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Detection of psychotropic substances in the blood by LC/MS/MS

Summary

Year on year, one can observe an increase in the use of addictive substances. This leads to occurring the problem of addiction as well as the use of psychoactive substances as a serious hazard to road users. The Regulation of the Minister of Health on agents acting similarly to alcohol and the conditions and manner of conducting research on their presence in the human body, requires adequate benchmarks for performing these tests. An important factor, from consultative point of view, is the knowledge of the chemical structure of substances belonging to different groups of drugs of abuse, their metabolic transformations that occur in the body as well as their influence on the body. This is to aid in the proper interpretation of the results of the analytical tests.

Keywords psychoactive substances, chromatography, mass spectrometry, identification

Introduction

The phenomenon of the use of addictive substances is, from year to year, more and more common. Taking narcotic substances can result in poisoning, including chronic poisoning. After a certain time, the use of psychoactive substances causes addiction of a physical and mental nature, characterized by, among others, the development of the occurrence of tolerance, increasing the doses of the substance, ineffective attempts to reduce the dose or discontinuing treatment and the occurrence of withdrawal symptoms. The problem of taking psychoactive agents poses a major threat to road users. Under the Criminal Code (art. 178a), it is prohibited to drive under the influence of a narcotic substance [1].

Traffic Law [2] and the Code of Offences [3] prohibit driving in the state after the use of an agent acting similarly to alcohol. It should also be noted that the Supreme Court, by a resolution dated February 27, 2007, ruled on the interpretation of the term "narcotic substance" within the meaning of Art. 178a of the Penal Code and the Act of 29 July 2005 on counteracting drug addiction (Journal of Laws no. 179 item 1485). According to the resolution, the concept of a narcotic substance includes not only drugs mentioned in the Act, but also other substances of natural or synthetic acting on the central nervous system, including psychotropic drugs, the use of

which reduces the efficiency in terms of driving. The Regulation of the Minister of Health on agents acting similarly to alcohol and the conditions and manner of testing on their presence in the human body requires adequate benchmarks for performing such tests. [4]. It is necessary to comply with the relevant analytical requirements that allow the determination of psychoactive substances in biological material in the relevant concentration limits. According to the above-mentioned regulation, in the blood sample, 5 groups of agents acting similarly to alcohol are drawn:

- morphine – which sets the limit of quantification (LOQ) of 20 ng/ml
- amphetamine and its analogues, including methylenedioxy – LOQ of 50 ng/ml
- cocaine and its metabolite benzoylecgonine – LOQ of 50 ng/ml
- delta-9-tetrahydrocannabinol – LOQ of 2 ng/ml
- benzodiazepine [4].

It is very important to harmonize the provisions relating to the interpretation of toxicology analysis of the tested biological material, including blood samples taken from the drivers on the presence of agents acting similarly to alcohol in relation to the states of – *after using* and *under the influence* of these agents. The basis to put forward the proposals to harmonize these provisions was Polish participation in the largest scientific research program of the European Union in the field of road traffic safety, which was the program

DRUID – *Driving under the Influence of Drugs, Alcohol and Medicines*, which ended in 2011. The program was attended by 19 member states, 37 different institutions and more than 200 experts. The main objectives of the program were the following:

- diagnosis of the problem of driving after consuming alcohol, drugs and some medications,
- assessment of the risks associated with the emergence of these substances in the road traffic,
- setting thresholds for different psychoactive substances, above which driving should be banned,
- evaluation and selection of the best methods for the detection of psychoactive substances in the drivers' systems,
- determining the best action strategy for the drivers who were arrested for driving after taking the banned psychoactive substance.

The purpose of the DRUID research in Poland was to determine the prevalence scale of alcohol, medication and drug use in the population of drivers, with reference to other countries participating in the DRUID [5]. Taking into account the results of the DRUID, the consultative practice and analysis limits being in use in 11 European countries, in November 2012, in Krakow, a team of analysts, doctors and lawyers established the Polish analyses limits of thresholds for the state *after using* and *under the influence* of agents *acting* similarly to alcohol, for the needs of expressing opinions for judicial purposes. The values were published in the conference proceedings of the Conference of Forensic Toxicologists XXX [6] (see Tab. 1).

