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ORIGINAL ARTICLE

Quantification of methylone and metabolites in rat and human plasma by liquid chromatography-tandem mass spectrometry

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Abstract Methylone is a commonly abused synthetic cathinone derivative marketed as a "legal" alternative to "ecstasy" or cocaine. Previous studies examined the metabolism of methylone in vitro and in vivo; 4-hydroxy-3-methoxymethcathinone (HMMC) was identified as the primary metabolite, with other reported minor metabolites, 3,4-methylenedioxycathinone (MDC) and 3,4-dihydroxymethcathinone (HHMC). However, limited information is known about methylone and its metabolites' pharmacokinetics. We developed and fully validated a method for the simultaneous quantification of methylone, HMMC, MDC and HHMC by liquid chromatography-tandem mass spectrometry in 100 µl rat and human plasma. β-Glucuronidase was utilized for plasma hydrolysis, followed by perchloric acid protein precipitation and solid-phase extraction utilizing cation exchange columns. Chromatographic separation was performed with a Synergi Polar column in gradient mode, and analytes were determined by two multiple reaction monitoring (MRM) transitions. Linear ranges of 0.5–1,000 µg/l (methylone, HMMC and MDC) and 10–1,000 µg/l (HHMC) were achieved. Bias and imprecision were generally acceptable, although quantification of HHMC exhibited variability (16.2–37 %). Extraction efficiencies and ion suppression were 89.9–104 % (for HHMC, 15.9–16.2 %) and < 11.4 %, respectively. Methylone and metabolites were stable in plasma for 24 h at room temperature, 72 h at 4 °C, and after three freeze–thaw cycles (except for a 60 % HMMC increase). Human and rat plasma were cross-validated, documenting that rat plasma quality control samples were accurately quantified against a human plasma calibration curve (−23.8 to 12 % bias). As proof of method, rat plasma specimens were analyzed pre-injection and after subcutaneous administration of methylone at 6 mg/kg from 15 to 480 min post-dosing. Methylone, HMMC, MDC and HHMC concentrations ranged from 1.1 to 1,310, 11.2 to 194, 1.9 to 152 and 24.7 to 188 µg/l, respectively.

Keywords Methylone · Synthetic cathinones · Novel designer drugs · Metabolites · 4-Hydroxy-3-methoxymethcathinone · LC–MS/MS

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Introduction

Abuse of novel psychoactive substances is an ongoing problem around the world. Synthetic cathinones appeared in the US market in 2010 as legal alternatives to 3,4-methylenedioxymethamphetamine (MDMA), amphetamine, or cocaine [1]. They are stimulant-like drugs derived from cathinone, the active ingredient of the khat plant *Catha*

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edulis. In 2011, the American Association of Poison Control Centers (AAPC) received 6,138 synthetic cathinone calls [2]. As a result of this increased presence, the synthetic cathinones 3,4-methylenedioxypyrovalerone (MDPV), 4-methylmethcathinone (mephedrone), and 3,4-methylenedioxymethcathinone (methylone) have been regulated as Schedule I controlled substances in the US. Despite scheduling, methylone remains one of the 25 most frequently identified drugs in the US [3].

Reports of synthetic cathinones from state and local forensic laboratories increased from 142 reports in the first half of 2010 to 7,997 reports during the first half of 2013 [1], with methylone detected in 65 % of reports in 2013. The Drug Enforcement Administration (DEA) and Customs and Border Patrol (CBP) also detected methylone in just over half (51 %) of the reported 518 synthetic cathinone cases from January to June 2013 [1]. A recent study administered a self-reported survey to students at a large university in the Southeastern US and found that 1.1 % of the population surveyed ($n = 2{,}394$) used synthetic cathinones at least once in their lifetime [4]. Another study analyzing 34,561 urine samples submitted for designer stimulant testing, between February 2011 and January 2013, reported methylone as the fourth-most detected synthetic cathinone ($n = 186$), behind only α-pyrrolidinovalerophenone (α-PVP), MDPV, and α-methylaminovalerophenone (pentedrone) [5].

From a chemical structure perspective, methylone only differs from MDMA by the presence of a keto moiety on the beta carbon (Fig. 1a). It was initially patented as an anti-depressant and anti-Parkinsonian drug in 1996; however, it was never developed into a pharmaceutical product [6, 7]. Drug users take methylone for its euphoric and psychostimulant properties, but its reported adverse effects include increased body temperatures, agitation, confusion, psychosis, seizures, and tachycardia [8–12]. Additionally, several fatalities have been linked to methylone consumption [8, 13–17].

Limited data are available on methylone's pharmacology. Recent preclinical studies demonstrated that like other stimulants, methylone interacts with the monoamine transporters [18–21]. Baumann et al. [19] reported that methylone acts as a fully efficacious transporter releaser at dopamine, norepinephrine, and serotonin transporters, to produce in vivo neurochemical effects resembling MDMA. The in vitro metabolism of methylone was studied in human liver microsomes [22, 23], with the identification of various metabolites, including dihydromethylone, normethylone or 3,4-methylenedioxycathinone (MDC), *N*-hydroxymethylone, 3-hydroxy-methylenedioxymethcathinone, and most notably 3,4-dihydroxymethcathinone (HHMC). In vivo studies analyzing rat urine after methylone administration, and human urine from acutely intoxicated individuals, revealed 4-hydroxy-3-methoxymethcathinone (HMMC) as the primary metabolite in both species [24, 25]. Other minor metabolites included the *N*-demethylated metabolite (MDC), and 3-hydroxy-4-methoxymethcathinone. These studies also reported that hydroxylated methylone metabolites were primarily conjugated. A recent study investigating oral and intravenous methylone administration in rats identified four metabolites in rat blood: HMMC, 3-hydroxy-4-methoxymethcathinone, MDC, and 3-hydroxy-methylenedioxymethcathinone. The intermediate metabolite HHMC, a precursor of the two hydroxylated metabolites (HMMC and 3-hydroxy-4-methoxymethcathinone), was not detected, potentially due to a relatively short half-life [26].

