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Research Article

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The In Vitro Stability of Four New Psychoactive Substances in Urine Samples Stored At Different Temperatures

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Abstract

Objective: In the last ten years New Psychoactive Substances (NPS) have become more frequent than the classic illicit drugs. The stability of these compounds during the different storage temperatures is less known, so the aim of our study was to investigate the extent of in vitro degradation in urine samples stored at room temperature (25 °C), in the refrigerator (4 °C) and in the freezer (-20 °C) for 21 days.

Methods: The analysis was performed on HPLC-DAD system. The confirmatory examination of synthetic cannabinoid parent compounds was carried out on SFC-MS/MS.

Results: N-Ethyl-Pentylone was stable at all three storage temperature. N-ethyl-hexedrone shows a significant ($p = 0.03$) decrease at room temperature, but was stable in the refrigerator and in the freezer. 5F-MDMB-PINACA and AB-FUBINACA metabolites seemed stable at lower temperatures, but at 25 °C the degradation was significant ($p = 0.04$).

Conclusion: The temperature greatly influences the stability, so based on our measures we conclude that the storage exclusively at -20 °C is satisfactory for the majority of NPS.

Keywords: New psychoactive substances; N-Ethyl-Pentylone; N-Ethyl-Hexedrone; Synthetic Cannabinoids; Stability; HPLC-DAD, SFC-MS/MS

Introduction

The consumption of NPS also known as designer drugs has shown a rapid and continuous increase in the last decade, which is an ongoing challenge for drug testing laboratories. Moreover, the legal status of these synthetic drugs is not cleared. The compounds are mainly produced in illegal Chinese and Indonesian laboratories by clandestine chemists who modify the chemical structure of already banned drugs to avoid legislation controls besides the preservation of the original effects. The trade of NPS differs from classic drugs as well. The distribution has been transferred from street drug dealers to headshops and websites, which offer NPS as bath salts, plant food, insect repellents, air fresheners or research chemicals with the warning label "not for human consumption" or "for research purposes only" [1-3].

NPS can be divided into groups according to their chemical structure. A high amount of them belongs to the synthetic cannabinoids and synthetic cathinones, while the others pertain to phenethylamines, tryptamines or piperazines. The most popular of them are the synthetic cannabinoids (e.g. 5F-MDMB-PINACA, AB-FUBINACA) which have similar effects as cannabis through the activation of CB1 receptors. Originally these compounds were developed for research purposes but later they conquered the drug market on various brand names like Spice in Europe or K2 in the United States. Typical symptoms of use are altered mood states, subjective changes in sensation, cognitive impairments, disorganized thought, paranoid and anxious reaction, depression, dissociation and hallucinations. The structure of synthetic cathinones (e.g. N-ethyl-pentylone, N-ethyl-hexedrone) are based on the structure of the alkaloid occurring in the khat plant (Catha edulis), which is consumed mainly on the Arabian Peninsula by chewing the leaves. These are β-keto analogues of amphetamines. Side effects of the use are mild agitation, aggression, euphoria, psychosis, hypertension, tachycardia and in some cases they can even be lethal [2,4-7].

Most of the NPS are not tested on humans before they appear on the market, so there are several risk factors related to the use. Thanks to the poor quality control in the production the chemical structure, the dosage and the possible contaminants are unknown, which can easily lead to a fatal poisoning or overdose. Furthermore, the metabolism and the interactions with other xenobiotics, including medicines are also unidentified. Traditional screening methods, like immunoassays are not optimal to identify NPS, so several analytical techniques must be applied, e.g. gas chromatography (GC), nuclear magnetic resonance (NMR) spectroscopy, high performance liquid chromatography (HPLC) and mass spectrometry (MS). To a reliable identification and quantification, the major challenge is the lack of analytical information and certified reference material [6-9]. While the stability of classic illicit drugs is already investigated and well known, the effect of storage temperature on the degradation of NPS in biological matrices, like blood or urine is mainly unidentified due to the large number of compounds. In addition, the samples often arrive after days or even weeks of the collection to the forensic toxicological laboratory and the transport conditions are also unknown, which factors can seriously complicate the examination of evidence specimens [10-13].

The aim of our study was to investigate to what extent the above mentioned representatives of NPS can be detected from forensic toxicological samples stored for 21 days at room temperature (25 °C), in the refrigerator (4 °C), and in the freezer (–20 °C).

