LABORATORY CHALLENGES OF DETECTING SYNTHETIC CANNABINOIDS IN URINE SAMPLES – A NEW SAMPLE PREPARATION METHOD

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Abstract
Synthetic cannabinoids (SCs) put the spotlight on the designer drugs' market due to their dangerous – in some cases lethal – biological effects and easy accessibility. They are more potent than the well-known Δ^9-tetrahydrocannabinol (THC) thanks to their special pharmacodynamic properties. The number of novel SCs on the market and of their users is growing which urges the forensic laboratories to use precise SCs detection methods routinely. Our aim was to develop a new sample preparation method for the newest 24 SCs analysis in urine samples achieving high recovery of SCs. Ethyl acetate was used instead of the traditional acetonitrile, and the targeted analysis of SCs was performed by use of liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS) method. The related matrix effect and process efficiency of sample preparation method were taken in consideration as well in our study.

Introduction
'Designer drug' is an almost 40-year-old term to describe those pharmaceuticals which trigger the same biological effect as well-known and banned substances of abuse do such as THC. Synthetic cannabinoids are a new subtype of the designer drug class which are aiming to mimic the recreational effects of marijuana by binding to the endogen cannabinoid receptors (CB_1 and CB_2), and they go by the street name of 'K2' or 'Spice'. Although SCs are often claimed to be the 'legal' and 'safe' analogues of marijuana it is far from the truth. As soon as the exact structure of a SCs is identified, it is immediately put on the illegal list of drugs, moreover, compared to marijuana, SCs have much stronger effect on the human body, because they are full (super) agonists of CB receptors while marijuana is a partial agonist [1]. This pharmacodynamic property of SCs is the key to understand why these are extremely dangerous. A hundred times lower concentration of SC is enough to trigger the same effect as can be obtained by using marijuana. [2] *(Figure 1)*
On the top of that, the endocannabinoid system crosses all over the human body and affects many different physiological functions; CB_1 receptors mainly can be found in the central nervous system (CNS) and on adipocytes – these receptors are involved in lipid metabolism, hence SCs originally were developed to treat obesity [3], while CB_2 receptors prominently play a role in immune modulation [4]. Clinical symptoms of acute SC intoxication are agitation, hallucinations, psychosis, anxiety, seizures and panic attacks [5]. Additionally, cardiovascular adverse effects are often present as well such as chest pain, hypertension and EKG abnormalities. [6] Lethal overdoses of SC are mostly linked to these cardiovascular effects. The pathophysiology behind its effects still remains unclear, but it is most likely linked to β-receptor activation in the heart. [7]