Currently, there are no published quantitative methods for the analysis of methylone and its main metabolites in plasma. It is critical to develop assays to detect methylone and metabolites to determine their presence and concentrations in forensic and clinical settings. Also, it is important to quantitatively characterize methylone pharmacokinetics and its metabolites in rodent models, since preclinical safety data are needed before controlled administration studies in humans can be conducted. Here, we report the development and full validation of a method for the simultaneous quantification of methylone, HMMC and MDC, and the dihydroxy intermediate HHMC by liquid chromatography-tandem mass spectrometry (LC–MS/MS). This method will be utilized to characterize the pharmacokinetics of methylone and its metabolites after controlled administration in rats.

Materials and methods

Chemicals and materials

Methylone (1 g/l) and methylone-d_3 (100 mg/l) were purchased from Cerilliant (Round Rock, TX, USA). HMMC, MDC and HHMC were synthesized and purified by the Drug Design and Synthesis Section of the National Institute on Drug Abuse (NIDA) Intramural Research Program, Baltimore, MD, USA, in powder form. Solid-phase extraction (SPE) was performed with SOLA SCX 10 mg × 1 ml cartridges (Thermo Scientific, Fremont, CA, USA). Glacial acetic acid, formic acid (FA), hydrochloric acid (HCl) (36.5–38 %), methanol, acetonitrile, water, methylene chloride, ethylenediaminetetraacetic acid (EDTA) and sodium metabisulfite (SMBS) were purchased from Fisher Scientific (Fair Lawn, NJ, USA); isopropranol and 4-methylcatechol from Sigma (Milwaukee, WI, USA) and ammonium hydroxide (28–30 %) and perchloric acid (69–72 %) from JT Baker (Phillipsburg, NJ, USA). All solvents employed in the extraction were of the high-performance liquid chromatography (HPLC) grade and LC–

MS grade in the chromatographic system. β-Glucuronidase from Red Abalone (> 100,000 U/ml) was obtained from Kura Biotec (Inglewood, CA, USA). Water for EDTA, 4-methylcatechol and SMBS solution preparation was purified in-house by an ELGA Purelab Ultra Analytic purifier (Siemens Water Technologies, Lowell, MA, USA).

Chemical synthesis of 3,4-dihydroxymethcathinone, 4-hydroxy-3-methoxymethcathinone, and 3,4-methylenedioxycathinone

The synthetic route to HHMC, HMMC, and MDC is shown in Fig. 1b. These compounds were synthesized according to the procedures of Kamata et al. [24], with modifications. Briefly, commercially available benzaldehyde (**1**) was treated with ethylmagnesium bromide (EtMgBr) to produce the desired alcohol (**2**) in 98 % yield. Oxidation of **2** was easily accomplished with manganase dioxide in refluxing chloroform to create the ketone **3** in 91 % yield. The ketone **3** was brominated to give a crude bromide. The crude bromide was treated with methyl amine to give crude **4**, which was converted to a pure HCl salt (**4**). Hydrogenolysis of the free base of **4** gave the desired HMMC in 63 % yield. HMMC was treated with 48 % HBr aq. to produce the HBr salt (HHMC-HBr) in 38 % yield. Reaction of a commercially available aldehyde (**5**) with EtMgBr created the desired alcohol (**6**) in 97 % yield. Oxidation of **6** was carried out with manganase dioxide in refluxing chloroform to give a ketone (**7**) in 80 % yield. Bromination of **7** provided a crude product that was treated with NaN_3 to give the desired azide derivative. Hydrogenolysis of azide resulted in the crude MDC that was converted to the pure HCl salt (MDC-HCl) in 68 % yield.

Instrumentation

LC–MS/MS was performed on a Shimadzu liquid chromatography (LC) system (Columbia, MD, USA) coupled with an ABSciex 3200 QTrap® mass spectrometer with a TurboIonSpray source (Foster City, CA, USA). The LC system consisted of a DGU-20A3 degasser, LC-20ADXR pumps, and a CTO-10AC column oven. SPE was performed with a negative pressure manifold. Evaporation under nitrogen was completed using a TurboVap LV® evaporator from Zymark (Hopkinton, MA, USA).

Preparation of standard solutions

Stock solutions (1 g/l) of all analytes were prepared in methanol. Calibrator working solutions were prepared in 3 % 250 mM SMBS, 3 % 250 mM EDTA in 0.01 M FA (SMBS + EDTA + FA mixture) at 0.5, 1, 5, 10, 25, 50, 100, 500 and 1000 µg/l. Quality control (QC) working solutions also were prepared in the same manner, on different days, at 1.5 µg/l (low 1), 30 µg/l (low 2), 300 µg/l (medium), and 750 µg/l (high). An internal standard solution (100 µg/l) of methylone-d_3 also was prepared in the SMBS + EDTA + FA mixture.