The important factor, in terms of consultative purposes, responsible for correct interpretation of the results of the analytical tests, is also the knowledge of the chemical structure of substances belonging to different groups of addictive substances, their metabolic processes occurring in the system and their actions on the body. The first part of the article deals with these issues. The second one, however, describes the analytical method developed in the Department

of Anti-Doping Research (ZBA) of Institute of Sport, used to analyse blood samples for the presence of drugs and stimulants. The method, based on high performance liquid chromatography coupled with tandem mass spectrometry allows the identification of nearly 30 substances.

Characteristics of the different groups of intoxicating substances

Opiates

Opium comprises about 20 alkaloids belonging to two groups, such as:

- phenanthrene alkaloids (morphine, codeine, thebaine),
- isoquinoline alkaloids (papaverine, narcotine [noscapine], narceine) [7].

Among the most important of these alkaloids are morphine, codeine, and thebaine, which has no narcotic effect, but is used to produce other psychoactive compounds [7].

People who are addicted take opiates by inhalation, smoking, subcutaneous and intravenous injections. The visible symptoms of their impact include the following:

- indifference, sleepiness, slowed movements
- motor disorders
- disturbance of consciousness (euphoria) called high (3–6 hours).
- the occurrence of seizures
- frequent licking of lips (dry mouth)
- extremely constricted pupils (pinpoint)
- drooping eyelids
- breathing disorders
- slurred speech.

Characteristics of morphine

Morphine is considered the main psychoactive component of opium. It induces the occurrence of tolerance and mental and physical addiction. It increases the effect of other substances acting as depressants on the central nervous system, such as sedatives, hypnotics, psychotropic medication and alcohol. The effect of morphine as a strong analgesic increases with the increase of dose. The duration of analgesic effect of morphine is different due to the manner of their administration so after oral administration it takes 4–6 hours, after subcutaneous and rectal administration – about 4 hours, and from 24 to 48 hours after the intrathecal one.[8] Morphine is present in the body in free and conjugated forms. In human urine it is identified in 10% in the free form and 75% in the form of morphine-3-glucuronide, of elimination half-life period up to 4 hours. The remainder is 6-glucuronide, 3-ether-sulfate and morphine 3,6-diglucuronide. These metabolites are

Table 1
Proposals for analysis limits and thresholds for the state after using and under the influence of a given substance

Substance	Limits analytic [ng/ml]	The value of the threshold concentration [ng/ml] for the state	
		after using	under
THC	1	1–2.5	≥ 2.5
Amphetamines and derivatives	25	25–50	≥ 50
Cocaine	10	10–20	≥ 20
Benzoyllecgonine	100	> 100	not established
Morphine	10	10–25	> 25

also present in the blood and plasma [7]. The lethal dose is 120–250 mg – orally, and about 70 mg in case of intravenous injection [8].

Characteristics of heroin

Heroin is more than twice stronger painkiller than morphine. The effect is much faster, but more short-lived than it is in the case of morphine, due to the better lipid solubility and easier passing through the blood-brain barrier. Heroin is also present as a mixture with other drugs, such as *speedball* (fast ball), or a mixture of heroin with stimulants – cocaine or amphetamines.

Heroin in the body is metabolised rapidly, and its half-life takes only 2–3 minutes. The metabolism of heroin includes the following:

- two-stage deacetylation to 6-acetylmorphine and morphine
- N-demethylation to normorphine (*morphine-like activity, but weaker*)
- conjugation with glucuronide and sulfuric acid.

The major metabolites of heroin occurring in urine for 20–40 hours after intravenous administration are: 3-O-glucuronide morphine (38.2% of the dose), 6-O-monoacetylmorphine (1.3%), free morphine (4.2%) and heroin unchanged (0.1%). Other glucuronides of morphine and normorphine are obtained from heroin in small amounts [7]. After taking heroin, codeine often also occurs in the urine. It is not a heroin metabolite but is formed as a result of deacetylation of acetylcodeine, which is a contaminant of heroin produced illegally. The use of heroin may attest to the presence of 6-O-monoacetylmorphine (MAM) in urine; however, there is a chance of detecting this metabolite only within 2–8 hours after taking heroin, as it is converted to free morphine very quickly. In the case of the administration of heroin, the intravenous dose for a novice is 5–10 mg, and for strong addiction it increases by 20–60 mg; a lethal dose is 70 mg when administered intravenously and 200–500 mg when taking orally [8].