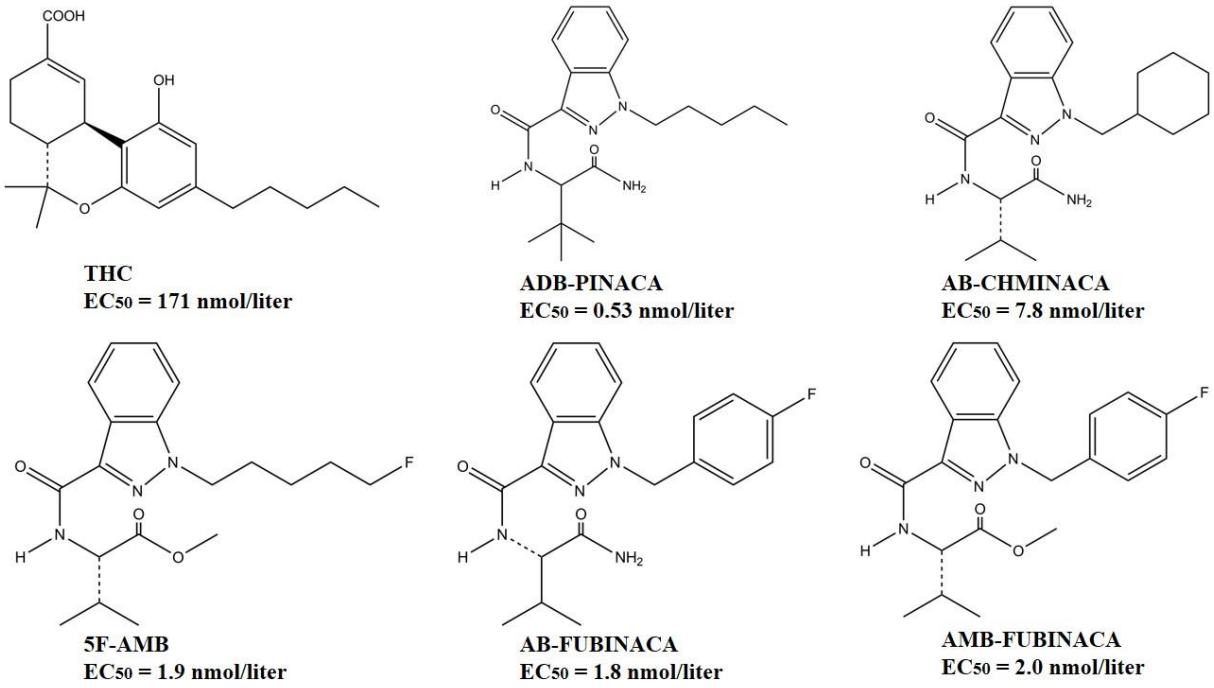

Figure 1 In vitro effective concentration required for 50% maximal response (EC50) and the molecular structure of SC are shown.

The number of SC users has been grown in the last decade [8], and it lead to a demand towards forensic laboratories to be able to analyze the newest SCs in low concentration accurately from biological samples. Sample preparation method using acetonitrile (ACN) became widespread in laboratory practice. Our aim was to challenge this method and try to develop a more effective sample preparation way to maximize the recovery of SCs from urine samples.

Experimental

Sample preparation

During sample preparation control urine with no drug or drug metabolite content was used. The mixture of 24 SCs was added to the samples in known concentration (**Fig. 2**). Three series of samples were prepared and measured in this study, one series was prepared with the use of the original method and two were prepared with the newly developed method. The original method uses ACN as organic solvent and ammonium sulphate for saturation of aqueous phase to obtain two phases, while the new method applies ethyl acetate (EtOAc) as solvent and ammonia for pH level setting (**Fig. 2**.). In order to determine the proper volume of EtOAc, 2, 4, 6, 8 and 10 mL were tested and no significant change was found over 4 mL in the recovery of SCs, therefore 4 mL was chosen for practical reasons. All other steps and the used chemicals were the same in both methods. After 1 min mixing, upon 5 min of incubation at room temperature, the sample was centrifuged at 2500 rpm for 8 min. In case of original method, 2 mL of the upper phase, while at new methods, 3 mL of top layer was collected. For double extraction procedure, the lower aqueous phase was re-extracted with 3 mL of EtOAc and 3 mL of upper phase was combined with the first 3 mL portion. The supernatants were dried by nitrogen stream at ambient temperature and the dried extracts were reconstituted in 200 µL ACN/water (1:1, v/v) containing 0.1 % formic acid mixture for LC-MS/MS measurement.

Determination of recovery, Matrix effect and process efficiency of methods were performed according to the protocol as described by Helga Trufelli and co- workers. [9]