Animals and surgery

Methylone and its metabolites were quantified in rat plasma specimens obtained after subcutaneous (s.c.) administration of methylone at 6.0 mg/kg. To obtain pooled plasma for hydrolysis optimization, twelve male Sprague-Dawley rats were anesthetized with 60 mg/kg pentobarbital, and indwelling jugular catheters were surgically implanted. One week after surgery, rats received a methylone injection in the home cage, and 4 h later, single blood specimens (1,200 µl) were withdrawn via the catheter and transferred to collection tubes. Blood was centrifuged at 3,000 rpm for 10 min at 4 °C. Plasma from all rats was combined into a single plasma pool and stored at −80 °C until analysis. For the time-course study, six male Sprague-Dawley rats received surgically-implanted jugular catheter under pentobarbital anesthesia as noted above. One week after surgery, rats were brought from the vivarium to the laboratory in their home cages, and polyethylene extension tubes were connected to the catheters. Rats received 0.5 ml intravenous 48 IU/ml heparin saline, and control blood specimens (300 µl) were collected via the catheter. Immediately thereafter, rats received s.c. injections of methylone at 6 mg/kg, with blood specimens collected at 15 min, 30 min, 1, 2, 4, and 8 h post-injection. Plasma was collected and specimens from each individual rat were stored at −80 °C prior to analysis, as described above. Animal use procedures followed the National Institutes of Health Guide for care and use of laboratory animals, and were approved by the National Institute on Drug Abuse Animal Care and Use Committee.

Sample preparation

Sample preparation was modeled after previously described methods [27, 28], with modifications. Twenty microliters of 250 mM SMBS, 10 µl of 250 mM EDTA, 50 µl of internal standard, and 100 µl of either standard or SMBS + EDTA + FA mixture for authentic specimens were added to 100 µl rat plasma in 1.5-ml microcentrifuge tubes and gently vortexed. Cleavage of metabolite conjugates was performed by incubating the specimens with 10 µl β-glucuronidase with the specimens at 50 °C for 60 min. Specimens were then cooled to room temperature before addition of 20 µl 4-methylcatechol. Protein precipitation was performed with 10 µl concentrated perchloric acid, followed by centrifugation (15,000×g, 10 min).

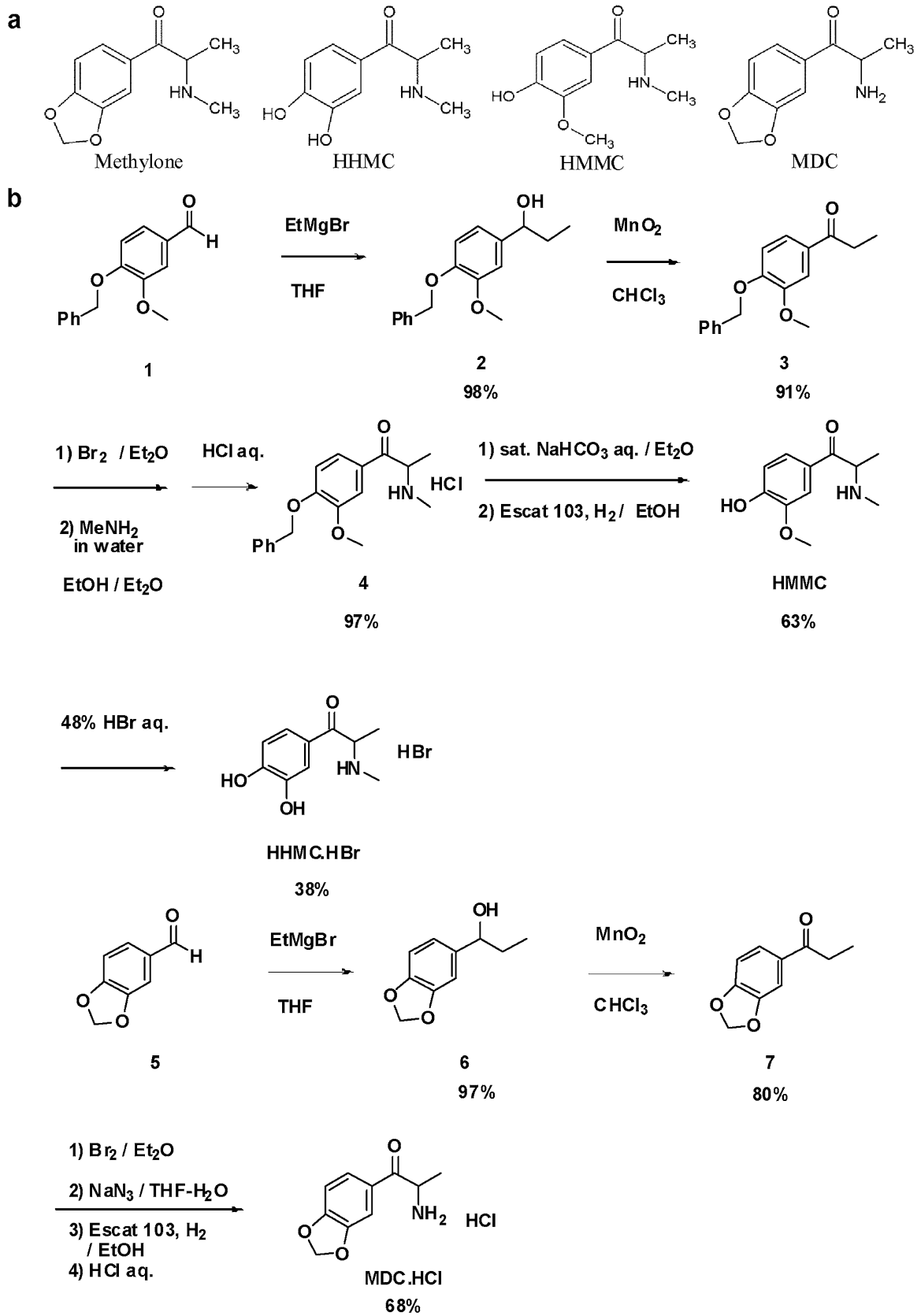

Fig. 1 **a** Structures of methylone, 3,4-dihydroxymethcathinone (HHMC), 4-hydroxy-3-methoxymethcathinone (HMMC), and 3,4-methylenedioxycathinone (MDC). **b** Chemical synthesis of HMMC, HHMC-HBr, and MDC-HCl

