]	**18 [6-40]**	53 [41-62]
Methylenedioxymethcathinone	100 [94-103]	101 [96-106]	74 [63-80]	83 [79-87]
N,N-dimethylcathinone	93 [90-96]	100 [96-107]	**22 [10-43]**	63 [52-74]
Methylephedrine	93 [89-96]	98 [95-104]	37 [27-51]	70 [65-77]
3-fluormethcathinone	85 [82-88]	102 [99-109]	**20 [7-44]**	44 [33-51]
4-fluormethcathinone	90 [80-95]	100 [94-105]	18 [10-35]	56 [44-67]
Ethcathinone	96 [92-98]	102 [97-107]	28 [14-47]	65 [57-73]
Methylenedioxyethcathinone	99 [94-103]	101 [97-107]	83 [77-88]	90 [84-96]
para-Methoxymethcathinone	100 [97-104]	102 [97-106]	69 [60-77]	83 [78-88]
α-pyrrolidionopropiophenone	97 [91-100]	100 [98-105]	64 [57-70]	85 [81-89]
Methylenedioxy-α-pyrrolidionopropiophenone	95 [92-97]	101 [97-106]	92 [86-99]	92 [86-95]
Butylone	94 [86-100]	101 [99-105]	84 [77-89]	90 [88-94]
Amfepramone	92 [88-96]	101 [97-108]	44 [35-56]	78 [72-85]
4-methylmethcathinone	100 [98-101]	101 [96-106]	39 [24-56]	75 [71-82]
4-methylethcathinone	100 [99-102]	100 [97-106]	51 [38-62]	81 [76-87]
Pentylone	100 [97-103]	100 [97-104]	88 [82-93]	93 [89-96]
3,4-dimethylmethcathinone	99 [95-103]	101 [96-106]	64 [53-71]	86 [80-90]
Methylenedioxypyrovalerone	95 [93-97]	101 [97-107]	94 [86-99]	92 [85-96]
Pyrovalerone	95 [93-98]	101 [98-107]	90 [84-96]	90 [84-94]
Naphtylpyrovalerone	93 [91-94]	101 [97-105]	87 [77-98]	85 [76-89]

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samples was reconstituted in 0.5 mL of a mixture of eluent B and eluent A (50:450) containing the analytes and IS. For set C, identical blank blood samples to those used for set B were spiked at described QC low and QC high concentrations and extracted as described previously.

Extraction efficiencies were estimated by comparison of the peak area of the samples of set B to those of set C. For the matrix effects, the peak area of the samples of set B was compared to those of set A, and for extraction efficiencies, set C was compared to set A. All values are reported in percentage. Values over 100% for matrix effects indicate ion enhancement, while values below 100% indicate ion suppression.

Results and Discussion

Preliminary experiments showed that a commonly used liquid–liquid extraction method in the author's laboratory could be applied for the detection of these designer cathinones (data not shown). This extraction procedure was also applied for the detection of another novel class of designer drugs, the synthetic cannabinoids. Table II shows mean values of extraction efficiencies and the corresponding variation over five different blood samples. Datasets in which the variation (minimum and maximum values in percentage) is greater than 20% difference of the mean value (not acceptable) are marked in bold type. Overall, the method showed satisfactory extraction efficiencies for most analytes. Despite the addition of 10% of isopropanol to 1-chlorobutane, the extraction efficiency for the hydrophilic cathinone derivatives and related ephedrines was low (some as low as 3–20%), but reproducible. Because the LC–MS-MS methods provided sufficient sensitivity, the low and reproducible extraction efficiency was acceptable. Table II also shows the mean values of matrix effects and the corresponding variation over five different blood samples. As described for the extraction efficiencies, datasets in which the variation (minimum and maximum values in percentage) is greater than 20% difference of the mean value (not acceptable) are marked in bold type. The described extraction procedure showed no significant matrix effects over five different blank blood samples. Matrix effect studies of the deuterated internal standards have been performed and showed similar results to their respective analogues; no significant matrix effects were observed over five different blank blood samples.

Antemortem blood was chosen as the matrix for the calibration standards and matrix effects studies rather than postmortem blood. Excess blank postmortem blood from deceased persons is difficult to obtain ethically for assay calibration purposes and matrix effects studies, whereas antemortem blood is readily available through blood banks.