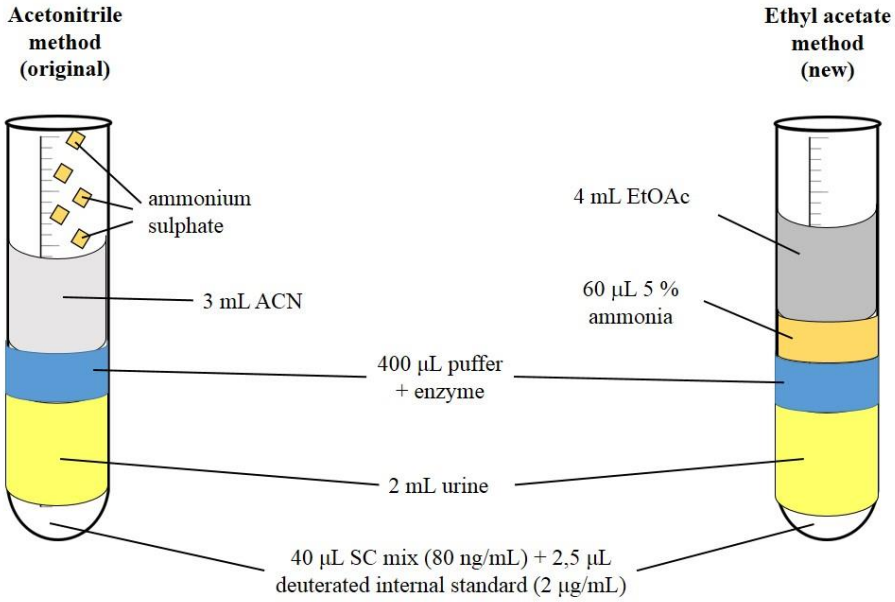

Figure 2 Comparison of the sample preparation methods

LC-MS/MS analysis

Separation was performed using an Agilent 1100 series HPLC system with Kinetex C18 column (100 x 2.1 mm, 2.6 µm) combined with a 50 x 2.1 mm guard column from Phenomenex. Injection volume was 20 □L. Elution was achieved within 6 min with a mobile phase composed of 0.1% FA in water (A) and 0.1% FA in ACN (B) at a flow rate of 0.4 mL/min. The gradient started with 50% B, ramped to 100% B within 4 min, held for 2 min. Autosampler and column oven temperatures were setting to 16 and 50 °C, respectively. The LC effluent was directed through the Agilent diverge valve to a Finnigan ESI interface on a Finnigan TSQ 7000 triple quadrupole mass spectrometer. The instrument was operated in the positive mode with a scan time of 0.3 s. The capillary heater was set at 250°C. The spray voltage was fixed at 4.5 kV. The collision gas (argon) pressure was set to 2.0 mTorr; the collision energy was optimized to two MS/MS transitions (quantifier, qualifier ions) per analyte. An electron multiplier voltage of 1900 V was used.

Results and discussion

The original acetonitrile method resulted in mean recovery of 66.3% for SCs, while the ethyl acetate method with single extraction improved to 75.2%, and the double extraction provided 91.8 % (Fig. 3).

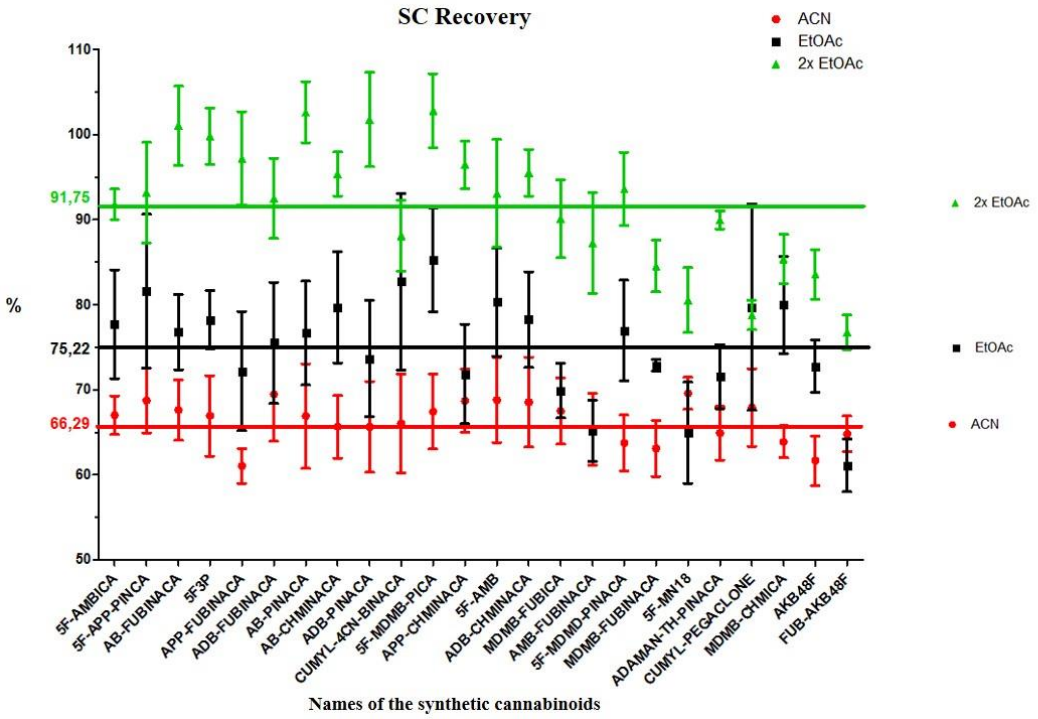

Figure 3 Recovery of 24 SCs with the acetonitrile and the single and double extraction ethyl acetate method

It was also evaluated if the matrix effect altered the recovery results. Matrix effects are caused by co-eluting matrix components (endogenous, exogenous) that alter the ionization of target analytes as well as the chromatographic behavior of target analytes, leading to reduced or increased sensitivity of the analysis. [10]