Characteristics of codeine

Codeine (methyilmorphine) is a natural alkaloid found in opium. It is used as an antitussive and in analgesic compounds (which indicate 20% of analgesic activity of morphine). As a medicine, codeine is administered orally and, as a component of “Polish heroin”, intravenously. It is well absorbed from the gastrointestinal tract. After intramuscular administration, it reaches peak concentration in blood after 15–60 minutes, and in the case of oral administration, approximately after 1–2 hours. Codeine is biotransformed to morphine (O-demethylation) and norcodeine (N-demethylation). Codeine and its metabolites are glucuronide conjugation.

Amphetamine, methamphetamine and their analogues: MDMA, MDEA, MDA

Characteristics of amphetamine

Amphetamine is a racemic mixture of two stereoisomers: a clockwise one, called D-amphetamine and a counterclockwise – L-amphetamine, which have different physiological effects. It is found in the form of tablets, capsules, pills, and white, yellow, pink or brown powder, depending on the type of impurities and counterfeits. It is taken orally, intranasally, intravenously and subcutaneously.

The symptoms of taking amphetamine include the following:

- motor anxiety and strong stimulation,
- talkativeness, nervousness, irritability,
- euphoria, but also mood swings,
- excessive self-confidence or unfounded fear,
- objective inability to concentrate (racing thoughts),
- inability to think logically,
- dilated pupils, showing no reaction to light,
- rapid breathing,
- sometimes gnashing of teeth, lockjaw,
- flushing,
- tremor, fatigue due to insomnia,
- significant decrease in weight (*with prolonged use*).

Amphetamine is easily and quickly absorbed from the gastrointestinal tract and readily penetrates through the placental barrier. Its biological half-life is from 4 to 6 hours [9]. It is taken in doses of 5–15 mg/day by the beginner recipients, and from 100–2000 mg/day by individuals chronically using it. Amphetamine starts working after 20 minutes after administering it orally and its effect lasts for 2–3 hours. The peak plasma concentration is usually reached within 4 hours after ingestion [10]. Not more than 20% of amphetamine binds to serum proteins [10], and is present in the urine within 20 minutes after ingestion. 20–30% of the amphetamine dose is excreted unchanged, and the remainder as phenylacetone, hippuric acid, benzoic acid – the products of deamination, hydroxylated metabolites – 4-hydroxyamphetamine and in conjugated form (25% of dose). 4-hydroxyamphetamine is further metabolised into 4-hydroxyephedrine [11].

The excretion rate of amphetamine and the size of non-metabolised fraction varies depending on the pH of urine. In the case of proper urine acidity (pH 5–7), it is excreted about 30% of the dose per day, if the urine is acidic, the excretion increases to about 78%, and when alkaline, it decreases to about 45%. Amphetamine oral dose at 10 mg corresponds to the concentration in the blood of 35 ng/ml, 25 mg – 41 ng/ml and 30 mg is equivalent to 111 ng/ml [8].

It should also be noted that amphetamine may arise in the body due to metabolism of some compounds belonging to prescribed drugs such as selegiline and also, not available in Poland, e.g. mefenorex or fenproporex.

Characteristics of methamphetamine

Methamphetamine is found in the form of hydrochloride which is a white powder of bitter taste, easily soluble in water. It is also available in the form of tablets. This drug is taken orally, intravenously, inhaled through the nose, or can be smoked. Smokable methamphetamine hydrochloride is found in the form of transparent, shiny crystals resembling ice (variety of *ice*). The efficiency of methamphetamine depends on the manner of ingestion. Immediately after the intravenous ingestion or smoking, intense stimulation comes, the so-called *rush* or *flash*. On the other hand, pulling through the nose or swallowing produces weaker euphoria, so called *high* which occurs 3–5 minutes after oral administration, and after 15–20 minutes after nasal ingestion. The initial symptoms of using the drug cause such reactions as stimulation, rapid breathing, decreased appetite, increased body temperature. In time, there are chest pains, hypertension, seizures, and damage to the small vessels of the brain, leading to a stroke. Methamphetamine abusers behave aggressively, suffer from insomnia, and feel constant anxiety. The use of the substance causes strong addiction.