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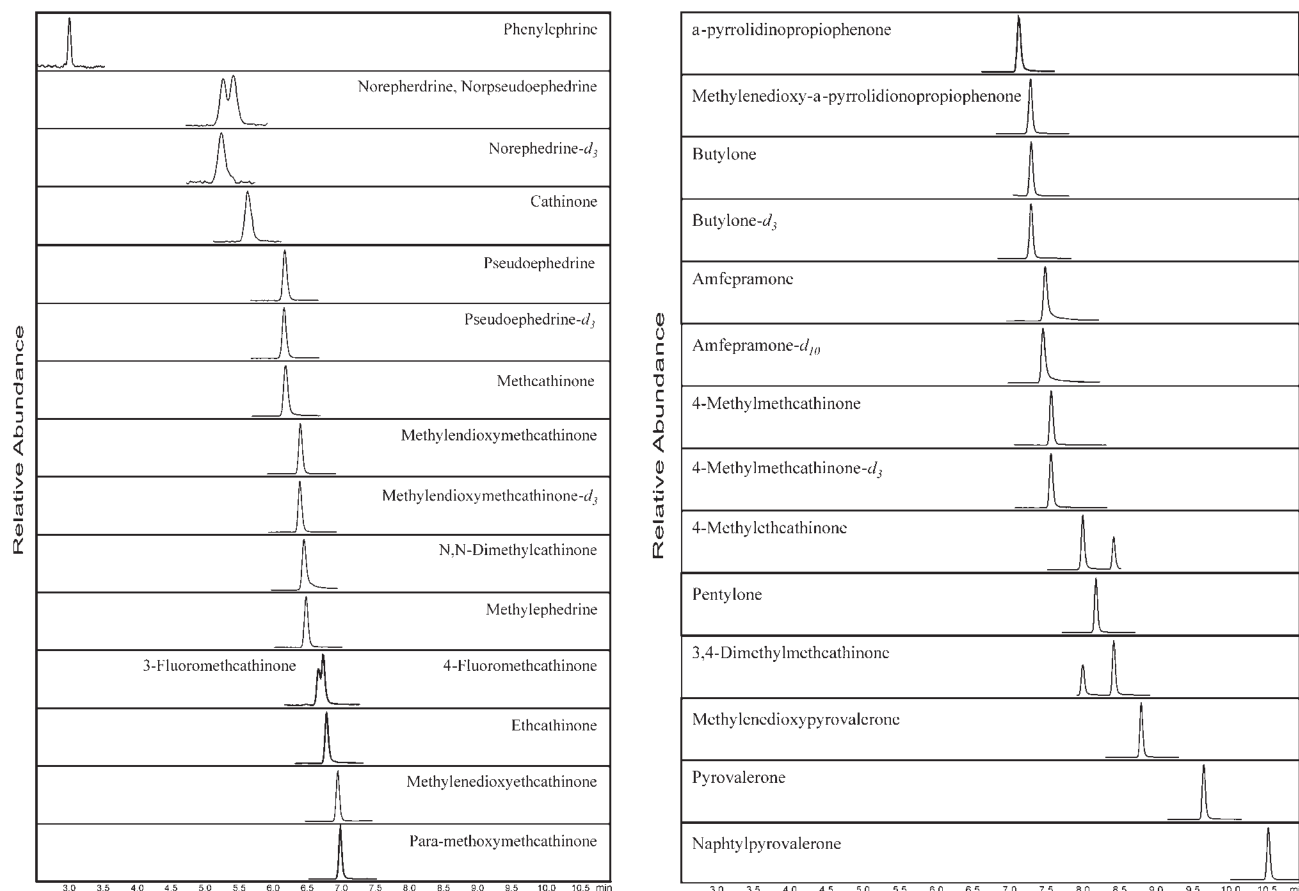

Figure 2. MRM chromatogram of all transitions recorded in positive mode of an extract of a calibrator at a concentration of 10 ng/mL.

In toxicological analysis, a lack of sample volume provided may reduce the possible number of tests able to be conducted. In this study, a small sample volume of 0.1 mL provided the required sensitivity. The LLOQs corresponded to the lowest concentrations used for the calibration curves with a signal-to-noise ratio of at least 10.