It was found that the matrix effect may cause more or less effective detection if each SC is evaluated individually. For example, the matrix effect decreased the peak area of MDMB-CHMICA, CUMYL-PEGACLONE, 5F-MDMB-PINACA, and 5F-AMBICA, especially with the ethyl acetate method, while it had a positive effect on the AMB-FUBINACA, 5F-AMB, CUMYL-4CM-BINACA, and 5F-MDMB-PICA. In some cases, the use of different solvents (ACN and EtOAC) resulted in opposite-signed matrix effect, e.g. the matrix effect with the use of ethyl acetate decrease the peak area of MDMB-FUBINACA and AB-PINACA, while the use of acetonitrile increased it. However, if the matrix effect was projected to the whole sample, meaning that all 24 SCs were considered together, it did not influence the effectiveness of the detection significantly.

The process efficiency was also measured. The mean process efficiency with the original acetonitrile method was 69.61%, with ethyl acetate, single extraction was 75.90%, and with ethyl acetate, double extraction was 89.10%.

Conclusion

Using double extraction with ethyl acetate solvent provided the highest recovery of SCs, and it is unambiguously more effective than the original ACN method in case of urine samples. Additionally, the new method is easier to conduct in the forensic laboratory environment. As a result of these two beneficial properties of the new sample preparation method, it has been introduced to everyday forensic laboratory practice in the Department of Forensic Medicine, University of Szeged.

New versions of SCs may appear any day on the market, this is why forensic laboratories should always use the best available sample preparation and detection methods, unless they will be unprepared for the challenges set by the designer drug market.

Acknowledgements

This study is supported by the ID number: *EFOP-3.6.1-16-2016-00008* EU project.

References

[1] *Pharmacological and Toxicological Effects of Synthetic Cannabinoids and Their Metabolites.* **Fantegrossi, Sherrica Tai and William E.** 2017, Curr Top Behav Neurosci., pp. 32: 249–262.

[2] *"Zombie" Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York.* **Axel J. Adams, B.S., Samuel D. Banister, Ph.D., Lisandro Irizarry, M.D., Jordan Trecki, Ph.D., Michael Schwartz, M.D., M.P.H., and Roy Gerona, Ph.D.** New England Journal of Medicine : s.n., 2017, Vols. 376:235-242.

[3] *Cannabis in fat: high hopes to treat obesity.* **Melody N. Hawkins, Tamas L. Horvath.** The Journal of Clinical Investigation : s.n., 2017, Vols. Volume 127, Issue 11.

[4] *Is lipid signaling through cannabinoid 2 receptors part of a protective system?* **P.Pachera, R.Mechoulamb.** Pages 193-211, Progress in Lipid Research : s.n., 2011, Vols. 50, Issue 2.

[5] *Toxicological Findings of Synthetic Cannabinoids in Recreational Users.* **Robert Kronstrand, Markus Roman, Mikael Andersson, Arne Eklund.** Journal of Analytical Toxicology : s.n., 2013, Vols. Volume 37, Issue 8, Pages 534–541.

[6] *Cardiotoxicity associated with the synthetic cannabinoid, K9, with laboratory confirmation.* **Young AC, Schwarz E, Medina G, Obafemi A, Feng SY, Kane C, Kleinschmidt K.** American Journal of Emergency Medicine : s.n., 2012, Vols. 1320.e5-7.

[7] *Receptors and Channels Targeted by Synthetic Cannabinoid Receptor Agonists and Antagonists.* **Pertwee, R.G.** Curr Med Chem : s.n., 2011, Vols. 17(14): 1360–1381.

[8] *A Survey of Synthetic Cannabinoid Consumption by Current Cannabis Users.* **Erik W. Gunderson, MD, Heather M. Haughey, PhD, Nassima Ait-Daoud, MD, Amruta S. Joshi, MS,and Carl L. Hart, PhD.** Subst Abus. : s.n., 2014, Vols. 35(2): 184–189.

[9] *An overview of matrix effects in liquid chromatography-mass spectrometry.* **Trufelli H, Palma P, Famiglini G, Cappiello A.** Mass Spectrom Rev. 2011, pp. 30(3):491-509.

[10] *Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring.* **Parinya Panuwet, 1,* Ronald E. Hunter, Jr.,1,3 Priya E. D'Souza,1 Xianyu Chen,1,2 Samantha A. Radford,1,2 Jordan R. Cohen,1 M. Elizabeth Marder, Kostya Kartavenka, P. Barry Ryan, and Dana Boyd Barr.** Crit Rev Anal Chem. : s.n., 2016, Vols. 46(2): 93–105.