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ORIGINAL ARTICLE

Evaluating Of Synthetic Cathinones in Human Urine Samples

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ABSTRACT

Objectives: Cathinone is the principal active constituent of the Khat plant (Catha edulis), and has similar stimulant properties to natural amphetamine. Substituted cathinones are derivatives of cathinone; some of them have medical uses as well, but some are strong psychoactive drugs and commonly sold in "bath salts". Their use may have very serious public health and safety consequences. We aimed to develope an easy and validated method for detecting synthetic cathinones in clinical and forensic toxicology cases.
Materials and methods: We used LC-MS/MS and certified standard solutions to create the method. We studied the linearity, LOD, LOQ, accuracy, imprecision, repeatability, reproducibility, recovery and carry-over as validation parameters and positive electrospray ionization in the MRM mode was used. The samples were obtained from emergency services.
Results and Conclusion: All validation parameters studied were found in acceptable analytical ranges. Alpha-PVP was the only cathinone detected in two samples. We developed an easy method suitable for analyzing synthetic cathinones and detected alpha-PVP in human urine for the first time in our country. The need for sensitivity in clinical and forensic toxicology determinations, LC-MS/MS is preferable for the determination of synthetic cathinones in toxicology cases.

Key words: Synthetic cathinones, urine, LC-MS/MS

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INTRODUCTION

Synthetic cathinones are a speedily growing group of designer drugs which are chemically similar to cathinone, the primary psychoactive component of khat (Catha edulis). They serve their activity by inhibiting the monoamine transporters of serotonin, norepinephrine and dopamine, and produce a complex order of adreno-serotonergic effects [1]. The recreational use (especially in Arabian Peninsula and East Africa) of cathinones are probably based on their psychostimulant effects similar to methamphetamine and cocaine and low cost and easy availability [1]. Beside the effects of increased energy, empathy, openness and libido, they can cause serious cardiac, neurological and psychiactric effects [2]. Generally synthetic cathinone products are made up with a mixture diluted with lidocain, caffeine and some other known adulterants along with the byproducts of their hidden synthesis. They are often noted "legal highs" and marketed as "plant food" or "bath salts" and marked as "not for human consumption" to bypass drug misuse legislations and they can be acquired through "head shops" (tobacco specialized retailers), online suppliers and local drug sellers. In most European countries (Germany, France, the Netherlands), Canada and the US, khat is a forbidden material whereas it is a licit substance in Yemen, Somalia, Ethiopia, Djibout and Israel [3]. Turkey included the khat plant itself and the active ingredients in the list of prohibited drugs and psychotropic substances by publishing in the Official Gazette in 2011 (No. 27845 Date 13.02.2011) and in the following year, synthetic cathinones have been also subjected to the Law on Control of Narcotic Drugs (22.03.2012).

Common administration ways of synthetic cathinones are oral ingestion and nasal insufflation. Ingestion of synthetic cathinones wrapped in paper (bombing) is mostly prefered to insufflation because of its nasal irritation. Cathinone peak concentration is achieved

within 1.5 hours after taking orally [4]. After consumption, the cathinones can be converted to distinct metabolites in the organism [1]. Hydroxylation, N- or O- methylation, demethylation, oxidation and conjugation reactions with sulfates and glucuronides are biotransformation processes of cathinones [5-8].

There are some limitations on the screening analysis by antibody-based immune- methods due to the structural variety of the synthetic cathinones, suggesting that advanced chromatographic screening techniques may be more appropriate. Considering the derivatization of samples and the potential for thermal instability during GC-MS analysis and the requirement for accuracy and sensitivity in forensic toxicology cases, alternative analytical techniques such as LC-MS, LC-MS/MS or LC-Q-TOF should be preferred for the detection of synthetic cathinones in biological samples. On the other hand, there are no synthetic cathinones in the routine drug screening panels performed by clinical laboratories in our country. Therefore, we aimed to develope a validated LC-MS/MS method for determining synthetic cathinones (Methedron, Buphedron, Mephedron, Alpha-PVP, Bupropion, d,l-4-etilmetcathinon) in human urine samples for evaluating clinical and forensic toxicology cases.