Biological half-life of methamphetamine is 10 hours, for all manners of administration. About 44% of the dose is excreted in the non-metabolised form. From 6 to 20% of the dose is N-demethylated to amphetamine, and about 10% hydroxylated to 4-hydroxyamphetamine. Also in the case of this substance, pH of the urine affects the rate and the percentage of the excreted substances from the body [11].

Psychoactive structural analogues of amphetamine and methamphetamine were created by the introduction of a cyclic dioxymethylene system consisting of two atoms of oxygen and a methylene group. This results in derivatives such as 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA) and 3,4-methylenedioxyamphetamine (MDA). In 1986, Nichols introduced the special term “entactogens” in order to identify these compounds. This term derives from the Greek word *endon* (inside) and Latin *generated* (born) and *tactus* (action), which means “action from the inside” [12, 13].

Characteristics of MDMA

MDMA is a synthetic analogue of methamphetamine and mescaline of stimulant properties as well as hallucinogenic ones, as it is the case of mescaline. It is most frequent in the form of hydrochloride. It is mainly taken orally in the form of white powder, and also multi-coloured tablets, capsules with embossed characters, symbols and inscriptions. The tablets and capsules may additionally include amphetamine, methamphetamine, cocaine, and acetaminophen. The main symptoms of taking MDMA are: euphoria, severe

agitation, loss of appetite, jaw clenching, dilated pupils, sudden increase of blood pressure, visual hallucinations, and attacks of panic and outbursts of aggression. MDMA is well absorbed from the gastrointestinal tract and easily penetrates the blood-brain barrier. A large part of the drug is excreted in the urine in unchanged form. The main pathways of metabolism of this substance are the following:

N-demethylation, O-demethylation, deamination, O-allylation [14].

The main MDMA metabolite is 4-hydroxy-3-metoxymethamphetamine (HMMA). As a result of N-demethylation, MDA is formed when a major metabolite is 4-hydroxy-3-methoxyamphetamine (HMA). Other metabolites are present in smaller quantities.

Dihydroxy metamphetamine (HHMA) and dihydroxy amphetamine (HHA) cannot be detected in plasma, because the substances are unstable and are rapidly converted to HMA and HMMA. The pH of the urine influences the rate of excretion of MDMA from the body. 70% of the dose is excreted unchanged when the pH of the urine is lower than 5 [8]. The effective dose of the drug is from 75 to 200 mg (1–2 tablets), and the effects usually appear after 30 minutes after ingestion and they increase within one hour. The effects of the drug disappear after about 4–6 hours. The MDMA oral dose of 50 mg corresponds to concentration in the blood of 106 ng/ml, 70 mg – 41 ng/ml, and 100 mg is equivalent to 180 ng/ml [8].

Characteristics of MDA

MDA is a synthetic drug, which in the sixties of the twentieth century gained great popularity among the so-called hippies as the *mellow drug of America* or *love drug*.

It is found as the hydrochloride in the form of powder, tablets or capsules. The effective dose of the drug is between 80 and 160 mg, and its action lasts for 6 to 10 hours. MDA produces stronger psychedelic and hallucinogenic effects than MDMA. MDA ingestion is followed by orientation disorder; obsessive thoughts appear, and strong inner anxiety is felt [9, 11, 15, 16].

Characteristics of MDEA

MDEA is found as the hydrochloride in tablets and pills and like MDA, it is added to ecstasy tablets. The average dose for an average MDEA recipient is about 150 mg and running from 3 to 5 hours. It causes symptoms similar to MDMA, but in contrast to MDMA it does not cause increased activity, but rather has a calming effect. After ingesting, symptoms such as hallucinations, paranoia, and madness appear. MDEA is excreted from the body mainly in unchanged form. Discussing structural analogues of amphetamine and methamphetamine, one should pay special attention to two psychoactive compounds: PMA – paramethoxyamphetamine and

PMMA – paramethoxymetamphetamine. PMMA is characterized by toxicity and its effects are similar to those of MDMA, but it has weaker stimulative effect. PMA is found most often in the form of tablets that look like *ecstasy*, which contains MDMA in its composition. PMA indicates much more toxic effect than amphetamine and it is characterized by a small difference between the dose inducing effects expected by the recipient and the lethal dose. This all makes the presence of PMA in tablets instead of MDMA can lead to accidental poisonings. PMA causes severe psychomotor agitation, significant pulse acceleration, lockjaw, convulsions, muscle spasms, sweating, and cardiac arrest. This drug also has hallucinogenic effects, more than five times stronger than mescaline [9].

