Changes in drug trends-challenges for clinical toxicology laboratories

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Theses of doctoral (PhD) dissertation

Clinical Medical Sciences

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1. Introduction

New psychoactive substances (NPS, designer drugs, research chemicals, potpourri, herbals, bath salts, plant food, etc.) have shown an alarming boost in popularity in the last few years and rate as the most common substances of abuse. The easy access to the new substances (through online trade), their similar psychoactive effects to those of the conventional drugs, their much lower price and the fact that they are legal led to the 'revolution' of recreational drugs. These transformations have also led to changes on the analytical side: in addition to traditional drugs, the accurate identification of hundreds of new psychoactive substances (and their metabolites) in the absence of reference standards is still a major challenge for clinical and forensic toxicology laboratories.

1.1. New psychoactive substances

New psychoactive substances have been in continuous production by clandestine chemists since as early as 1910, when MDA (3,4-Methylenedioxyamphetamine) was first produced.

Though they are defined as substances designed to replicate the effects of traditional illegal drugs such as cocaine, ecstasy, amphetamines and cannabis, it has been suggested that these mimics are extensively more potent than their counterparts. Beyond the limits of current legislation, clandestine chemists have set out to manufacture these substances for profit, whilst benefitting users in areas of affordability, availability and purity. The manufacturing of these substances includes a slight change of chemical-structural qualities of an already known drug. These small modifications (structural analogues, stereoisomers, derivatives, etc.) of a 'basis' drug may lead to great variations in effects and side-effects of the new drug. In some cases however, there is the development of new designer drugs with similar effects to other drugs with completely diverse chemical structures.

The concept of the new psychoactive substance is described in the Criminal Code in Hungary (Act XCV of 2005 on Medicinal Products for Human Use and on the Amendment of Other Regulations Related to Medicinal Products, Section 1, paragraph 37): *'new psychoactive substance' shall mean a new substance or compound never before used in the field of medicine that, acting on the central nervous system, functions as a mood altering drug, having the capacity to change human behaviour and perception, and that may consequently pose a comparable threat to public health as the substances listed in Tables I and II of the Single Convention on Narcotic Drugs signed in New York on 30 March 1961, promulgated by Law-*

Decree No. 4 of 1965, or in Lists I and II of the Annex to the Convention on Psychotropic Substances signed in Vienna on 21February 1971, promulgated by Law-Decree No. 25 of 1979, or in the lists of psychotropic substances contained in Annex 2, and that has been classified on that basis as a psychoactive substance by the minister in charge of the healthcare system by means of a decree'.

Though several short-term effects of NPS are known such as, agitation, paranoia, psychosis, hypertension, elevated temperature and seizures, many long-term harms are unknown and though re-occurring death rates demonstrate obvious indicators of their danger, users continue to play 'Russian-roulette' with their lives.

Organizations dealing with the emergence, distribution, seizure of new psychoactive substances and drug-related crime:

- UNODC (*United Nations Office on Drugs and Crime)* - on global scale;
- EMCDDA *(European Monitoring Centre for Drugs and Drug Addiction),* EWS (*Early Warning System*)- in the European Union;
- HIFS *(Hungarian Institute for Forensic Sciences)* -in Hungary.

1.2. Groups of new psychoactive substances according to their chemical structure

New psychoactive substances (with a few exceptions) can be classified into four major groups based on their chemical structure. A common feature of the very different compounds is that they do not have a specific antidote, and therapy is just symptomatic treatment.

1.2.1. Synthetic cathinones

Semi-synthetic and synthetic variants of the natural (plant-derived) psychostimulant cathinone (*Catha edulis*) are the members of this group. The first synthetic cathinone derivative was Mephedrone (4-MMC, 4-methylmethcathinon). It was first synthesized in 1929 but was rediscovered in 2003 and is reportedly manufactured in China. They are distributed as 'bath salts', 'research chemicals' and 'plant food' and show similar psychotropic effects to MDMA, cocaine and because of their structural similarity to amphetamines

Chemical structure:

The general structure of a cathinone derivative (Figure 1.) shows substitution patterns at three locations of the cathinone molecule.

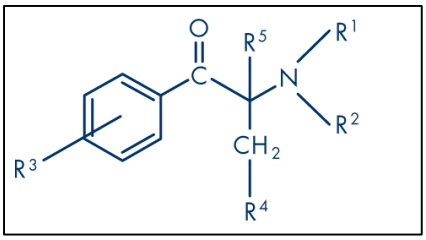


Figure 1. Cathinone skeleton

Groups of cathinones:

a. N-alkylated cathinone derivatives; includes N-alkyl compounds or those with an alkyl or halogen substituent at any possible position of the aromatic ring. The majority of the first synthetic cathinones fall into this group.
b. Methylenedioxy-substituted compounds with substituents at any given position of aromatic ring. In terms of their structure and pharmacological effect, these compounds are quite similar to 3,4-methylenedioxymethamphetamine (MDMA).
c. Analogs of natural cathinone with an N-pyrrolidinyl substituent.
d. Compounds which include both methylenedioxy and N-pyrrolidinyl substituents.

1.2.2. Synthetic cannabinoid receptor agonists

Synthetic cannabinoid receptor agonists (SCRAs) are heterogeneous compounds originally intended as probes of the endogenous cannabinoid system or as potential therapeutic agents. The first ‘classical cannabinoid’ analogue of THC that was synthesised in Israel in 1988 was ‘HU-210’ (Figure 2.). Major limitations in the development of new cannabinoids have been undesirable psychoactive properties and public perception of cannabinoid use.

The SCRAs were first reported as new psychoactive substances in Europe in 2008. There has been a steady rise in the availability of a wide range of different SCRAs since this time and have been the most common new psychoactive substances reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) with 169 SCRAs reported from 2008 to the end of 2016. Synthetic cannabinoids are among the most popular NPSs besides synthetic cathinones.

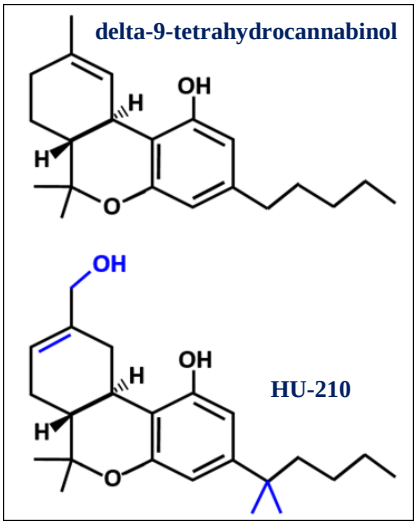


Figure 2. Δ^9-THC and the fist synthetic analogue

Chemical structure:

The cannabinoid receptor agonists form a diverse group, but most are lipid soluble and non-polar, and consist of 22 to 26 carbon atoms. A common structural feature is a side-chain, where optimal activity requires more than four and up to nine saturated carbon atoms.