Materials and Methods

Chemicals and reagents for HPLC analysis

Water and acetonitrile were sourced from Fisher Chemical (Pittsburgh, PA, USA). Potassium dihydrogen phosphate and o-phosphoric acid (w = 85%) were obtained from Molar Chemicals (Budapest, Hungary). Potassium hydroxide was purchased from Lach-Ner (Neratovice, Czech Republic). Steritop Threaded Bottle Top Filter (500 ml process volume, 0.22 µm pore size) was from Merck Milipore (Burlington, Canada). Internal Standard for TOX.I.S (2 µg/ml N-ethyl-

oxazepam) was purchased from Recipe Chemicals (Munich, Germany). The previously analyzed and certified reference materials to the NPS identification and quantification were obtained from the Hungarian Institute for Forensic Sciences (Budapest, Hungary).

For the preparation of potassium phosphate buffer (pH = 2.3) 33.3 g potassium dihydrogen phosphate was transferred into a 5000 ml measuring flask filled with water. The mixture was stirred for approximately 10 minutes with a magnetic stirrer without heat. 22.4 g o-phosphoric acid was added and stirred for another 5 minutes. The pH was adjusted to 2.25–2.35 with
o-phosphoric acid, and then the measuring flask was filled to the mark. For the other potassium phosphate buffer (pH = 9.0) 13.61 g potassium dihydrogen phosphate was dissolved in 900 ml water and mixed approximately 10 minutes with a magnetic stirrer without heat. Before the filling of the measuring flask to 1000 ml, the pH was adjusted to 9.0 with potassium hydroxide solution. Both buffers were filtered and stored in amber-glass bottles in the refrigerator, the durability was at least 6 months.

HPLC analysis

For the sample preparation 100 µl urine was transferred into a 1.5 ml SafeSeal tube (Sarstedt, Nümbrecht, Germany), thereafter 500 µl water, 250 µl potassium phosphate buffer (pH = 9.0) plus 250 µl Internal Standard (2 µg/ml N-ethyl-oxazepam) was added, vortexed and centrifuged for 4 minutes at 8360 RPM. The injection volume was 1.0 ml. The analysis was performed on Shimadzu Prominence TOX.I.S II. HPLC system coupled with diode array detection (Shimadzu, Duisburg, Germany). The HPLC was performed with a system controller (CBM-20A), a ternary pump system (LC-20AB), a solvent degasser (DGU-20A), a manual injector (Rheodyne 7725i) with a 1000 µl loop, an oven (CTO-20AC), a gradient unit (FCV-20AH), four flow-line selection valves (two FCV-12AH; two FCV-14AH), and a diode array detection (DAD) system (SPD-M20A) The data were evaluated using Shimadzu LC Solution software (version 1.21). The extraction column (Strata-X On-Line Extraction Cartridge) and the analytical column with guard cartridges (Gemini-NX 3u C18 110A, 5 µm) were obtained from Phenomenex (Aschaffenburg, Germany). The oven was set to 40 °C, the detection was performed at 205 and 235 nm, and the mobile phase flow rate was 1.0 ml/min. The compounds were separated with the following eluents: the loading buffer was 0.01 M potassium phosphate buffer (pH = 9.0; solvent 1), the first wash solvent was acetonitrile-water (90/10, v/v; solvent 2), and the second wash solvent was 0.05 M potassium phosphate buffer (pH = 2.3; solvent 3). The mobile phase was a mixture of solvent 2 and 3 (15/85, v/v) [14].

Confirmatory Examination

The HPLC system which was used to investigate the stability of NPS is not able to identify the parent compound of synthetic cannabinoids, therefore in most of the processed cases only the metabolite was detectable on the chromatogram. In such instances the identification was based on the similar spectrum of the parent compound and metabolite. Because there are no certified reference materials available for metabolites, in such cases the extent of the degradation was concluded from the changes of under the curve areas.

To identify the parent substances confirmatory examination were carried out in the Department of Forensic Medicine. After salting-out assisted liquid-liquid microextraction, measurements were performed by ACQUITY UPC2 supercritical fluid chromatography system (Waters, MA, USA) coupled with a Xevo TQ-S Triple Quadrupole Mass Spectrometer (Waters, MA, USA). Data were recorded by MassLynx software (version 4.1) and evaluated by TargetLynx XS software. Separation of the compounds was performed on a 3.0 mm × 100 mm, 1.7 µm particle size, AQUITY UPC2BEH analytical column (Waters, MA, USA). The mobile phase consisted of the mixture of carbon dioxide (A), 30 mM ammonium hydroxide and 15 mM acetic acid in methanol (B) with a flow rate of 1.5 ml/min. The following gradient profile was used: 99.9% A at 0.0 min and 68% A at 8 min. Pre-equilibration period lasting 2.5 minutes was applied before each injection. Constant 200 bar back pressure was used to maintain the supercritical state. The temperature was set at 45 °C and the volume of injection was 1.5 µl. To sustain a suitable electrospray, an additional solution consisting of 5 mM acetic acid in methanol was applied with a flow rate of 0.15 ml/min. This makeup solvent was delivered by a Waters 515 HPLC pump.