Preliminary experiments showed that the reconstitution of samples was critical for the performance of the assay. The composition of the reconstitution solvent usually matches the start conditions of the mobile phase to avoid chromatographic changes for early eluting drugs. A pre-mixed solution of eluent A and eluent B (90:10) did not offer the lipophilic properties to re-dissolve some compounds. Therefore, 0.05 mL of eluent B were used to dissolve the designer cathinones. This solution was diluted using 0.45 mL of eluent A to provide the constitution required for the chromatographic conditions. Although the relatively hydrophilic designer cathinones eluted within a time-frame of 10.5 minutes, a total run time of 20 minutes was required to avoid the build-up of lipohilic compounds found in the extracted blood samples.

After extraction from blood, the drugs were separated using gradient elution on an XBD C18 column. Preliminary experiments showed increased chromatographic robustness using a large size column and considerably high flow rates (data not shown). The increase of flow rate over the run improved the separation and peak shape of lipophilic compounds. For the detection of the designer cathinones and their related ephedrines, three MRM transitions were used for each analyte; their use and their respective peak area ratios enabled unambiguous identification of all drugs included in the assay and showed no interference in 20 drug-free samples studied in the selectivity experiments.

The MRM settings described in Table I were chosen by the Shimadzu LC−MS software and additionally critically reviewed. The review process ensured that all fragment ions were explainable as possible fragments of the respective chemical structure. The dwell times were optimized depending on the signal response of each individual designer cathinone.

In the class of designer cathinones, structural isomers are common. It is therefore not surprising to encounter isobaric compounds with similar fragmentation. To avoid misidentifications, chromatographic separation of these isobaric compounds needed to be achieved. A typical sample chromatogram showing the chromatographic separation of all analytes acquired in positive mode is given in Figure 2. The structural isomers of norephedrine and norspeudoephedrine, as well as 3-fluoromethcathinone and 4-fluoromethcathinone, were not baseline separated; however, sufficient separation could be achieved under the described conditions. A peak splitting integration had to be applied for quantitative analysis. The determination of retention times within a batch of analysis using positive samples containing both isomer pairs is necessary to avoid misidentifications.

Validation experiments

The described procedure was validated according to internationally accepted recommendations (30, 31, 33). The assay was found to be selective for all tested compounds, and no interfering peaks were observed in the extracts of the different postmortem and antemortem blank blood samples. A typical chromatogram of a blank sample is given in Figure 3.

Calibration curves were linear in the range described previously. All analytes were visually checked for a linear fit, a

Table III

Accuracy, precision (time-different intermediate precision) and repeatability (within-day precision) of the LC-MS-MS method for all analytes. IS for quantification is given in parenthesis. Datasets outside required limits are marked in bold type