Structural classification of synthetic cannabinoids:

1. *Naphthoylindoles, Naphthylmethylindoles, Naphthoylpyrroles, Naphthylmethylindenes: JWH-007, JWH-018, JWH-073, JWH-200, JWH-398, AM-1221, AM-2201 (Fluoroalkyl derivative from JWH018), AM-694, Win-55,212-2.*
2. *Phenylacetylindoles (i.e., benzoylindoles): JWH-250, RCS-8.*
3. *Cyclohexylphenols: CP-47947, CP-55940.*
4. *Tetramethylcyclopropylindoles: UR-144, XLR-11 (Fluoroalkyl derivative from UR-144).*
5. *Adamantoylindoles: 5F-AKB-48, STS-135.*
6. *Indazole carboxamides: AB-PINACA, AB-FUBINACA.*
7. *Quinolinyl ester: PB-22.*

1.2.3. Substituted phenethylamines

Phenethylamines include a wide range of natural or synthetic substances which own psychostimulant, entactogenic, and hallucinogenic effects.

Phenethylamines represent a class of compounds with documented psychedelic and stimulant effects. The 2C family, which includes structural analogues such as 2C-B and 2C-I, has been well known since the 1970s from the synthetic work of Alexander Shulgin and the

influence of his 1991 book *PIHKAL: A Chemical Love Story*. Other related phenethylamine groups consist of the ring-substituted D amphetamines (e.g., DOI, DOB) and the dibenzofurans (e.g., BromoDragonFLY, 2C-B-FLY). The first seizure data for these substances are from 2009 (both in Europe and the United States).

Chemical structure:

Phenethylamines are those NPS whose core chemical structure consists of a 2-phenyl-ethylamine (Figure 3.)

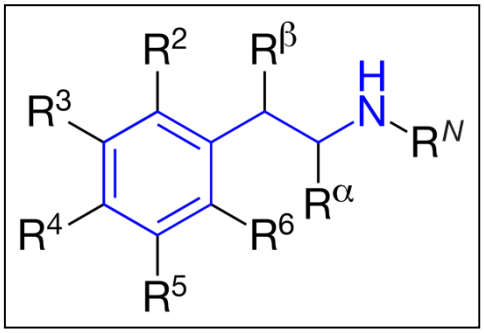


Figure 3. The structural formula of phenethylamine with marked substitution points

Structural classification of substituted phenethylamines:

1. *mescaline-type phenethylamines:* e.g., mescaline, 2,5-dimethoxy-4-methylamphetamine (DOM).
2. *amphetamine-type stimulants:* e.g., 3-Fluoroamphetamine (3-FA), para-Methoxyamphetamine (PMA).
3. *MDMA-like drugs:* e.g., 3,4-Methylenedioxy-N-ethylamphetamine (MDEA), N-methyl-1-(1,3-benzodioxol- 5-yl)-2-butanamine (MBDB).
4. *N-benzyl substituted phenethylamines:* e.g., 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe).
5. *benzofurans:* e.g., 5-(2-Aminopropyl)Benzofuran (5-APB).
6. *2C-series compounds:* e.g., 2,5-Dimethoxy-4-ethylphenethylamine (2-CE).
7. *Bromo-dragonFLY:* 1-(8-bromobenzo[1,2-b; 4,5-b′]difuran-4-yl)-2-aminopropane.

1.2.4. Tryptamines

Tryptamines have been classified in three groups: (1) ‘simple tryptamines,’ structurally derived from tryptamine, (2) ‘ergolines,’ structurally related to the semisynthetic lysergic acid diethylamide/LSD and (3) phenethylamines.

Alexander Shulgin synthesized several hundred substituted tryptamines, of which about 50 are known to be psychoactive and currently used for recreational purposes. Their synthesis, doses and adverse effects are described in his book- *TIHKAL :Tryptamines I Have Known and Loved* (Shulgin and Shulgin, 1997.).

Chemical structure:

The wide variety of synthetic tryptamine analogues may exhibit different modifications on the nitrogen atom of the side chain, on the α position of the side chain and/or in the aromatic ring (Figure 4.).

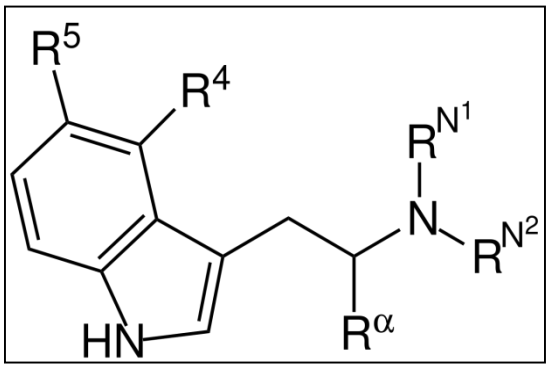


Figure 4. The structure of substituted tryptamines

1.2.5. Other new substances

Arylcyclohexylamines: structural analogues of ketamine and phencyclidine (e.g., methoxetamine).

Synthetic opioid-like drugs: e.g., new fentanyl analogues (furanylfentanyl, methoxyacetylfentanyl, benzoylfentanyl).

Piperidinek: following the control of methoxetamine, several new dissociative piperidine derivatives, antagonists of NMDA receptors, including diphenidine and 2-methoxydiphenidine (MXP, 2-MXP), have appeared on the recreational drug market.

Aminoindanes, piperazines, and pipradrol derivatives: these compounds are novel psychoactive substances found in ‘Ecstasy’ tablets as replacements for 3,4-methylenedioxymethamphetamine (MDMA) or substances sold as ‘ivory wave’.

Designer benzodiazepines: The first designer benzodiazepines available online were diclazepam, flubromazepam and pyrazolam. Recently, five others became readily available (e.g., clonazolam, des-chloroetizolam, flubromazolam, nifoxipam and meclonazepam), none of which has been approved for medicinal use in any country.

Synthetic Cocaine Derivatives: Different synthetic cocaine derivatives sold as 'research chemicals' have been identified as potential pharmacological drugs, two of which are under tight observation because of their high abuse potential. Among these substances are 3-(p-fluorobenzoyloxy)tropane (pFBT), with chemical structure very similar to cocaine, and dimethocaine, with chemical structure very similar to procaine, a local anaesthetic lacking psychoactive property.