To further improve analyte recovery and reduce matrix effects, supernatants were loaded onto SOLA SCX cartridges preconditioned with methanol (500 µl) and water (500 µl), utilizing a negative pressure manifold. Columns were washed with 1 M acetic acid (500 µl) and methanol (500 µl) before drying columns under nitrogen using negative pressure at 10 psi for 5 min. Elution was performed with 5 % ammonium hydroxide in methylene chloride/isopropanol (60:40 v/v). Acidic methanol (1 % HCl) (50 µl) was added to eluates prior to evaporating to dryness under nitrogen for 20 min at 40 °C. Specimens were reconstituted in 200 µl mobile phase A (0.1 % formic acid in water), vortexed briefly, and centrifuged (4,000×*g*, 5 min).

Hydrolysis optimization and performance

Pooled rat plasma specimens were utilized to optimize hydrolysis parameters, as conjugated methylone metabolite reference materials were not commercially available. Temperature (37, 50, and 70 °C), incubation time (60, 90, and 120 min) and amount of enzyme (10, 20, and 40 µl) were optimized to maximize the recoveries of methylone and its metabolites. One-way analysis of variance (ANOVA) followed by Tukeys post hoc test evaluated hydrolysis conditions. Data were considered significant if $P < 0.05$. In addition, after hydrolysis conditions were optimized, authentic specimens were analyzed with and without hydrolysis, and evaluated with a paired *t*-test for significance.

Liquid chromatography-tandem mass spectrometry

Chromatographic separation was achieved at 40 °C with a Phenomenex Synergi Polar-RP column (100 × 2 mm, particle size 2.5 µm) and identically packed defender guard cartridges (10 × 20 mm, particle size 2.5 µm). Gradient elution was performed with 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B) at 0.3 ml/min of flow rate. Initial composition (5 % B) was increased to 50 % over 5 min, from 50 to 95 % over 0.5 min, held at 95 % for 1 min, and returned to initial conditions over 0.5 min. A 2-min equilibration followed, yielding a total run time of 9 min.

MS conditions were: interface, electrospray ionization (ESI) in positive mode; IonSpray voltage, 3.5 kV; capillary temperature, 600 °C; curtain gas, 50; ion source gas 1 and 2, 50 and 30, respectively; nitrogen collision gas, medium for all experiments; dwell time, 50 ms; collision cell exit potential (CXP), 4. Analyst software version 1.5.1 was used for data collection and processing. Multiple reaction monitoring transitions and MS parameters for methylone, methylone-d_3, HMMC, MDC, and HHMC are outlined in Table 1.

The following criteria identified compounds: presence of two characteristic transitions, ion ratio of the quantifying ion/qualifying ion within ±20 % of the average of all calibrators, and retention time (RT) within ±0.2 min.

Method validation

Method validation was performed based on the standard practices for method validation of the Scientific Working Group for Forensic Toxicology (SWGTOX) [29]. Validation parameters included linearity, limits of detection (LODs), limits of quantification (LOQs), bias and imprecision, ionization suppression/enhancement, extraction efficiency, process efficiency, interference studies, carryover, dilution integrity and autosampler and short-term stability studies. Linearity (r^2) was determined by least squares regression with ≥ 6 nonzero calibrators on 5 days. Acceptable linearity was achieved when $r^2 \geq 0.99$ and calibrators quantified within ±15 %. LODs and LOQs were evaluated with decreasing analyte concentrations; LOD was the lowest concentration with acceptable chromatography, signal/noise ratio ≥ 3, with the ratio of ions within ±20 % of the average ratio of the calibrators. LOQs were the lowest concentrations that met LOD criteria, a signal/noise ratio of at least 10, bias and imprecision within

Table 1 Parameters for analysis of methylone, its metabolites and internal standard by liquid chromatography-tandem mass spectrometry

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	DP (V)	EP (V)	CEP (V)	CE (V)	RT (min)
HHMC	196.1	**160.1**, 132.2	31	3.5	12	21, 31	1.93
HMMC	210.1	**160.2**, 132.3	26	5.5	18	25, 39	2.87
MDC	194.1	**146.0**, 118.1	31	4	14	21, 35	3.39
Methylone	208.2	**160.2**, 132.2	36	5	14	23, 35	3.67
Methylone-d_3	211.1	**163.1**, 135.1	26	11	18	25, 35	3.67

Quantification ions are in bold

DP declustering potential, *EP* entrance potential, *CEP* cell exit potential, *CE* collision energy, *HHMC* 3,4-dihydroxymethcathinone, *HMMC* 4-hydroxy-3-methoxymethcathinone, *MDC* 3,4-methylendixoycathinone, *RT* retention time

±20 % of target concentrations. LOD and LOQ were evaluated in triplicate on three different days ($n = 9$).

Assay bias and imprecision were determined at four concentrations [1.5 μg/l (low 1), 30 μg/l (low 2), 300 μg/l (medium) and 750 μg/l (high)] in triplicate over 5 days (overall $n = 15$). Bias was evaluated for each concentration as the percent error. Acceptable bias was ±20 % of target. Imprecision was expressed as percent coefficient of variation (%CV), and was determined by the one-way analysis of variation (ANOVA) approach to calculate combined within-run and between-run imprecision. Acceptable imprecision was ≤ 15 %CV.