Cocaine and its metabolites

Cocaine is the ester of ecgonine and methyl alcohol, as well as benzoic acid and it belongs to tropane alkaloids derived from tropane which is the product obtained by condensation of piperidine and pyrrolidine rings. On the market the drug is available in the form of cocaine hydrochloride (“coke”, “charlie”) and a crystalline form of the freebase cocaine as, so called, crack cocaine (*crack*) [17]. Cocaine hydrochloride is a white crystalline substance of characteristic bitter taste, which, in the melting temperature (about 197°C), decomposes. Cocaine base, like hydrochloride, also has the form of a white crystalline powder, with a melting point is slightly lower (96–98°C). Considering that at higher temperatures it evaporates without degradation, there is the possibility of burning it in a pipe or as a cigarette. Cocaine formulations sold on the open market are counterfeited by various substances, such as phenacetin, lignocaine, benzocaine, procaine, caffeine, acetaminophen (*paracetamol*), sugars, and atropine [18].

Cocaine may be taken by various routes: through the nose (“*sniffing*”, “*snorting*”) or as intravenous injections and intramuscular injections. Also, it is administered orally, sublingually, vaginally, rectally. Visible signs of cocaine use include the following:

- red nose, often with pustules or symptoms of eczema,
- dilated pupils,
- pale face,
- low body weight, general weakness,
- talkativeness, racing thoughts,
- frequent dizziness and vomiting.

Cocaine causes very strong psychological dependence [19]. In large doses, it acts as depressant on the central nervous system, which can lead to the respiratory and circulatory systems arrest [20]. Cocaine in the body is subject to many changes that lead to the creation of derivative products. Its average half-life in the body depends on the dose and lasts

20–90 minutes, and in the case of constant use, it can even increase up to 110 hours [21].

Cocaine is extensively metabolised, only 1–5% of the dose of the drug is excreted in the urine as unchanged drug. The size of this fraction increases with increasing acidity of urine. 25–45% of the administered dose of cocaine is hydrolyzed into benzoylecgonine, and the 18–22% into ecgonine methyl ester. Within 3 days, about 70% of the dose is excreted in the urine, and only 4–6% in the faeces [9]. The first step in the metabolic transformation of cocaine is hydrolysis of one or both of the ester groups to pharmacologically inactive metabolites: benzoylecgonine and ecgonine methyl ester, and ecgonine at last [22]. As a result of N-demethylation of cocaine, norcocaine is formed, which indicates pharmacological activity similar to that of cocaine. As for the cocaine addicts who drink alcohol, the enzymatic transesterification of cocaine originates, in the presence of ethyl alcohol, which occurs in the liver under the influence of non-specific carboxylesterases, which results in the production of the ethyl ester of benzoylecgonine or cocaethylene, otherwise called ethylcocaine. In comparison with cocaine it has a longer biological half-life which takes about 2–2.5 hours [9].

The effects of cocaine on the body depend primarily on the way of its administration and the ingested dose. The maximum concentration of cocaine in the blood plasma occurs 50–60 minutes after oral administration, 30–40 minutes after the intranasal one, five minutes after smoking and 2–3 minutes after intravenous injection. The effects of these interactions can be divided into three phases of action [20]:

Phase I: Stimulation, euphoria or “high”: the recipient is in a good mood, talkative, cheerful, euphoric, has reduced feeling of hunger, reduced need for sleep with the accompanying increase in blood pressure and heart rate acceleration. After taking a higher dose, restlessness appears as well as aggressive and violent behaviour, accompanied by headaches, abdominal pains, vomiting, sweating, chills. Due to the rapid metabolism of cocaine the status of Phase I is usually short-termed.

Phase II: prolonged stimulation, so-called exhilaration after cocaine intake: then anxiety and negative perception of the environment can appear.