4,4'-Dimethylaminorex: a synthetic substituted oxazoline derivative, psychostimulant. Between June 2013 and June 2014, thirty-one deaths associated with the consumption of this new drug have been registered across Europe.

1.2.6. Summary table of new psychoactive substances

Psychoactive effect	Chemical classification	Receptor-targeting	Clinical symptoms of intoxication
Depressants	Designer benzodiazepines	$GABA_A$	drowsiness, respiratory depression
	Synthetic opioids	opioid receptors (μ-opioid)	respiratory depression
	Synthetic cannabinoids	CB_1 and CB_2	nausea, vomiting, conjunctival injection, agitation, arrhythmia, convulsion
	Arylcyclohexylamines	NMDA-receptors	vomiting, nystagmus, increased muscle tone
	GHB	$GABA_B$	amnesia, bizarre involuntary movements, agitation, convulsion
Stimulants, Empathogens	Synthetic cathinones-mephedrone group	SER=DA, NA	sleeplessness, hyperthermia, arrhythmia, convulsion, rhabdomyolysis, renal failure
	Synthetic cathinones-pentylone group	DA, NA>SER	
	Synthetic cathinones-valerone group	DA, NA>>>SER	
	Piperidines	DA, NA	chest pain, palpitation, hyperthermia, difficulty in breathing, convulsion
	Piperazines	DA, NA	
	Synthetic cocaine derivatives	DA>>>NA, SER	hypertension, tachycardia, anxiety, chest pain
	Phenethylamines	SER>>>DA, NA	hyperthermia, arrhythmia, convulsion, rhabdomyolysis, renal failure, DIC, hyponatraemia, hypoglycemia
	Aminoindanes	SER	
Hallucinogens	Phenethylamines	$5\text{-}HT_{2a}$	vasospasm, vomiting, diarrhea, bizarre involuntary movements
	Arylcyclohexylamines	NMDA-receptors	vomiting, nystagmus, increased muscle tone
	Tryptamines	$5\text{-}HT_{2A}$ and $5\text{-}HT_{1A}$	hypertension, tachycardia, rhabdomyolysis, renal failure
	Synthetic cannabinoids	CB_1 and CB_2	nausea, vomiting, conjunctival injection, agitation, arrhythmia, convulsion

Table 1. Summary table of new psychoactive substances

1.3. Legal aspects of new psychoactive substances in the European Union and in Hungary

In Hungary, various trials have been taken to control new substances, and three broad types of legal responses can be identified. The individual list regulation (which is the most widespread based on UN treaties) puts the exactly identified substance or group of substances on the list in standard, extraordinary or accelerated way. In the generic regulation, the substance group (the base formula) and its derivatives are also included in the list. Besides the United Kingdom and Ireland, Hungary applies this kind of regulation as well. The analogue regulation is based on chemical and/or biological similarity (USA, Latvia, and Norway).

2. Aims of the study

New psychoactive substances pose a potential danger to consumers, besides we have had to face a number of challenges on the analytical side over the years. The aim of the study is to present the problems that arise due the NPS and how we solved them, as well as the research done with the new materials, such as:

1. difficulties due to lack of standards,
2. cross-reactivity of NPS in immunoassays,
3. diagnostic dilemmas in detection of synthetic cannabinoids,
4. determination of serum catecholamine levels in new psychoactive substances users,
5. the in vitro stability of new psychoactive substances in urine samples stored at different temperatures.

3. Materials and methods

3.1.Materials

Biological specimens (including urine, blood and body tissues), unknown powders, tablets, liquids, plant materials sent to the Department of Laboratory Medicine for toxicological analyses in the period 2010-2019. The investigated samples can be divided into two groups according to their origin:

1. Clinical samples (n_C=7253)
2. Forensic samples (n_F=4604).

3.2. Methods

3.2.1. MALDI-TOF

We used MALDI-TOF Autoflex (Bruker) mass spectrometer to identify active ingredients in unknown powders (for later use as a standards).

3.2.2. FPIA

For the cross-reactivity measures ABBOTT AxSYM® fluorescence polarization immunoassay (FPIA) was used.

3.2.3. KIMS

A semiquantitative immunochemical method (Kinetic Interaction of Microparticles in a Solution - KIMS - Roche, Cobas® Integra 400 Plus) was used to detect benzodiazepines and THC and its metabolites in urine.

3.2.4. HPLC- DECADE II. SCD

We applied Shimadzu Prominence HPLC to determine catecholamines in serum with '*Catecholamines in Plasma'* (Chromsystems Instruments & Chemicals GmbH, Germany) kit. Detection was performed with an electrochemical detector (DECADE II. SCD-Antec Scientific-Netherlands).

3.2.5. HPLC-DAD

For the qualitative and quantitative detection of traditional drugs and new psychoactive substances in urine, Shimadzu Prominence TOX.I.S., and followed by an upgrade (from 2013) TOX.I.S. II. HPLC-DAD system was used.

Sample preparations were determined by the types of samples, which were the follows:

- *urine,*
- *serum,*
- *whole blood (post mortem),*
- *powders, tablets,*
- *liquids,*
- *plant materials,*
- *other post mortem samples e.g. liver-, bile-, stomach-extracts.*

The relatively simple sample preparations were due to the online extraction.

Statistical analyses were performed using Microsoft Office16 (Excel).

4. Results

4.1. Identification of active substances in unknown powders

The presence of new psychoactive substances, implies perhaps the biggest problem for toxicology laboratories: the lack of reference standards. In many cases, we found the new compound in a biological sample before the standard would have been available. In 2010-11, we used powders / tablets obtained from the Addiction Center of Pécs or purchased on the Internet (still legally available at the time of ordering) as standards. Due to the uncertain source /origin, we had to determine the active ingredients. Measurements were performed on a MALDI-TOF mass spectrometer, and substances were identified by their mass (mass to charge ratio; m/z). Using this method, we identified ten new psychoactive substances:

- 5 synthetic cathinones (mephedrone, MDPV, 4-FMC, 4-MEC, pentedrone);
- 2 phenethylamines (4-fluroamphetamine and 5-APB);
- 2 synthetic cannabinoids: (JWH-122 and AM-2201);
- 1 arylcyclohexylamine (methoxetamine).

After the mass determination, the samples were also analysed by HPLC-DAD system to define the retention time, relative retention time, and UV spectra of the new compounds.