Ion suppression/enhancement, extraction efficiency, and process efficiency for each analyte were measured at low (30 μg/l) and high (750 μg/l) QC concentrations, as described by Matuszewski et al. [30]. Ion suppression/enhancement was assessed by comparing analyte peak areas of neat samples ($n = 6$) (Set 1) to peak areas of ten different blank samples fortified with analyte and internal standard after extraction (Set 2). Extraction efficiency was examined by comparing analyte peak areas of five different samples fortified at low and high concentrations with internal standard before extraction (Set 3), to peak areas of Set 2. Process efficiency examined the overall effect of extraction efficiency and ion suppression/enhancement on quantification of analytes, and was calculated by comparing mean peak areas of Set 3 with mean peak areas of Set 1 at low and high concentrations.

Endogenous and exogenous interferences were evaluated. Interferences from endogenous matrix components were investigated by analyzing plasma samples from nine individuals and one rat without the addition of internal standard. Exogenous interferences including an amphetamine mixture [amphetamine, methamphetamine, MDMA, 3,4-methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA), and phentermine], a stimulant mixture (benzoylecgonine, cocaethylene, cocaine, and ecgonine methyl ester), and a benzodiazepine mixture (alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, and temazepam) were analyzed in neat samples with interferences equivalent to 1,000 μg/l. Interferences were considered insignificant if analytes of interest were less than LOD. Lack of carryover was demonstrated by injecting triplicate internal standard-fortified blank samples after a sample fortified with analytes at 2,500 μg/l. Carryover was considered negligible if the measured concentration was less than LOD.

Dilution integrity was evaluated by diluting 2,500 and 1,500 μg/l samples in SMBS + EDTA + FA mixture to achieve tenfold and twofold dilutions, respectively. After internal standard addition, samples were extracted as previously described. Dilution integrity was maintained if samples quantified within ±20 % of target. Autosampler stability was investigated by reinjecting low (30 μg/l) and high (750 μg/l) QC samples stored 48 h at 4 °C on the autosampler ($n = 3$) and calculating results against the original calibration curve. In addition, short-term stability was evaluated with plasma fortified at low and high QC concentrations and stored for 24 h at room temperature ($n = 3$), 72 h at 4 °C ($n = 3$) and −20 °C ($n = 3$), and after three freeze–thaw cycles ($n = 3$). Internal standard was added to each sample just prior to extraction and processed as described. Stability was considered acceptable if QC samples quantified within ±20 % of freshly prepared QC samples ($n = 3$).

Results

Hydrolysis optimization and performance

Hydrolysis conditions, including temperature, time, and enzyme amount, were evaluated for optimal recovery of methylone and metabolites. Overall significant differences between hydrolysis conditions were observed for methylone, HMMC, and MDC, with significant decreases in peak areas when specimens were incubated at 70 °C versus 50 °C ($P < 0.05$). No significant differences were observed between amounts of enzyme utilized and times of incubation. Therefore, to save on cost and time, 10 μl enzyme and 60 min incubation were utilized for hydrolysis. No significant differences were observed between hydrolysis conditions for HHMC. Therefore, optimal recovery of methylone and metabolites was achieved when specimens were incubated at 50 °C for 60 min with 10 μl enzyme.

Methylone and metabolite concentrations in rat plasma, with and without hydrolysis ($n = 3$ each), were examined 4 h after rats were administered 6 mg/kg methylone subcutaneously. Methylone (with hydrolysis 26.5 μg/l; without hydrolysis 26.4 μg/l) and MDC (with hydrolysis 42.7; without hydrolysis 41.6 μg/l) mean concentrations did not change significantly with or without hydrolysis; however, HMMC (with hydrolysis 183.0 μg/l; without hydrolysis 2.8 μg/l) and HHMC (with hydrolysis 38.4 μg/l; without hydrolysis <LOQ) mean concentrations significantly decreased ($P < 0.05$) without the deconjugation step, to concentrations near or below their respective LOQs.

Method validation

Linearity of analyte-to-internal standard peak area ratio versus theoretical concentration was verified in plasma samples from 0.5 (methylone, HMMC and MDC) or 10 (HHMC) to 1000 μg/l with $1/x^2$ weighted linear regression. The $1/x^2$ weighting was selected for this method as correlation coefficients were acceptable and data demonstrated

homoscedasticity, or random distribution of the individual residuals around the zero line, suggesting that this linear model was optimal in comparison to other models. Calibration curves from five separate days yielded determination coefficients (r^2) above 0.99 ± 0.002 with residuals within ± 15 %, except HHMC (0.986 ± 0.014). LODs were 0.25 μg/l for all analytes except HHMC (10 μg/l), and LOQs were 0.5 μg/l for all analytes except HHMC (also 10 μg/l). Figure 2a shows a chromatogram of a human plasma sample with all analytes at LOQs.

Bias and imprecision results using human blank plasma are presented in Table 2. For all analytes, bias was between −16.8 and 9.5 %, and within-run and between-run imprecision was <8.8 %, except for HHMC, which displayed greater imprecision (16.2–37.0 %). Extraction efficiencies and process efficiencies were 89.9–104 % and 83.3–96.9 %, respectively, for methylone, HMMC, and MDC (Table 2). HHMC exhibited extraction efficiencies between 15.9 and 16.2 % and process efficiencies between 15.2 and 15.6 %. Ion suppression was less than 11.4 % for all analytes with %RSD <12.7 (Table 2).