The MS measurement was performed in positive ion mode. The ESI source was operated with a spray voltage of 3.00 kV, cone voltage was 30 V. The source was set at 150 °C. Both desolvation and cone gasses were nitrogen delivered at 300 and 150 l/min respectively. Desolvation gas was temperatured at 300 °C. Collision gas was argon with flow rate of 0.13 ml/min. MS/MS experiments were performed in MRM (multiple reaction monitoring) mode, monitoring three fragments with optimal collision energies [15].

Statistics

Statistical analysis was performed with MS Office Excel software (version 2016). Student's t-test was used to compare the mean values of two groups (room temperature samples to refrigerated/frozen samples) in each NPS type. Differences were considered as significant when the p value was < 0.05.

Results

The stability of synthetic cathinones is shown in Table 1 and 2, while the changes of the synthetic cannabinoid metabolites are in Table 3, where only under the curve areas are listed because of the previously mentioned absence of certified reference materials. The first analysis data are from the first measures, after the samples arrived at the laboratory. Further data are from the measures performed on the 21st day of the storage. The small increases of all examined NPS could have been put down to the analytical variation.

N-ethyl-pentylone was stable at all storage temperatures. The comparison of the first analysis data to the room temperature ($p = 0.38$), to the refrigerated ($p = 0.44$) and to the frozen ($p = 0.41$) samples resulted in no significant degradation in spite of the fact that in some samples the decrease exceeded the 50%. The mean values of the compounds are on (Figure 1).

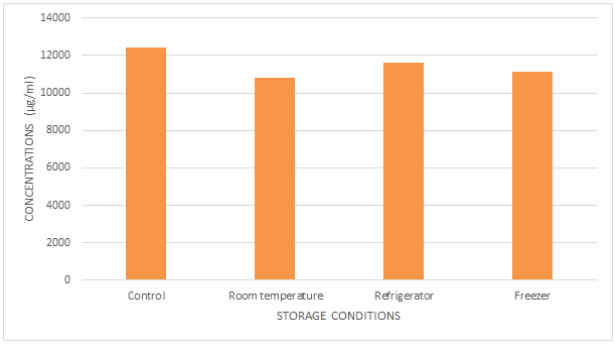

Figure 1: The mean values of N-ethyl-pentylone (n=6) measured on different storage temperatures.

First analysis data	Room temperature	Refrigerator	Freezer
20800	17200	18900	15200
100	**-17**	**-9**	**-27**
19300	16800	17200	17800
100	**-13**	**-11**	**-8**
16900	15200	17700	17700
100	**-10**	**5**	**5**
17100	15500	15600	15800
100	**-9**	**-9**	**-8**
30	10	10	20
100	**-67**	**-67**	**-33**
400	0	400	395
100	**-100**	**0**	**-1**

Table 1: Changes of the concentrations of N-ethyl-pentylone in µg/ml (white rows) and in percent (bold Values).

In the degradation of N-ethyl-hexedrone the statistical analysis eventuated no significance both in the samples stored in the refrigerator ($p = 0.27$) and in the freezer ($p = 0.28$), but in the room temperature a significant ($p = 0.03$) decrease was provable. The mean values of the compounds are on (Figure 2).

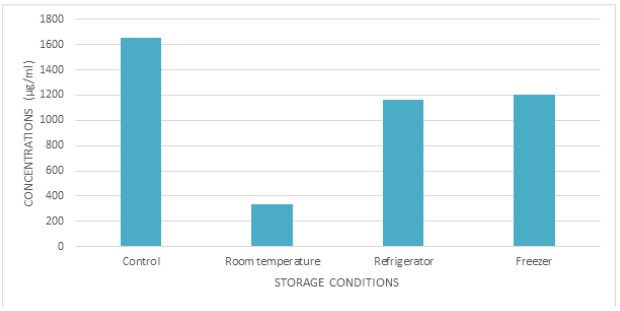

Figure 2: The mean values of N-ethyl-hexedrone (n=12) measured on different storage temperatures.