4.2. Cross-reactivity of new psychoactive substances with the FPIA immunoassays developed for conventional drugs (Amphetamine / Methamphetamine; Cannabinoids)

Indications for measurement:

a) new psychoactive stimulants: several forensic urine samples tested by police showed amphetamine positivity, then, as determined by immunochemical test (FPIA), amphetamine concentrations exceeded 8000 ng / ml in many cases, but we could not detect amphetamine by HPLC-DAD analysis of the same samples.
b) synthetic cannabinoid receptor agonists: in some clinical urine samples where the use of ‘Herbal’ was reported to occur, we obtained values around the sensitivity of the ABBOTT AxSYM® Cannabinoids Test (13 ng/ml).

4.2.1. Measurement of urine samples spiked with different stimulant types of new psychoactive substances by ABBOTT AxSYM® Amphetamine / Metamphetamine II. (FPIA) assay

Before the measurements, we tested all levels (low, medium, high) of controls, and we used 98% pure amphetamine powder as 'own' control.

The results of the measurements are summarized in Table 2.

Sample conc. / Name	100 µg/ml	80 µg/ml	40 µg/ml	20 µg/ml	10 µg/ml
1. Mephedrone	*1405*	*1109*	*398*	*225*	*<100*
2. 4-FMC	*807*	*756*	*335*	*171*	*<100*
3. 4-MEC	*1502*	*1236*	*644*	*426*	*198*
4. Pentedrone	*<100*	*<100*	*<100*	*<100*	*<100*
5. 4-FA	*107*	*<100*	*<100*	*<100*	*<100*
6. MDPV	*<100*	*<100*	*<100*	*<100*	*<100*
7. 5-APB	*>8000*	*>8000*	*>8000*	*>8000*	*>8000*
8. Meteoxetamine	*<100*	*<100*	*<100*	*<100*	*<100*

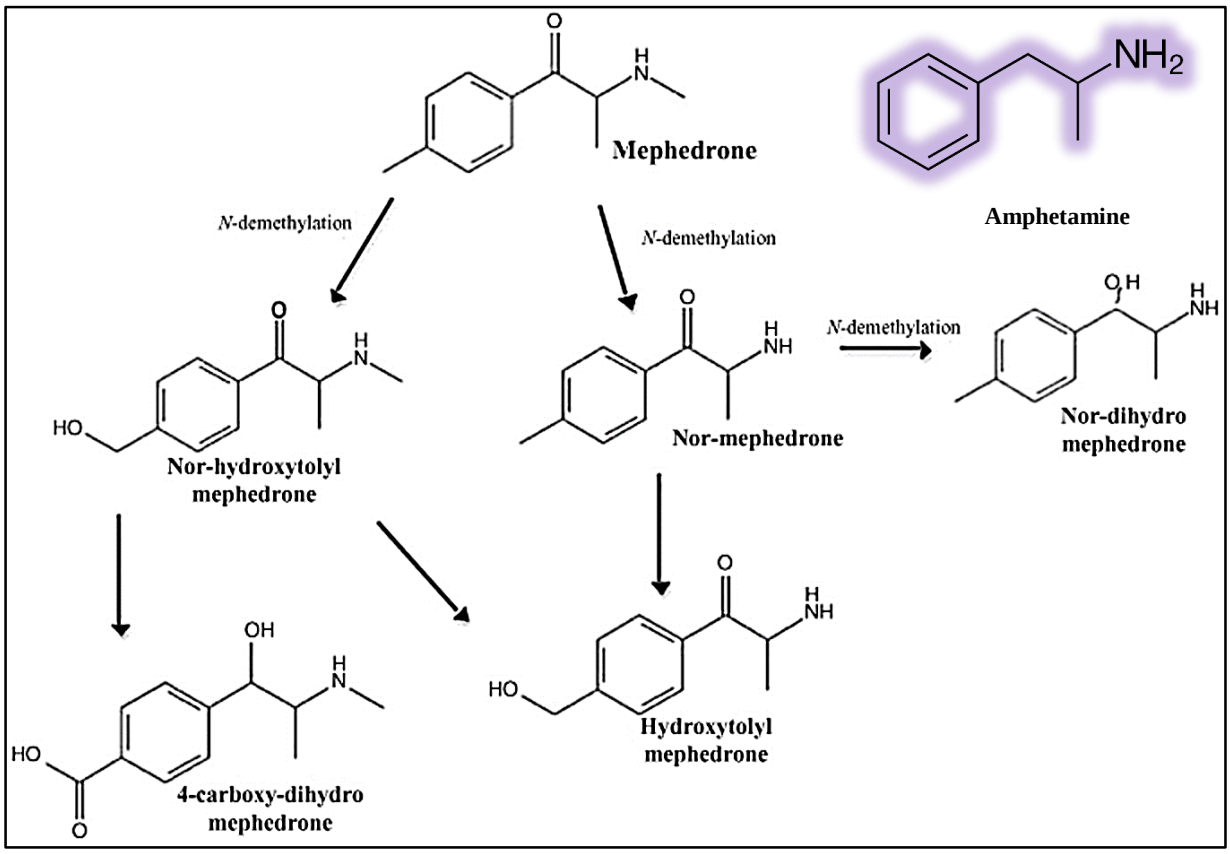
Sample conc. / Name	10 µg/ml	5 µg/ml	2,5 µg/ml	1 µg/ml	0,5 µg/ml
5- APB	**>8000**	**4426**	**2300**	**677**	**206**

Table 2. Measurement results of urine samples spiked with different stimulant types of new psychoactive substances by ABBOTT AxSYM® Amphetamine / Metamphetamine II. (FPIA) assay

Results:

- there was no cross-reactivity with pentedrone, 4-FA, MDPV, methoxetamine,
- minimal cross-reactivity with mephedrone, 4-FMC, 4-MEC,
- strong cross-reactivity with 5-APB.

The reason of the high amphetamine concentration measured in forensic urine samples (except the 5-APB), is presumably not due to the parent compound but to the cross-reaction of metabolites (Figure 5).



Fugure 5. Structural similarities between the amphetamine and the major metabolites of mephedrone

4.2.2. Measurement of urine and serum samples spiked with JWH-122 by ABBOTT AxSYM® Cannabinoids (FPIA) assay

Before the measurements, we tested all levels (low, medium, high) of controls.

Result: there was no cross-reactivity between JWH-122 and the Cannabinoids test. In our experience, the synthetic cannabinoid receptor agonists do not produce a positive result for cannabis on any rapid test either, so we did not investigate further synthetic cannabinoids in this direction.