MATERIALS and METHODS

Chemicals

We used certified standard materials with an internal standard solution and gradient grade chemicals. Methedron, alpha-PVP, buphedron, bupropion, mephedron (4- metihylmethcatinone (MMC)), d,l-4-ethylmethcatinone (EMC) and the internal standard (IS) mephedron-D3 were purchased from Lipomed (Swiss Health Care Company); acetonitrile and methanol from Merck and ammonium formate from Sigma Aldrich. Ultra-pure water (18.1 MΩ) was produced by a Mes Mp Mınıpure water system (MPMINIPURE, Turkey).

Calibrators

Calibrator samples were prepared daily by adding all standards into blank urine at different concentrations (10, 25, 50, 75, 100, 150, and 200 ng/mL). Each calibrator was spiked with IS to the final concentration of 100 ng/mL.

Samples

Urine samples (n= 16) sent from pediatric emergency service due to suspected abused drug toxicity during the first 6 months in 2016 were investigated in our forensic toxicology laboratory with the approval of clinical ethic committee of the related hospital (Ankara Child Health and Disease Hematology Oncology Training and Research Hospital) upon optimization and validation of the LC-MS/MS cathinone method. Eleven patients were men aged between 10 and 17; five of them were woman aged between 13 and 17.

Sample Preparation

Freshly obtained urine samples were first mixed with acetonitrile (v/v=1/1), then centrifuged for 5 min at 14000rpm. The supernatant (200µL) was transferred to the auto sampler vial and fortified with IS (100ng/mL) before LC/MS-MS injection.

Control Samples

Positive urine quality control (QC) samples (25ng/mL and 150 ng/mL) were prepared daily and freshly by using the stock standard sources, separately from calibrators. Negative urine control was prepared from the mix of clear urine samples (blank urine) obtained from volunteers (n=5) in our department that have not been using any drug or medication. Both positive and negative control samples were spiked with IS and analyzed at each batch with the real samples.

İnstrumentation

The analytical column was Shim-Pack Column FC-ODS 150X2.0 (Shimadzu). The aqueous mobile phase (phase A) consisted of 10 mM ammonium formate in water, while the organic mobile phase (phase B) consisted of methanol. The analytical column was maintained at 40 °C, and the flow rate was 0.4 mL/min with the injection volume of 10µL. The initial composition increased to 95% B over 7 min, held at 95% for 1 min, decreased from 95% to 5% and returned to initial conditions over 4 min, lasting a total run time of 12 min. The MS/MS system used was Shimadzu 8030-Plus, with an ESI (electrospray ionization) source. All analytes were ionized in positive mode. All data were collected and quantitated using Lab Solutions Version 5.80 software. Multiple reaction monitoring (MRM) method parameters were optimized by direct injection of standard solutions. The most abundant MRM transition was selected for quantification and the retention times were determined for schedule time of all substances.

Validation

LOD (limit of detection), LOQ (limit of quantification), linearity, recovery, accuracy (bias), imprecision (RSD),

selectivity and carryover of the assay are studied according to international method validation guidelines in forensic toxicology [9,10]. Coefficient of determination at least 0.99 and calibrator samples within ±15% of target, except LOQ (± 20%), were acceptable for linearity. The blank urine (mix of 5 volunteers) was fortified with standards to determine the LOD and LOQ. LOD was designated as the lowest concentration with suitable chromatography, where the signal-to-noise ratio (S/N) was at least 3. LOQ was the lowest concentration where S/N was at least 10, RSD below 20%, bias below ±15%, and also met the LOD criteria. Three different mix standard concentrations (low, medium and high) with 5 replicates for 5 days, were used to define imprecision and accuracy. Recovery for each analyte was measured at each QC concentration with three replicates. Blank urine samples were fortified with QCs and IS before and after extraction. Percent recovery was calculated from the mean analyte area of samples fortified before extraction divided by mean area of samples fortified after extraction.Interference effect of endogenous matrix components was evaluated by analyzing real urine samples (n=10) comparing to IS added blank urine. Specificity of the method was defined by adding potentially interfering drugs to blank urine samples in high concentrations and analyzed. Evaluated drugs as interfering agents were methamphetamine, amphetamine,3,4-methylenedioxyamphetamine (MDA),3,4-methylenedioxymethylamphetamine (MDMA),3,4-methylenedioxyethylamphetamine (MDEA), and N-methyl-1-(3,4-methylenedioxyphenyl)-2-butylamine (MBDB). Results below LOD was acceptable for appropriate selectivity.Carryover was assessed by measuring IS added blank samples just after the IS and mix standard solution (400 ng/mL) added samples (with 3 replicates). Carryover was considered insignificant, if the calculated results were under the LOD level.