Under the described conditions, no interference from any extractable endogenous plasma compound was observed in nine human plasma samples and one rat plasma sample. The addition of potentially interfering drugs and metabolites at 1,000 μg/l to neat samples did not produce any interfering peaks. For all four analytes, no carryover was observed after a sample fortified at 2,500 μg/l.

Dilution integrity was evaluated for 1:2 and 1:10 dilutions. Diluted samples (750 μg/l targeted) were quantified within −16.9 to 4.1 % of target ($n = 2$) for a 1:2 dilution, and within −7.4 to 0.6 % of target (250 μg/l) ($n = 2$) for a 1:10 dilution for methylone, HMMC, and MDC. Extracted analytes were stable in the autosampler for 48 h with % differences between −11.5 and 3.9 % (Table 3). When stored at room temperature for 24 h, at 4 °C for 72 h, and after three freeze-thaw cycles, methylone, HMMC, and MDC were stable (−15.8 to −0.2 %); however, HHMC was not stable (−14.1 to 60.6 %).

Human-rat plasma cross validation was investigated by assaying rat plasma from drug-naïve rats fortified at all QC concentrations ($n = 5$) against a calibration curve fortified into human plasma. Imprecision (% CV) was < 11.5 and % bias was between −16.9 and 12.1 % for methylone, HMMC, and MDC (Table 4). For HHMC, bias ranged from −23.8 to −17.1 %, with % CV < 25.7 %. Due to the poor validation performance of HHMC, concentrations were considered semi-quantitative.

Authentic specimens

As proof of method, plasma specimens from individual rats were analyzed for methylone and metabolites following s.c. administration of methylone at 6 mg/kg, from 15 to 480 min post-dosing. In the specimen collected before dosing, all analytes were absent. After s.c. dosing, all specimens were positive for methylone, HMMC, MDC and HHMC, with concentrations ranging from 1.1 to 1,310, 11.2 to 194, 1.9 to 152, and 24.7 to 187 μg/l, respectively.

Discussion

A sensitive and specific LC–MS/MS method for the detection of methylone and its metabolites HMMC, MDC and HHMC in plasma was developed and validated. Currently, there are no published quantitative methods for the analysis of methylone and its main metabolites in plasma. A previous study quantified methylone only in rat plasma specimens obtained via repeated venipuncture under isoflurane anesthesia, with a 10 μg/l LOQ in 100 μl plasma [26]. In the present study, we obtained repeated plasma specimens from unanesthetized, freely behaving rats bearing indwelling jugular catheters. Our LOQs were 0.5 μg/l for methylone, HMMC and MDC, and 10 μg/l for HHMC, in 100 μl plasma. Lower LOQs may be achieved with a larger specimen volume; however, a limitation with pharmacokinetic animal studies is the low amount of specimen available for analyses. Methylone blood concentrations from reported intoxication and fatality cases ranged from 7 to 3,400 μg/l [8, 13–17]; therefore, LOQs in this new method are more than adequate to detect methylone and metabolites in authentic cases, even with low specimen volume.

Sample preparation for this method required three steps: deconjugation of possible phase II metabolites, protein precipitation, and solid-phase extraction. Deconjugation was performed because prior metabolism studies demonstrated that methylone metabolites are primarily conjugated [24, 25], and commercial conjugated reference standards were unavailable. HHMC and HMMC had significant decreases in concentrations when there was no hydrolysis procedure, similar to MDMA metabolites, 3,4-dihydroxymethamphetamine and 4-hydroxy-3-methoxymethamphetamine [31, 32]. These results indicate that HHMC and HMMC are present in rat plasma primarily as conjugated metabolites, which coincides with previous reports [24, 25, 31, 32]. Protein precipitation was achieved with perchloric acid after the addition of 4-methylcatechol, which was added to reduce possible adsorption of the dihydroxy-metabolite to precipitated proteins. Cold acetonitrile also was evaluated for protein precipitation; however, analyte recoveries were lower and interference/background noise was larger than observed with perchloric acid.