Phase III: depression: strong feeling of fatigue appears, low mood and desire for taking the next dose.

Delta 9-tetrahydrocannabinol and its metabolites

Cannabinol are compounds of 21 carbon atoms of dibenzopyrane structure occurring only in cannabis. Currently, we know over 60 specimens of cannabinol, since the highest biological activity indicates 9-tetrahydrocannabinol (Δ^9 -THC). In a large number,

cannabinol is found in blooming and fruiting tops and leaves of the plant of *Cannabis* (drug type). Cannabis preparations are ingested mostly by smoking, and less frequently orally. During burning, a part of Δ^9 -THC is decomposed and only 20–70% of the active compound, contained in marijuana, enters into the lungs, wherein its content in the smoke depends on the burning technique.

The symptoms of Δ^9 -THC include the following:

- sweetish odour of breath,
- talkativeness and jocular attitude, euphoria,
- conjunctival redness, and sometimes swelling of eyelids, nystagmus, photophobia,
- drying of the mucous membranes of the mouth, coughing,
- general excitement, psychomotor activity,
- ataxia, dizziness, shakiness,
- memory impairment (short-lived), concentration and attention disorders,
- impaired sense of time, confusion, impaired ability of critical thinking,
- sweating, pale skin [9].

In order to induce feelings of euphoria, occasional marijuana smokers need only 2 to 3 mg of Δ^9 -THC, and with one manually prepared "twist", 2 to 4 beginner smokers [23] can be introduced in a state of intoxication. THC is extensively metabolised in the body [9] and less than 1% of unchanged compound passes into the urine. During smoking marijuana, metabolic processes begin in the lungs, and after oral application, mainly in the liver. Within 72 hours after smoking, nearly 50% of the inhaled THC is excreted as metabolites and the remaining 50% is distributed in different tissues. It is particularly strongly retained by the adipose tissue, from which it is released very slowly, over a number of days. The excretion occurs mainly in the feces (65%) and by the kidneys (25%). THC biological half-life is 20 hours and indicates slow elimination of the compound from the body.

The main metabolic pathway in the biotransformation of Δ^9 -THC is the oxidation of carbon 11 to form 11-hydroxy- Δ^9 -tetrahydrocannabinol (11-OH- Δ^9 -THC), which has an activity similar to Δ^9 -THC. 11-OH- Δ^9 -THC is then converted to the inactive metabolite, 11-nor- Δ^9 -tetrahydrocannabinolacid (THC-COOH), which together with glucuronic acid forms glucuronide. Both THC-COOH and its glucuronide are present in the urine in the largest quantities. The product of oxidation of Δ^9 -THC at carbon 8, which is 8 β -hydroxy- Δ^9 -THC, exhibits approximately 4% of the activity of the parent compound, while 8 α -hydroxy- Δ^9 -THC, differing only in the configuration of the hydroxyl group, actually is lacking in any activity [9].

Smoking marijuana causes a rapid rise of concentration of Δ^9 -THC in plasma, which then is reduced so after 2–3 hours it becomes undetectable, while there is an increase in the concentration of THC-COOH, which can be detected for approximately 6

hours. There is also difference in metabolic profile Δ^9 -THC in blood. It depends largely on the way the compound was ingested. For example, after smoking marijuana cigarettes and after the intravenous ingestion of Δ^9 -THC, the concentration of the inactive metabolite 11-OH- Δ^9 -THC makes up 10–15% concentration of Δ^9 -THC, however after the oral intake – about 50%. It happens due to the result of the, so-called, first pass effect, which affects Δ^9 -tetrahydrocannabinol in the liver before reaching the systemic circulation. Therefore, the active metabolite of Δ^9 -THC: 11-hydroxy- Δ^9 -tetrahydrocannabinol and 8 β -hydroxy- Δ^9 -THC are in a small degree responsible for the effects observed after intravenous and intrapulmonary taking Δ^9 -THC, and can significantly influence these effects after the oral administration.