RESULTS

Total ion chromatogram of synthetic cathinones investigated in our study is expressed in Figure 1. MRM conditions are presented in Table 1. Validation results are expressed in Table 2 and 3. We measured the average bias and RSD for repeatability and reproducibility between 0.09 - 4.02 and 1.11 - 3.18 respectively at three different concentrations. Both bias and RSD values were found in acceptable analytical ranges (below ±20% and 20% respectively) whereas the mean bias of low concentration was significantly high (2,34) than those of medium and high concentrations (0,96 and 049 respectively). Depend on this data the calculated F value in ANOVA analysis was greater than the critical one when compared the average bias values between three concentration levels. Mean recovery was in the range of 85.5 - 100.1%, and linearity was around 0.999. LOD and LOQ values were between 0.48 - 0.97 and 1.61 - 5.98 respectively. No interference from urine matrix and other interferable compounds was observed. No carryover was detected after the injection of a sample at the high concentration of 250 ng/mL.

Two samples were positive for synthetic cathinone alpha-PVP (Figure 2), whereas THC, amphetamines and opiates were the primary drugs found in the remaining positive samples. Urine alpha-PVP levels were measured as 56.9 ng/mL (with positive blood result), and 4.49 ng/mL

DISCUSSION

Synthetic cathinones are the second largest group of abused substances monitored by The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). They were first identified in Europe in 2004, and 103 new cathinones has been identified since then and 26 in 2015 (Figure 3). Synthetic cathinones are usually sold as 'legal' replacements for stimulants such as amphetamine, MDMA and cocaine. Case-level seizure data reported to the EU Early Warning System indicate that the new drug market continues to grow steadily. Synthetic cathinones were the second largest group with over 8000 seizures (8.343), weighing over 1 ton and the most seized five cathinones were mephedron (222 kg) and its isomers 3-MMC (388 kg) and 2-MMC (55 kg) along with pentedron (136 kg) and alpha-PVP (135 kg) [11]. Especially, young people are unwittingly being used as subjects for these items where the possible health risks are hardly unknown at all. Alpha-PVP, similar to MDPV (3,4-methylenedioxypyrovalerone), is a strong psychostimulant synthetic cathinone. According to the European Drug Report, in November 2015, a risk assessment for alpha-PVP (alfapyrrolidinopentiophen) has been carried out at the European level. It has been existing in the European Union drug market since at least February 2011 and is available to all 28 Member States. Alfa-PVP has been detected in 191 acute poisoning and 115 deaths. In 20% of deaths, it is either the cause of death or has been reported as a contributory substance to death and in five of the cases, alpha-PVP is the only substance detected [11]. One of the most life-threatening effect of synthetic cathinones is the risk

of acute heart attack [3]. Tachycardia, hyperthermia, hypertension, agitation, sedation, agitation, psychosis, rhabdomyolysis, myocardial infarction and death are severe clinical effects of MDPV expressed in various reports [12, 13]. There are several methods described by authors for detecting synthetic cathinones using distinct sample preparation processes with various chemicals and devices including NMR, DARTMS, GCMS and LC-MS/MS [6, 14-18]. Considering recently published papers, LC-MS/MS is a preferable method for determination of illicit drugs as synthetic cathinones in biological samples. We validated a simple and easy method for cathinone detection in human urine with a total run time of 12 min by using LC-MS/MS and we found two patients positive for the synthetic cathinone alpha-PVP among all samples sent from emergency service due to suspected drug use. Although synthetic cathinones use is a growing aspect overall the world, in Turkey they are not mentioned among frequently used illicit substances in the National Drug Monitoring Center Report 2014 [19]. Based on this knowledge most clinical laboratories do not screen synthetic cathinones routinely among abused drugs. Also, looking at the published papers there are no data about cathinones detected in human materials in Turkey. Only three of 1200 herbal compound samples were found positive for cathinone and cathine analyzed between 2010 and 2012 in the Istanbul Narcotic Department of the Council of Forensic Medicine at the request of the judicial authorities [20]. So, this article represents the first positive synthetic cathinone results detected in human materials from patients admitted to the emergency department. Limitations of our study may be the small size of samples and the small number of analytes. We chose only six synthetic cathinones (methedron, alpha-PVP, buphedron, bupropion, mephedron, d,l-4- EMC) most commonly mentioned in literature because of our inadequate budget for acquiring more standard materials.