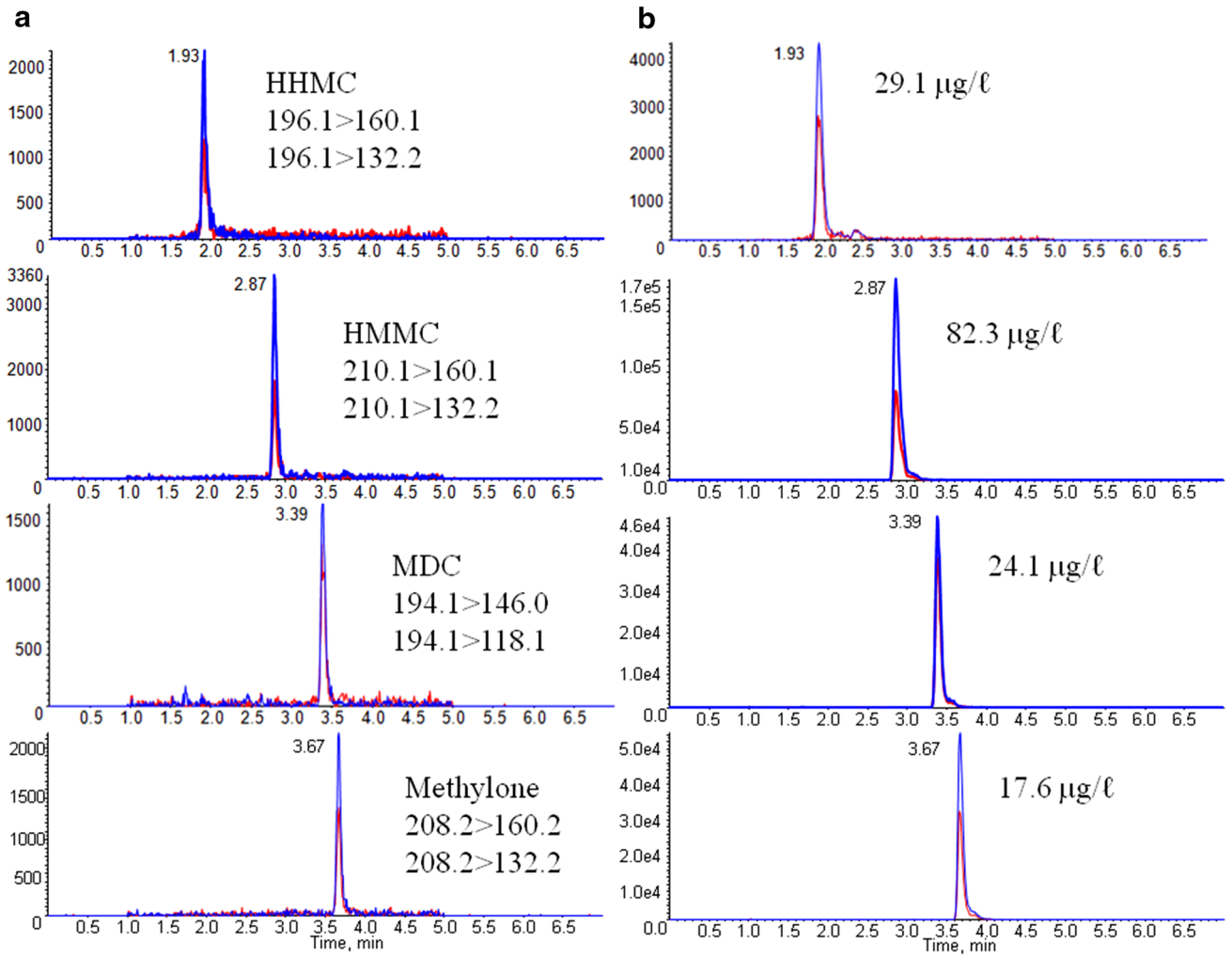

Fig. 2 Multiple reaction monitoring chromatograms for **a** limits of quantification for HHMC (10 µg/l), HMMC, MDC, and methylone (0.5 µg/l) fortified in human plasma; **b** authentic rat plasma specimen for HHMC, HMMC, MDC and methylone after subcutaneous administration of methylone at 6 mg/kg, collected 4 h post-dosing

Despite adequate analyte recoveries with perchloric acid protein precipitation, large matrix effects and endogenous interferences were observed during method development; therefore, SPE was employed to reduce these effects. The SPE method employed in this study minimized ion suppression, as less than 11.4 % suppression was observed for all four analytes; however, recovery of the intermediate dihydroxy metabolite, HHMC, was difficult due to its polarity. Several different elution solvents were evaluated during method development, including 2 % ammonium hydroxide in methylene chloride/isopropanol (80:20 v/v), 5 % ammonium hydroxide in methylene chloride/isopropanol (60:40 v/v), and methanol/ammonium hydroxide (95:5 v/v). Optimal HHMC recovery was accomplished with methanol/ammonium hydroxide (95:5 v/v) (49.2 %), although recovery of the three remaining analytes was poor (64.6–81.7 %). Taking into account that HMMC and MDC are the main metabolites of methylone, 5 % ammonium hydroxide in methylene chloride/isopropanol (60:40 v/v) was utilized to recover all four compounds from the SPE cartridges. It also is important to note that SMBS and EDTA were utilized to prevent analyte oxidation, similar to MDMA and MDPV metabolite quantification in plasma [27, 28].

Validation results of methylone and its main metabolites, HMMC and MDC, were acceptable. Extraction efficiencies and process efficiencies were between 89.9 and 104 %, and 83.3 and 96.9 %, respectively. The performance of the intermediate dihydroxy metabolite (HHMC), however, was not optimal. This is most likely due to its low extraction (15.9–16.2 %) and process efficiencies (15.2–15.6 %), and analyte instability. Percent bias was acceptable for HHMC (−12.8 to 6.7 %), although imprecision was greater than 20 %. Similar

Table 2 Within-run and between-run imprecision and % bias for methylone, HMMC, MDC and HHMC in human plasma at low_1 (1.5 µg/l), low_2 (30 µg/l), medium (Med) (300 µg/l), and high (750 µg/l) concentrations; and analyte extraction efficiency, process efficiency, and ion suppression/enhancement in human plasma at low (30 µg/l) and high (750 µg/l) concentrations

	Methylone				HMMC				MDC				HHMC			
	Low_1	Low_2	Med	High	Low_1	Low_2	Med	High	Low_1	Low_2	Med	High	Low_1	Low_2	Med	High
Within-run imprecision ($n = 15$, %CV)	5.8	4.2	2.7	3.2	5.9	5.8	1.7	2.9	5.3	5.0	3.4	4.6	–	28.5	20.0	26.2
Between-run imprecision ($n = 15$, %CV)	5.4	4.3	3.2	5.7	8.8	5.5	2.4	3.0	5.0	6.5	4.3	5.8	–	37.0	21.2	26.8
% Bias ($n = 15$)	0.2	1.8	−5.2	−4.0	−6.7	9.5	−13	−16.8	−4.1	−3.0	−1.5	4.1	–	−12.8	−5.2	6.7
Extraction efficiency % ($n = 5$)	–	104	–	97.2	–	104	–	89.9	–	102	–	90.7	–	15.9	–	16.2
Process efficiency % ($n = 5$)	–	91.7	–	88.7	–	96.9	–	87.8	–	90.1	–	83.3	–	15.2	–	15.6
Ion suppression/enhancement % (%RSD) $n = 10$	–	−11.4	–	−8.7	–	−6.8	–	−2.4	–	−11.3	–	−8.2	–	−4.6	–	−3.4
		(12.7)		(5.5)		(8.7)		(1.1)		(10.5)		(4.4)		(11.1)		(3.8)