Name	Accuracy			Precision			Repeatability		
(LoQ for all compounds: 10 ng/mL)	30 ng/mL	500 ng/mL	900 ng/mL	30 ng/mL	500 ng/mL	900 ng/mL	30 ng/mL	500 ng/mL	900 ng/mL
Phenylephrine (Norephedrine-d_3)	-12.5	-7.9	-8.3	14.6	10.7	8.5	14.6	7.1	8.7
Norepherdrine (Norephedrine-d_3)	-2.9	-2.3	3.0	7.4	6.9	6.8	7.4	4.6	6.8
Norpseudoephedrine (Norephedrine-d_3)	-9.4	1.5	4.6	13.4	7.3	8.1	11.9	7.3	7.3
Cathinone (4-methylcathinone-d_3)	-0.7	-2.1	14.2	12.4	10.5	11.0	8.3	8.6	11.0
Pseudoephedrine (Pseudoephedrine-d_3)	-6.5	0.0	1.0	5.1	3.4	5.0	5.0	3.4	5.0
Methcathinone (Diethylcathinone-d_{10})	10.2	10.8	11.6	10.4	7.6	9.0	5.5	5.9	6.0
Methylenedioxymethcathinone (Methylenedioxymethcathinone-d_3)	0.8	0.9	1.9	4.7	4.8	5.0	3.1	3.8	5.0
N,N-dimethylcathinone (Diethylcathinone-d_{10})	7.1	9.7	14.3	10.7	9.5	8.7	7.1	6.1	5.4
Methylephedrine (4-methylmethcathinone-d_3)	-7.3	-1.9	3.7	8.7	5.8	6.7	8.7	3.2	5.8
3-fluoromethcathinone (Diethylcathinone-d_{10})	2.3	-1.0	0.2	9.9	7.3	10.1	8.8	7.3	10.1
4-fluoromethcathinone (Diethylcathinone-d_{10})	-1.7	11.6	12.2	9.4	7.1	5.8	6.1	5.3	5.6
Ethcathinone (Diethylcathinone-d_{10})	1.9	5.4	6.7	6.3	5.5	5.6	6.3	4.1	5.6
Methylenedioxyethcathinone (Butylone-d_3)	-12.3	-2.0	1.1	5.3	4.8	6.5	2.1	2.6	5.5
para-Methoxymethcathinone (Butylone-d_3)	-10.0	-1.3	4.0	3.9	4.7	6.6	3.9	2.7	6.6
α-pyrrolidionopropiophenone (Methylenedioxymethcathinone-d_3)	-5.0	4.7	5.0	9.6	10.1	9.4	6.8	6.6	7.3
Methylenedioxy- α-pyrrolidionopropiophenone (Methylenedioxymethcathinone-d_3)	-4.8	-2.2	3.5	5.9	9.6	8.2	5.0	6.2	4.1
Butylone (Butylone-d_3)	-7.9	1.2	1.6	4.8	3.2	5.1	4.6	2.5	5.1
Diethylcathinone (Diethylcathinone-d_{10})	-0.8	-0.6	-0.9	4.9	3.8	4.9	4.3	2.1	4.9
4-methylmethcathinone (4-methylmethcathinone-d_3)	-12.9	4.8	4.0	9.5	12.2	7.8	5.7	5.4	5.4
4-Methylethcathinone (4-methylmethcathinone-d_3)	-10.4	0.1	-1.7	5.3	4.7	5.5	5.3	4.7	5.2
Pentylone (Butylone-d_3)	1.0	0.0	0.7	4.8	2.6	7.7	4.0	1.9	7.7
3,4-dimethylmethcathinone (Methylenedioxymethcathinone-d_3)	-6.7	-1.7	0.8	5.4	5.4	7.5	4.6	3.7	7.5
Methylenedioxypyrovalerone (Butylone-d_3)	-3.8	-3.6	-1.8	8.9	10.9	9.3	6.2	6.7	7.7
Pyrovalerone(Butylone-d_3)	-12.1	-2.2	-0.7	10.4	7.4	9.2	8.3	4.6	9.2
Naphtylpyrovalerone (Butylone-d_3)	-5.8	1.8	-0.4	8.5	10.8	13.3	8.5	6.6	13.3

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weighted second order model fit and a quadratic fit. Linear regression was applied to all studied analytes. The calibration fit showed a coefficient of determination of $r^2 > 0.99$ for all drugs.

In extracts, the analytes were stable at low and high concentrations for a period of more than 24 h at 4°C. In the freeze/thaw and long-term stability experiments, the ratio of means (stability versus control samples) was within 90–110%, whereas the 90% CIs for stability samples were within 80–120% of the respective control means, therefore fulfilling the acceptance criteria for all analytes at both concentrations.

Accuracy data were for all analytes within the acceptance interval of ±15% (±20% at the LLOQ) of the nominal values for all drugs. Within-day (repeatability) and intermediate precision data were within the required limits of 15% RSD (20% RSD at LLOQ). The results are summarized in Table III, including the IS for each drug used for quantification purposes. The internal standards for analytes other than the non-labeled equivalent were chosen based on retention time.

Conclusion

The LC–MS-MS assay presented is a suitable procedure for separation, detection and quantification of designer cathinones and related ephedrines in blood samples. It has proven to be selective, linear, accurate and precise for all studied drugs. The method will be applied to authentic antemortem and postmortem samples to evaluate pharmacodynamic and pharmacokinetic data that will help to gain knowledge about the toxicological significance of the detection of the designer cathinones in blood.

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