As a conclusion, in this study, we wanted to share the initial experiences of our newly founded laboratory on the developing of a method for synthetic cathinones which is not used in routine drug screen analysis in clinical laboratories and to determine the cathinone abuse cases that lead to urgent health problems. Our method validation parameters have been found in acceptable analytical ranges indicating that this method is a suitable and easy method for analyzing designer cathinones in clinical and forensic toxicology cases. Furthermore, this study repots the use of cathinone (alpha-PVP) by using human materials for the first time in our country pointing out that there is a need to add these substances to routine drug screen analysis especially at emergency laboratories. But further investigations with more human samples and drug analytes should be performed to monitor novel drug abuse pattern in Turkey.

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Table 1: MRM conditions of analytes

Analyte	Mode +/-	RT (min)	Q1 (m/z)	Q3 (m/z)	CE (V)
Methedron	+	4.636	194.20	161.20 146.20	-18.0 -28.0
Alpha-PVP	+	6.180	232.30	91.20 77.20	-16.0 -29.0
Buphedron	+	4.943	178.20	91,20 77.20 184.00	-15.0 -30.0 -15.0
Bupropion	+	6.876	240.30	166.10 131.20 204.10	-30.0 -23.0 -18.0
Mephedron	+	5.117	178.20	145.20	-26.0
Mephedron-D3	+	5.084	181.20	148.20 91.20 144.20	-27.0 16.0 -26,0
d,l-4-EMC	+	5.891	192.20	145.20 144.20 136.80	-26.0 -25.0 -15.0