Describing substances from the group of *cannabis*, should also cannabimimetics be taken into account, the active substances which are part of the, so-called, highs, which use is associated with the risk to health and life. The analysis of the so far identified compounds allows dividing them into groups, accordingly to the chemical structure, i.e. derivatives of the following:

- naphthoylindole (e.g. JWH-015, JWH-018, JWH-073),
- naphthylmethylindole,
- naphthoyl pyrrole,
- naphthoylindole,
- phenylacetylindole (e.g. JWH-250),
- cyclohexylphenol (e.g. CP47, 497),
- dibenzopyran (structural analogues of Δ^9 -THC – HU-210) [6].

The chemical structures of some synthetic cannabinoids are shown in literature [24].

As a result of taking the synthetic cannabinoids, the effects are very similar to those caused by the products put into the body, other than fibrous hemp, such as herbs or resin, commonly known as marijuana and hashish. These include a change of mood and a feeling of bliss, euphoria, hallucinations, sometimes depression, apathy and the presence of delusions. Taking these substances causes an increase in blood pressure, tachycardia, bloodshot eyes, impaired motor coordination and attention, dizziness, drying out of the mucous membranes. Similar effects are the result of the influence of synthetic cannabimimetics on the same receptors, which are influenced by the active ingredient of the cannabis plant, delta-9-tetrahydrocannabinol.

Benzodiazepines

Benzodiazepines are a class of compounds counting nearly 3,000 substances, of which about 50 are used in medicine. The wide range of clinical applications of benzodiazepines is closely related to their broad therapeutic profile, associated with its functions:

- hypnotic (facilitating and deepening sleep) (flunitrazepam, estazolam, midazolam, triazolam, temazepam),
- anxiolytic, treating anxiety disorders (diazepam, medazepam, oxazepam, chlordiazepoxide, halazepam, lorazepam),
- sedative (nordiazepam),
- myorelaxant, reducing the skeletal muscle tone (tetrazepam),
- anticonvulsant (clonazepam).

Benzodiazepines involve a different size of a therapeutic dose, which is for example 1–2 mg of flunitrazepam, alprazolam 1–3 mg, estazolam 2–6 mg, diazepam 5–30 mg, 7–30 mg of temazepam, medazepam 5–25 mg, oxazepam 10–60 mg.

The binding level of benzodiazepines with blood proteins is very high, an average of 70–99%. Therefore, only a small portion of the drug introduced into the body determines the pharmacodynamic effect. Benzodiazepines are also characterized by different values of their biological half-life periods. The following table displays the values of half-lives of selected benzodiazepines and their metabolites – tab. 2.

Benzodiazepines are lipophilic compounds, they easily pass through the blood-brain barrier. Their abuse may lead to the occurrence of different side effects, which include among others, the following:

- drowsiness, slowing down motor functions, visual impairment,
- urinary retention, constipation,
- agitation and aggressive behaviour, tachycardia.

In terms of chemical structure in the classical benzodiazepines, the basic structure is the 1,4-benzodiazepine system, wherein the benzene ring (ring B) is attached to 1,4-diazepine (ring A). The exception is clobazam as derivative of 1,5-benzodiazepine and tofisopam – a derivative of 2,3-benzodiazepine [26].

Benzodiazepines are metabolised primarily in the liver and only in a little degree is excreted unchanged. In the body, the following metabolic processes take place:

- hydroxylation of aliphatic chains and aromatic rings,
- dealkylation (it is usually disconnecting the methyl group),
- reduction of the nitro group,
- acetylation,
- conjugation with glucuronic acid.

The diagram of metabolic transformation of benzodiazepines into diazepam and nordiazepam shows Fig. 1 (Metabolism of benzodiazepines [9]; see Polish version).

In the centre of the metabolic transformation, there is nordiazepam, into which other benzodiazepine drugs are converted, such as diazepam, clonazepam, chlordiazepoxide, Oxazolam, pinazepam, halazepam, prazepam. While nordiazepam is hydroxylated to oxazepam. Because the elimination half-life of

Table 2
Half-lives of selected benzodiazepines and their metabolites by [25]