It is important to highlight the limitations of using immunochemical tests (e.g., drug rapid tests) that have been well-established for screening traditional drugs. In the cases of the new substances, neither rapid tests nor immunoassays developed for conventional drugs are not suitable for screening; due to the high incidence of false-positive (e.g., synthetic cathinones and amphetamine) or false-negative (e.g., synthetic cannabinoids and THC) results. Thus, in order to prove the drug consumption, a large-scale confirmatory test is required in each case.

4.3. Detection problems for synthetic cannabinoids

Urine is the most common matrix for drug testing. *All previously investigated SCRAs were extensively metabolized, with little to no unchanged parent drug found in human urine.* Generally, phase I metabolites are the best SC marker metabolites to document intake because they have higher mass spectrometry responses and are more stable than phase II metabolites over time. Therefore, metabolism studies on novel emerging SCRAs are essential, but only a few relevant literature data exist. It is important to emphasize that one metabolite can be the metabolic product of several parent compounds, therefore in the Hungarian legal system (currently) only the identification of the parent compound is suitable to justify the consumption. A suitable device for identification of SCRAs is commonly a separation technique (GC, HPLC, supercritical fluid chromatography) coupled to mass spectrometer (MS) or tandem mass spectrometer (MS / MS).

For the above reasons, we did not attempt to detect SCRAs by HPLC-DAD until 2014, but in this year we observed the presence of a substance with a similar UV spectrum and retention time in several urine samples (either forensic or clinical), which later we could identify as a metabolite of SCRA.

Diode array detection allows to be classified materials with similar molecular configuration into groups based on their UV spectrum similarity. After the reference standards of the SCRAs were run, the similarity between the parent compound and the metabolites became clear (Figure 6.). The differences are between the retention times. Confirmatory examination of the same samples by SFC-MS / MS (PTE, Medical School, Department of Forensic Medicine) confirmed the consumption of SCRA.

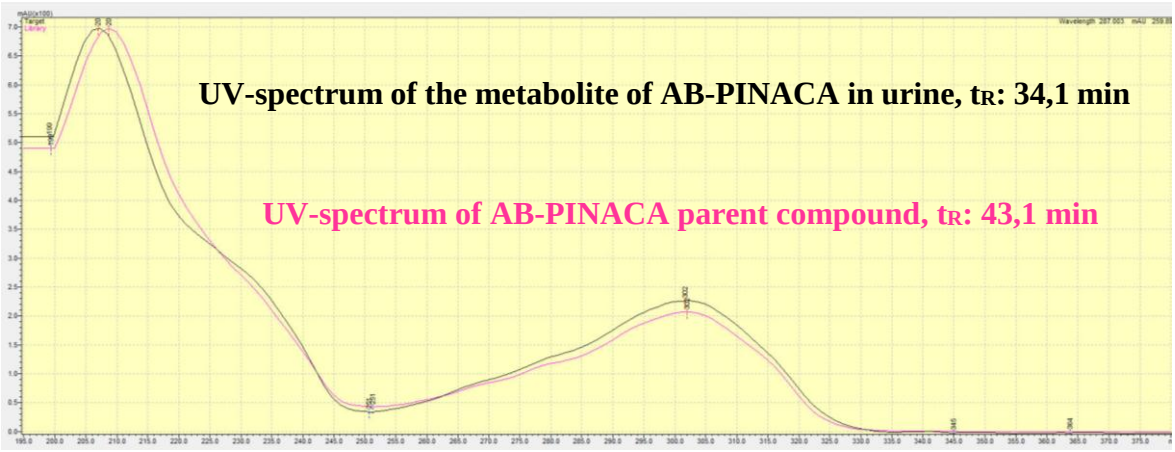


Figure 6. UV spectrum of AB-PINACA metabolite in urine and the parent compound

By HPLC-DAD system we can detect indole- (e.g., MDMB-CHMICA, 5F-MDMB-PICA, 4F-MDMB-BICA) and indazole- (e.g., AB-PINACA, ADB-FUBINACA, ADB-CHMINACA) based SCRA (Figure 7.) metabolites (rarely the parent compound).

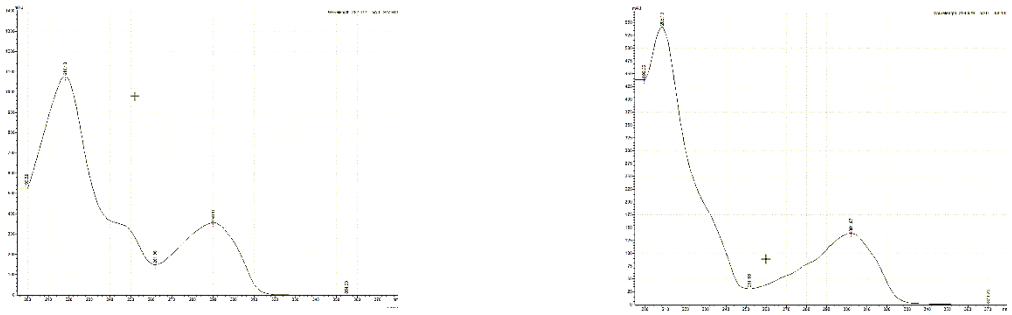


Figure 7. UV-spectrum of indol based SCRA (left side); UV-spectrum of indazole based SCRA (right side)

In clinical specimens, the SCRA metabolite detected in urine *alone* is sufficient to justify the consumption.

4.4. Determination of serum catecholamine levels in samples of stimulant-type novel psychoactive substance users

The catecholamines adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine are biogenic amines, which play a central role in the body as hormones and neurotransmitters.

The determination of catecholamines has clinical significance in the diagnosis of pheochromocytomas and other tumours affecting the nervous system. Furthermore, the

concentrations of adrenaline and noradrenaline are important indicators for cardiac insufficiency, cardiac diseases and arteriosclerosis.

The aim of our research was to determine the catecholamine concentrations in the serum samples of stimulant-type new psychoactive substance users. Beside the target group serum catecholamine levels testing, catecholamine concentrations were measured in 6 healthy, drug-negative serum samples (control group) too. The criteria for selection in the target group was: detection of a new stimulant-type psychoactive substance in urine (by HPLC-DAD) and that no other drugs and / or narcotics be present in the urine. Serum catecholamine levels were determined by HPLC / EC (DACADE II. SCD). The reference ranges for serum catecholamine concentrations (Chromsystems) were the follows:

- Noradrenaline: 80-499 ng/l (0,47-2,95 pmol/l),
- Adrenaline: 3-82 ng/l (0,02-0,45 pmol/l),
- Dopamine: 2-58 ng/l (0,013-0,379 pmol/l).