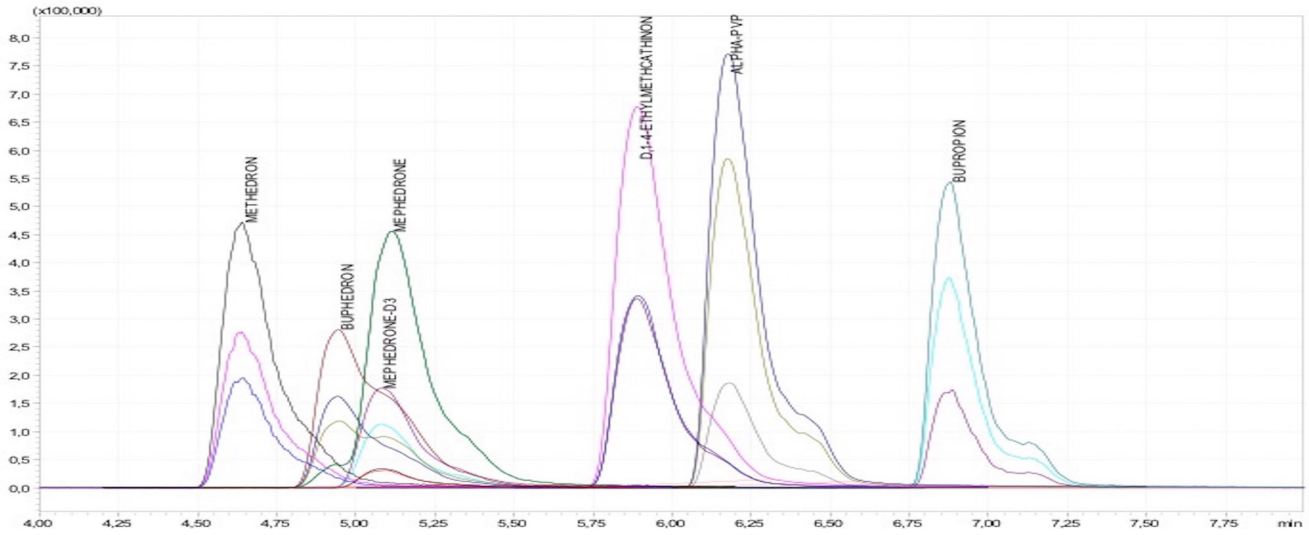

Figure 1. Total ion chromatogram of synthetic cathinones

Table 2. Validation results; recovery, LOD, LOQ, and linearity (r^2)

Analyte	Recovery (%)	LOD (ng/mL)	LOQ (ng/mL)	r^2 (10-200 ng/mL)
Methedron	94.41	0.72	2.41	0.9972
Buphedron	85.50	0.48	1.61	0.9971
Mephedron	100.1	0.80	2.68	0.997
d,l-4-EMC	95.89	0.97	3.24	0.9966
Alpha-PVP	96.51	0.70	2.35	0.9963
Bupropion	96.96	1.80	5.98	0.9962