First analysis data	Room temperature	Refrigerator	Freezer
400	17	190	240
100	**-96**	**-52**	**-40**
4300	2180	4090	3800
100	**-49**	**-5**	**-12**
1470	790	1520	1580
100	**-46**	**3**	**7**
110	50	105	112
100	**-55**	**-5**	**2**
800	240	230	700
100	**-70**	**-71**	**-12**
7600	0	4500	4300
100	**-100**	**-41**	**-43**
900	0	10	70
100	**-100**	**-99**	**-92**
90	0	30	40
100	**-100**	**-77**	**-66**
1180	770	1090	1060
100	**-35**	**-8**	**-10**
700	0	600	600
100	**-100**	**-14**	**-14**
400	0	170	400
100	**-100**	**-57**	**0**
1860	0	1430	1520

100	**-100**	**-33**	**-18**

Table 2: Changes of the concentrations of N-ethyl-hexedrone in µg/ml (white rows) and in percent (Bold Values).

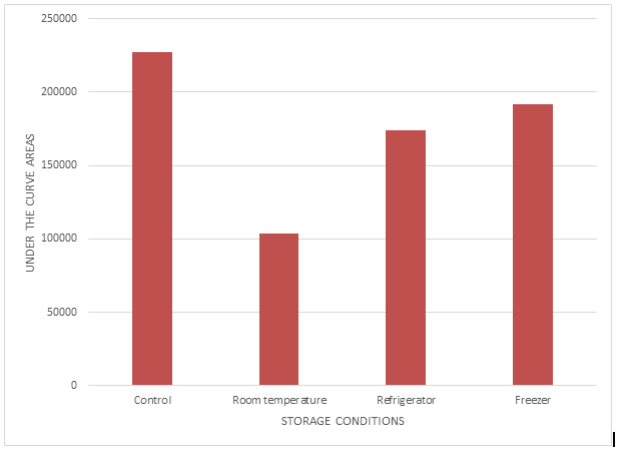

Figure 3: The mean under the curve areas of synthetic cannabinoid metabolites (n=16) measured on different storage temperatures.

We detected two types of synthetic cannabinoid metabolite: 5F-MDMB-PINACA in 14 and AB-FUBINACA in 2 samples. These NPS seem stable in the refrigerator (p = 0.23) and in the freezer (p = 0.31) as well, but in the urines stored in 25 °C the degradation was significant (p = 0.04). The mean values of the compounds are on (Figure 3).

First analysis data	Room temperature	Refrigerator	Freezer	Detected metabolites NPS
684839	683696	683756	683960	5F-MDMB-PINACA
100	**0**	**0**	**0**	
166889	134129	189076	180839	5F-MDMB-PINACA
100	**-20**	**13**	**8**	
123524	62204	125059	116547	5F-MDMB-PINACA
100	**-50**	**1**	**-6**	
106415	90922	109921	105978	5F-MDMB-PINACA
100	**-15**	**3**	**0**	
541270	14359	369716	277276	5F-MDMB-PINACA
100	**-97**	**-32**	**-49**	
614640	270898	316661	425626	5F-MDMB-PINACA
100	**-56**	**-48**	**-31**	
158905	0	0	132294	5F-MDMB-PINACA
100	**-100**	**-100**	**-17**	
77420	24718	40989	51514	5F-MDMB-PINACA
100	**-68**	**-47**	**-33**	
142912	68385	76368	89941	5F-MDMB-PINACA
100	**-52**	**-47**	**-37**	
34679	0	32852	34001	5F-MDMB-PINACA
100	**-100**	**-5**	**-2**	
185768	4123	190164	184256	5F-MDMB-PINACA
100	**-98**	**2**	**-1**	
238856	51207	160820	257611	5F-MDMB-PINACA
100	**-79**	**-33**	**8**	
410242	251289	339392	376055	5F-MDMB-PINACA
100	**-39**	**-17**	**-8**	
149906	0	144529	149102	5F-MDMB-PINACA
100	**-100**	**-4**	**-1**	
905	33	268	316	AB-FUBINACA
100	**-96**	**-70**	**-65**	
712	0	0	0	AB-FUBINACA
100	**-100**	**-100**	**-100**	

Table 3: Changes in under the curve areas (white rows) and in percent (grey rows) of synthetic cannabinoid metabolites.

Discussion`

The spreading of NPS in the last few years brings up serious problems like the impact of these chemicals on human health, the legislation controls or the detection from biological matrices. The degradation is also less observed during long term storage, but as we expected the temperature greatly influences the stability. Based on our measures we conclude that the storage exclusively at -20 °C is satisfactory for the majority of NPS.

The rapid changes on the drug market make it necessary to extend the study to substances appearing in the near future. It will be also justifiable to work out protocols for sample transport and storage to reduce the degradation as much as possible.

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