Serum catecholamine concentrations of the target group are shown in Table 3.

Number of sample	***Noradrenaline [ng/l]***	***Adrenaline [ng/l]***	***Dopamine [ng/l]***
1.	188	**293**	**334**
2.	404	**200**	**226**
3.	**2913**	**966**	**385**
4.	237	**215**	**265**
5.	**2873**	**175**	**290**
6.	358	**370**	**203**
7.	**1084**	**212**	**396**
8.	**1098**	**225**	**382**
9.	**589**	**180**	**603**

Table 3. Serum catecholamine concentrations of the target group

Our hypothesis, that the catecholamine concentrations of the target group for all three tested compounds differ significantly from the control group was tried to support by statistical analysis (Student's two tailed heteroscedastic T test). The results showed that the concentrations of dopamine ($p = 4.44x10^{-5}$) and adrenaline ($p = 0.019$) in the control and target groups differed significantly, while in the case of noradrenaline ($p = 0.054$) there was no significant difference between the two groups.

4.5. The in vitro stability of new psychoactive substances in urine samples stored at different temperatures

The number of international literatures on the stability of new psychoactive substances is extremely small, and there is no Hungarian one at all. The aim of our research was to determine the stability of several representatives of new psychoactive substances from urine samples (sent for forensic or clinical toxicological examination) stored at room temperature (25 °C), refrigerator (4 °C) or freezer (-20 °C) at specified time intervals. Based on our preliminary hypothesis, drug concentrations measured in samples stored at room temperature will be significantly lower compared to the concentrations detected in frozen samples.

We performed, precision and accuracy measurements within one day and between days of four psychoactive substances (two conventional: amphetamine and MDMA and two new ones: N-butylpentylone and ADB-FUBINACA) in spiked urine samples before the analyses. A total of 18 urine samples were examined, of which in 8 cases the same urine was measured at three different temperatures, and 10 samples were re-analysed that were stored only at room temperature for 5-9 months.

The results of the first analysis served as a control, which we compared to the concentrations (areas under the curves) of the drugs measured later.

Results: no significant loss of the traditional analytes under study we observed at any of the investigated conditions, in contradistinction to the examined new psychoactive substances, which degradation was significant.

4.6. New psychoactive and conventional stimulants in biological samples between 2010 and 2019

According to the EMCDDA data, more than 730 new psychoactive substances appeared on European drug markets between 1997 and 2018, 90% of them were reported after 2008. By December 2019, that number has risen to 950.

Both the drug market and the type of the drugs preferred by the users have dramatically changed; conventional drugs have become less popular; they have been replaced by the new substances with extraordinary variability. An obvious method to monitoring the changes is the distribution of conventional and new agents detected in the biological samples of consumers, which is presented below in relation to Baranya county.

For the research we used forensic samples (n = 4604) sent to the laboratory for toxicological screening between 2010 and 2019. We examined eight type of samples: 4194 urines, 370 blood extracts, 22 liver extracts, 13 bile extracts, 3 liquors, 1 stomach extract, 1 spleen extract and 1 kidney extract. Most of the relevant cases concerned possession of drugs (81.5%), driving under the influence (2.9%), drug trafficking (2.7%), as well as post-mortem cases (12.9%) including fatal intoxication (Figure 8.).

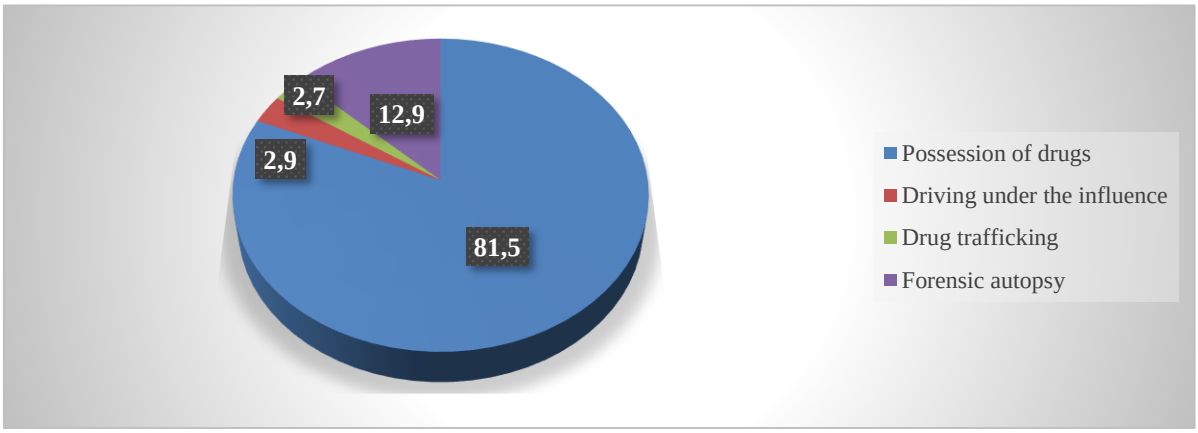


Figure 8. Reasons for investigation

We used HPLC-DAD system for screening and supercritical fluid chromatography coupled to tandem mass spectrometry (SFC-MS/MS) for confirmatory examination. Between 2010 and 2019, a total of 72 new stimulants (Table 4.) were analysed by the methods.

2C-E	4-CMC	5-BPDI	eutylone	N-butylpentylone
2C-TFM	4-EMC	5-DBFPV	isopropylphenidate	N-ethylbuphedrone
2-MPA	4-fluoro-PHP	alpha-PBP	MDPHP	N-ethylheptedrone
3,4-CTMP	4-FMC	alpha-PHP	MDPV	N-ethyl-hexedrone
3-CMC	4F-pentedrone	alpha-POP	mephedrone	N-ethylpentedrone
3F-phenmetrazine	4-FA	alpha-PPP	MDMA	N-ethylpentylone
3,4-dimethoxy-α-PHP	4-FEA	alpha-PVP	MDPHP	NM-2AI
3-Chloroephedrine	4F-PV9	alpha-PVT	metamphetamine	pentedrone
3-MMC	4-MA	amphetamine	methamnetamine	pentylone
4,4'-dimethylaminorex	4-MC	AMT	methoxetamine	propylphenidate
4-BMC	4-MEC	BMDP	methyl-MMDA-2	PV8 (alpha-PHPP)
4-CDC	4-methyl-pentedrone	buphedrone	methylone	TH-PVP
4-CEC	4-methylbuphedrone	butyl-hexedrone	mexedrone	
4-chloro-α-PVP	4-methyl-N,N-DMC	dibutylone	MMMP	
4-Cl-PPP	4-methyl-TMP	dipentylone	MPHP	

Table 4. The analysed new substances

4.6.1. Drug frequency

During the ten-year period between 2010 and 2019, we found new stimulants in 973 (21.1%) cases. Regarding their chemical structure, synthetic cathinones were the most popular ones (98.66%), followed by phenethylamines (0.83%), and finally a tryptamine derivative (0.51%).