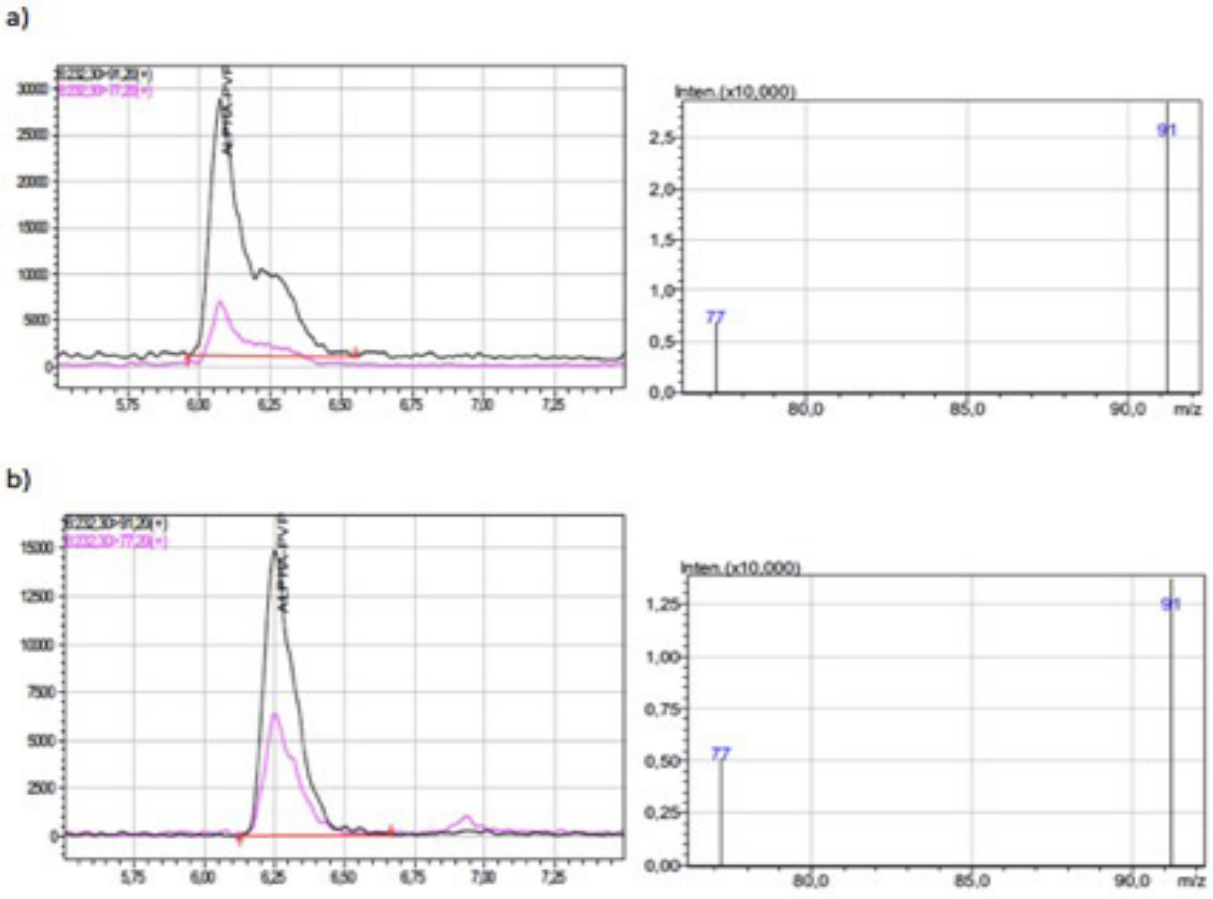

Figure 2. Chromatogram and MS spectrum of alpha-PVP of Sample 1(a) and Sample 2 (b)

Table 3. Validation results; accuracy (bias, %) and imprecision (RSD, %)

Analyte	Level 1 25ng/mL		Level 2 75ng/mL		Level 3 200ng/mL	
	BIAS	RSD	BIAS	RSD	BIAS	RSD
Methedron	2.56	3.18	1.22	1.69	0.45	2.16
Buphedron	4.02	2.04	1.14	1.83	1.16	1.52
Mephedron	3.52	1.75	1.18	2.25	0.25	1.51
d,l-4-EMC	0.81	1.43	0.97	1.67	0.50	1.73
Alpha-PVP	1.75	1.42	1.11	1.62	0.54	1.46
Bupropion	1.39	3.09	0.15	1.49	0.09	1.11

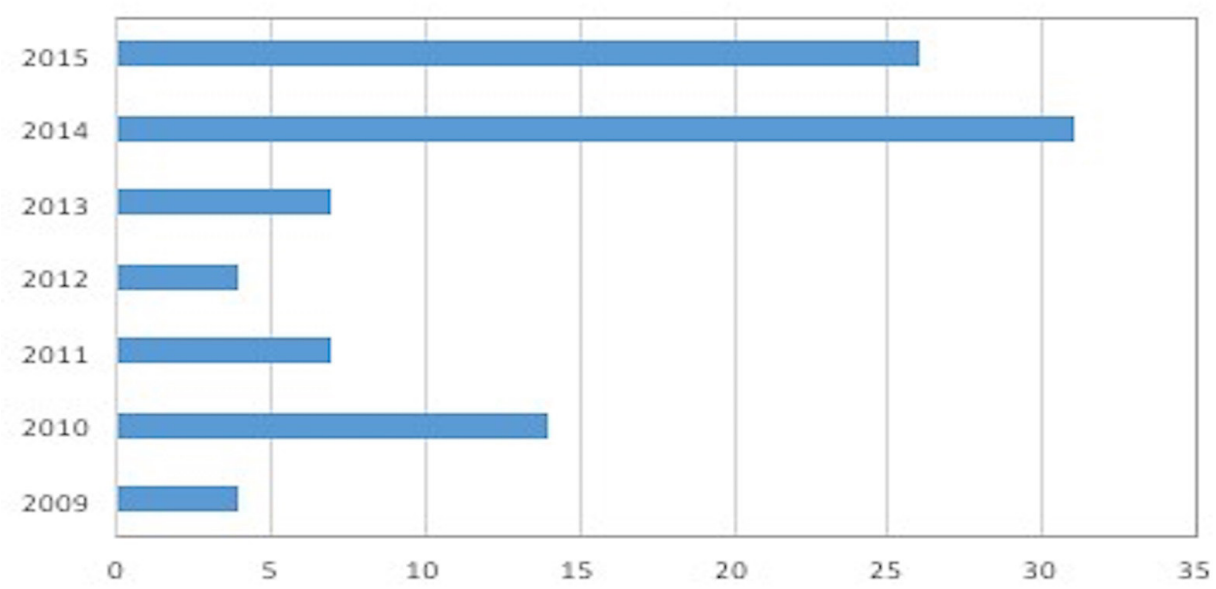

Figure 4. New cathinones informed for the fisrt time by the EU Early Warning System

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