process and extraction efficiencies were recently reported with the dihydroxy MDPV metabolite, 3,4-dihydroxypyrovalerone (39–44.9 and 35.6–45.3 %, respectively) [27]. Authors attributed this loss to partitioning of the polar analyte into the aqueous layer after centrifugation and protein precipitation. Due to poor HHMC performance in the method validation, concentrations are considered semi-quantitative. No previous studies reported HHMC identification, potentially due to the analyte's short half-life in blood [26].

Another important issue to consider with synthetic cathinones when quantifying them in various matrices is their stability. Methylone, HMMC, and MDC were stable under each short-term stability condition tested: 24 h at room temperature, 72 h at 4 °C, and after three freeze–thaw cycles. Other studies examining synthetic cathinone stability in plasma demonstrated varying results. Johnson and Botch-Jones [33] reported MDPV to be stable at room temperature, 4 °C, and −20 °C for up to 14 days, whereas mephedrone exhibited a 30 % loss after 14 days at 4 °C, and was not detected after 7 days at room temperature. Soh and Elliot [34] detected a 54 % loss of 4-methylethcathinone (4-MEC) in plasma after 14 days at room temperature. The stability of methylone, HMMC, and MDC observed in the present study may be attributed to SMBS and EDTA use during sample storage and preparation, as it prevents oxidation, combined with the fact that methylone does not readily reduce to its corresponding alcohol [5, 35]. HHMC demonstrated varying results in terms of stability (−10.6 to 60.6 % difference). It is difficult to determine if this was due to analyte instability, or poor extraction and process efficiencies.

The human-rat plasma cross-validation results demonstrated that it is possible to quantify methylone and metabolites in rat plasma utilizing human plasma for calibration, as volumes of drug-free rat plasma are often limited. The results of this study demonstrate that this assay can be utilized for preclinical, clinical or forensic applications. Proof of concept was demonstrated with plasma from rats dosed with 6 mg/kg methylone subcutaneously. The highest concentrations observed for methylone, HMMC, MDC and HHMC were 1,310 µg/l (15 min post-dosing), 194 µg/l (60 min post-dosing), 152 µg/l (60 min post-dosing) and 185 µg/l (60 min post-dosing), respectively. Plasma concentrations of methylone, HMMC, MDC and HHMC were 1.1, 11.2, 1.9 and 24.7 µg/l at 480 min post-dose, respectively. These preliminary data indicate that methylone plasma C_{max} is higher than metabolites, although metabolites appear to have longer windows of detection. This suggests that, in addition to the parent compound, the metabolites could be good markers to detect methylone abuse; however, further investigation is warranted.

Table 3 Stability data (% difference) for methylone, HMMC, MDC, and HHMC at low (30 μg/l) and high (750 μg/l) concentrations after storage in autosampler at 4 °C for 48 h, at room temperature for 24 h, at 4 °C for 72 h, and after 3 freeze–thaw cycles (over 72 h)

Stability conditions ($n = 3$)	Methylone		HMMC		MDC		HHMC	
	Low	High	Low	High	Low	High	Low	High
48 h at 4 °C in autosampler	0.9	−11.5	3.5	−7.2	3.9	−8.5	3.5	3.2
24 h at room temperature	−6.2	−14.2	−3.1	−11.2	−8.1	−14.0	33.6	−14.1
72 h at 4 °C	−6.5	−15.8	−4.4	−10.9	−5.2	−13.2	−3.1	−10.6
3 freeze–thaw cycles	−1.3	−14.6	−0.2	−10.6	−5.5	−11.3	60.6	37.3

Table 4 Human-rat plasma cross validation data for methylone, HMMC, MDC, and HHMC, including % bias and % CV of fortified rat plasma at low 1 (1.5 μg/l), low 2 (30 μg/l), medium (300 μg/l), and high (750 μg/l) concentrations against a human plasma calibration curve

Analyte	% Bias (% CV) ($n = 5$)			
	Low1	Low2	Med	High
Methylone	12.1 (11.5)	−2.9 (3.7)	−0.3 (4.1)	−5.3 (3.9)
HMMC	−9.7 (10.7)	4.0 (2.3)	−12.5 (4.9)	−16.9 (2.0)
MDC	5.3 (9.0)	−11.8 (5.2)	1.1 (5.3)	−0.2 (1.7)
HHMC	–	−17.1 (15.1)	−23.8 (12.4)	−18.3 (25.7)

Conclusions

A sensitive and specific LC–MS/MS method for simultaneous quantification of methylone and the metabolites HMMC, MDC and HHMC in plasma was developed and validated. Only 100 μl specimen was required for LOQs of 0.5 μg/l for all analytes except HHMC (10 μg/l). Quantification methods are needed to characterize the pharmacokinetics of methylone and its metabolites for preclinical, clinical and forensic studies. Methylone remains one of the most commonly used synthetic cathinones in the United States despite scheduling efforts, and as such, quantification data for this analyte and its metabolites will aid in the interpretation of results in forensic and clinical settings, and may suggest mechanisms of methylone toxicity.

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