4.6.2. The annual distributions of the new and conventional psychoactive stimulants

In the examined time period, we could identify stimulating substances in 1631 cases in the samples. Conventional stimulants were detected in 40% of the samples (n=658), the others were new psychoactive stimulants (n=973). We listed amphetamine, methamphetamine and MDMA as conventional drugs. (Figure 9.).

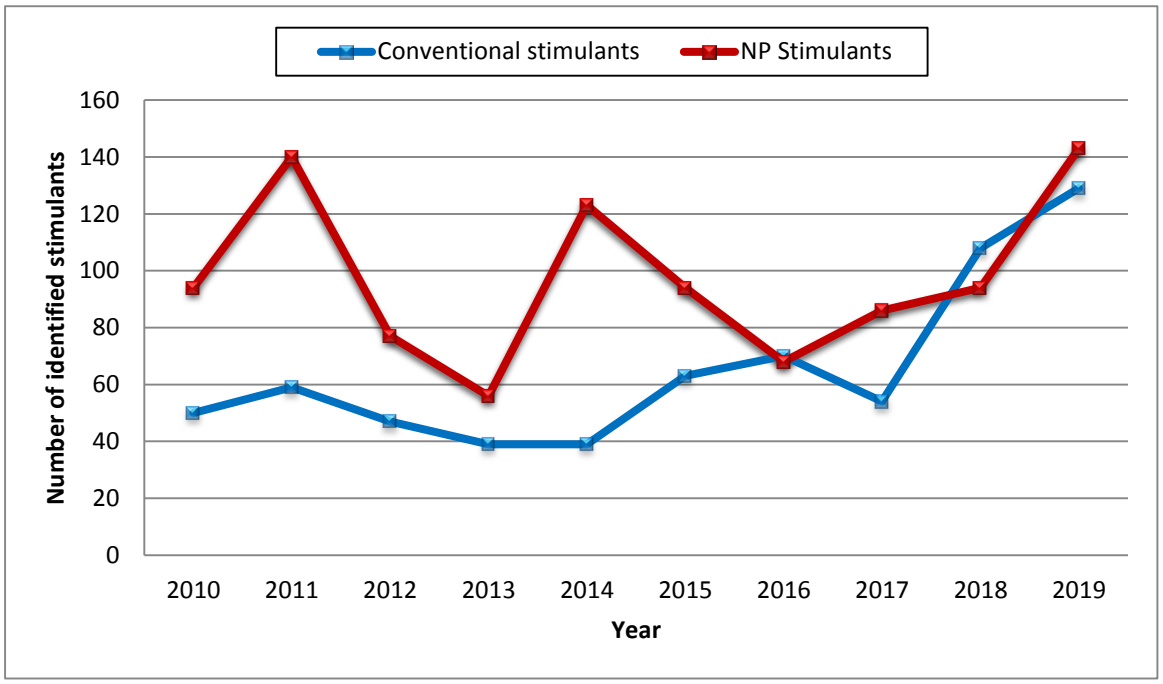


Figure 9. The annual division of the new and conventional psychoactive stimulants in samples.

4.6.3. The annual distributions of the most common new psychoactive stimulants and the conventional stimulants

The main reason for the appearance and then disappearance of new psychoactive stimulants is the change in their legal state. The most common new psychoactive stimulants in 2010 was mephedrone, in 2011 were mephedrone and MDPV, in 2012 was pentedrone, in 2013 was

pentedrone again. According to Hungarian law, from 01 January 2011, mephedrone, from 01 January 2012 MDPV, from 01 January 2015 alpha-PVP and pentedrone have been defined as illicit drugs in the criminal law. N-ethylpentylone, N-ethylhexedrone, 4-methyl-N-ethyl-norpentedrone and 4-Cl-PVP, which appeared in 2016 and showed an increasing trend in 2017, have been defined as 'New Psychoactive Substances'. In 2019 mephedrone returned to the Hungarian illicit drug market again and in many cases, we could detect 4-CMC and N-ethylheptedrone as new psychoactive stimulants. Out of the traditional stimulants, the number of MDMA usages has continuously increased (Figure 10.).

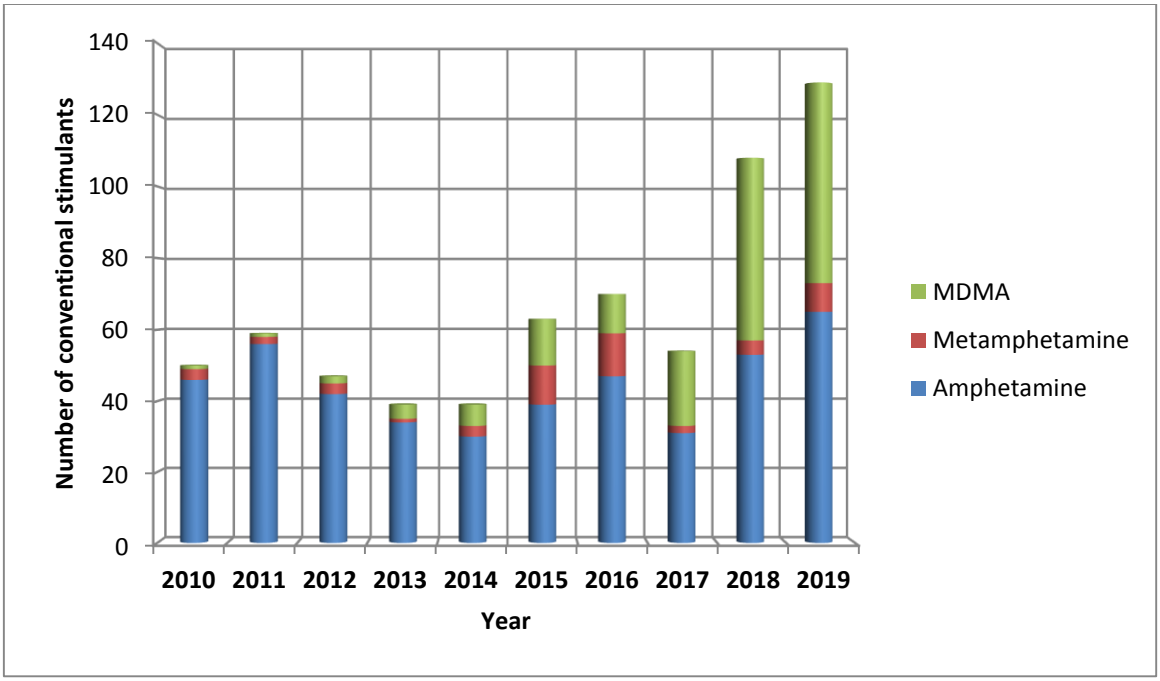


Figure 10. Annual division of the conventional stimulants in samples (n=658).

4.6.4. New psychoactive stimulants in post-mortem cases

12.9% (594) of all cases were post-mortem analyses. We found new psychoactive stimulants in eight cases of them. In one case, the drug was the primary cause of death, in the other cases drugs caused indirect deaths. The causes of the indirect deaths were the following: fatal hangings (2), carbon monoxide poisoning (2), drowning (1), circulatory failure (1), hypothermia (1), and fatal intoxication (1). The most prevalent new stimulant was pentedrone (with alcohol in one case, and with amphetamine and MDMA in two cases), followed by mephedrone (alone in one, with amphetamine in another case), N-ethylhexedrone (alone in two cases) and 4,4'-dimethylaminorex (alone in one case-fatal intoxication).

4.6.5. New psychoactive stimulants and other drugs

In many of our cases, we revealed the presence of more than one new psychoactive stimulant, or the presence of new stimulants together with conventional drugs, like amphetamines, cannabinoids, cocaine, and benzodiazepines. One new stimulant was the sole agent in 65% of all the positive cases (973), and two or more new stimulants were present in 20% of cases. One new psychoactive stimulant with conventional stimulants were detected in 11%, two or more new stimulants with conventional stimulants were present in 4% of the cases. Benzodiazepines (alprazolam or clonazepam) were found in 135 samples (13.8%), THC or THC-COOH in 141 samples (14.5%), and cocaine in 17 samples (1.74%) in addition to new psychoactive stimulants.

4.7. Novel findings of the study, conclusions:

In the dissertation we presented the results of the initial research with the new substances and the conclusions that can be drawn:

1. we identified the active ingredients from a number of unknown materials, but the quantitative analysis is not possible without knowledge of the pure active substance content, so the lack of reference standards is still a problem, especially for clinical toxicology laboratories,
2. in the study of cross-reactions, we concluded that immunochemical tests developed for conventional drugs (neither immunoassays nor rapid tests) are not suitable for the detection of new psychoactive substances,
3. in the clinical toxicology practice the detection of metabolites of indole and indazole-based SCRA compounds in urine by HPLC-DAD system *confirms* the fact of consumption,
4. serum catecholamines levels of new psychoactive stimulant users showed significant differences in adrenaline and dopamine concentrations compared to the control group,
5. the in vitro stability study we concluded that conventional stimulants (amphetamine, MDMA) in urine samples were stable at room temperature storage, in contrast to the tested new psychoactive substances, where degradation was significant,

6. changes in drug trends entail changes in drug use patterns, what we supported by regional data by analyzing the prevalence of new and traditional stimulants in biological samples over the past ten years.

List of publication

Articles related to this thesis

1. Lajtai Anikó, Mayer Matyas, Lakatos Agnes, Kuzma Mónika, Miseta Attila. (2020). New psychoactive versus conventional stimulants - a ten-year review of casework in Hungary. Legal Medicine. 47. 101780. 10.1016/j.legalmed.2020.101780. **IF: 1,195**

2. Mayer Matyas, Benkő, Andras, Huszar Andras, Sipos Katalin, Lajtai Anikó, Lakatos Agnes, Porpáczy, Zoltán. (2012). Simultaneous determination of 4-substituted cathinones (4-MMC, 4-MEC and 4-FMC) in human urine by HPLC-DAD. Journal of chromatographic science. 51. 10.1093/chromsci/bms183. **IF: 1,026**

3. David Hesszenberger, Aniko Lajtai, Matyas Mayer, Agnes Lakatos, Attila Miseta. (2020) The In Vitro Stability of Four New Psychoactive Substances in Urine Samples Stored At Different Temperatures. J Forensic Toxicol Pharmacol 2020, 9:2 doi: 10.37532/jftp.2020.9(1).167.

Articles not related to this thesis

1. Lajtai Anikó, Mayer Mátyás, Lakatos Ágnes, Porpáczy Zoltán, Miseta Attila. Embutramide, a component of Tanax® (T-61) as a new drug of abuse? J. Forensic Sci, March 2016, Vol. 61, No. 2. doi: 10.1111/1556-4029.13010. **IF: 1,127**

2. Lajtai Anikó, Lakatos Ágnes, Kuzma Mónika dr., Mayer Mátyás dr., Miseta Attila dr.. (2020) A paracetamolterápia és az idült májbetegség tragikus következményei. Orv Hetil. 2020; 161(40): 1720–1723. doi: 10.1556/650.2020.31752. **IF: 0,497**

3. Nagy C, Bajzik G, Skobrak A, Csorba E, Lajtai A, Balogh G, Nagy F, Vajda Z. Gyermekkori sporadikus hemiplegiás migrén arteria cerebri media hipoperfúzióval [Childhood sporadic type of hemiplegic migraine with arteria cerebri media hypoperfusion]. Ideggyogy Sz. 2017;70(9-10):343-348. doi:10.18071/isz.70.0343

4. Pogáts Rebeka, Péter István, Laufer Zsófia, Lajtai Anikó, Hollódy Katalin. Benzodiazepin-intoxikáció gyors diagnózisa elektroenkefalográfia segítségével. GYERMEKGYÓGYÁSZAT 70: 6 pp. 434-438., 5 p. (2019)

5. Gábor Simon, M.D.; Veronika Heckmann, M.D.; Anikó Lajtai; Mátyás Mayer, PharmD; Mónika Kuzma, PharmD, PhD. (2020) Suicide by potassium infusion and review of the literature. Forensic Science Medicine and Pathology. (Under review) **IF: 1,61**

Summary of IFs: 5,455.

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