Benzodiazepine	The parent compound and metabolites	T _{1/2} [h]
Alprazolam	-Alprazolam -α-hydroxyalprazolam	9–30 1–2
Bromazepam	-bromazepam -3-hydroxybromazepam	9–19
Chlordiazepoxide	-chlordiazepoxide -nordazepam -oxazepam	5–30 50–99 5–15
Diazepam	-diazepam -nordazepam -oxazepam	20–50 50–99 5–15
Estazolam	-estazolam -4-hydroxyestazolam	12–18
Flunitrazepam	-flunitrazepam -7aminoflunitrazepam	11–25
Flurazepam	-flurazepam -desalkylflurazepam	2–3 50–100
Ketazolam	-ketazolam -diazepam -nordazepam -oxazepam	1.5 20–90 50–99 5–15
Clobazam	-clobazam -norklobazam	10–50 40
Clonazepam	-clonazepam	10–50
Lorazepam	-lorazepam	8–25
Lormetazepam	-lormetazepam -lorazepam	10 8–25
Midazolam	-midazolam -α-hydroxy-midazolam -4-hydroxy-midazolam	1–5 1 1
Medazepam	-medazepam -diazepam -nordazepam -oxazepam -temazepam -normedazepam	1–2 22–50 50–99 5–15 3–38
Nitrazepam	-nitrazepam	18–38
Nordazepam	-nordazepam -oxazepam	50–99 5–15
Oxazepam	-oxazepam	5–15
Oxazole	-oxazole -nordazepam	50–99
Prazepam	-prazepam -nordazepam -3-hydroxyprazepam -oxazepam	3 50–99 5–15
Temazepam	-temazepam -oxazepam	3–38 5–15
Tetrazepam	-tetrazepam -3-hydroxytetrazepam	13–44
Triazolam	-triazolam -α-hydroxytriazolam -4-hydroxytriazolam	1–4 4 4

nordiazepam is long, the benzodiazepines, which are transformed into it, are considered drugs with a long duration of action.

Description of the analytical method used for the analysis of blood samples for the presence of psychotropic substances

Materials and methods

Biological material

The biological material for the research were control blood samples, that was needed for the development of the method, and intravital blood collected by the relevant judicial authorities in order to determine the presence of drugs.

Reference substances

The following standard substances were used [27]:

- alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4]benzodiazepine)
- bromazepam (7-bromo-5-pyridin-2-yl-1,3-dihydro-1,4-benzodiazepin-2-one)
- chlordiazepoxide (7-chloro-4-hydroxy-N-methyl-5-phenyl-3H-1,4-benzodiazepin-2-imine)
- clonazepam (5-(2-chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one)
- clobazam (7-chloro-1-methyl-5-phenyl-1,5-benzodiazepine-2,4-dione)
- diazepam (7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one)
- estazolam (8-chloro-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4]benzodiazepine)
- lorazepam (7-chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-1,4-benzodiazepin-2-one)
- lormetazepam (7-chloro-5-(2-chlorophenyl)-3-hydroxy-1-methyl-3H-1,4-benzodiazepin-2-one)
- midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine)
- nitrazepam (7-nitro-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one)
- nordiazepam (7-chloro-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one)
- oxazepam (oxazepam, 7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one)
- temazepam (7-chloro-3-hydroxy-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one)
- zaleplon (N-[3-(3-cyanopyrazolo[1,5-a]pyrimidin-7-yl)phenyl]-N-ethylacetamide)
- zopiclone ([6-(5-chloropyridin-2-yl)-5-oxo-7H-pyrrolo[3,4-b]pyrazin-7-yl]4-methylpiperazine-1-carboxylate)
- amphetamine (1-phenylpropan-2-amine)
- metamfetamina (metamphetamine, (2S)-N-methyl-1-phenylpropan-2-amine)
- hydroxyamphetamine (hydroxyamphetamine, 4-(2-aminopropyl)phenol)

- MDMA (1-(1,3-benzodioxol-5-yl)-N-methylpropan-2-amine)
- MDA (4-[(4-aminophenyl)methyl]aniline)
- cocaine (cocaine, methyl (3S,4R)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate)
- morphine (morphine, (4R,4aR,7S,7aR,12bS)-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol)
- LSD ((6aR,9R)-N,N-diethyl-7-methyl-6,6a,8,9-tetrahydro-4H-indolo[4,3-fg]quinoline-9-carboxamide).

The above-mentioned formulae came from the companies Cerilliant, EDQOM, SIGMA and NARL.

- benzoylecgonine (benzoylecgonine, (1R,2R,3S,5S)-3-benzoyloxy-8-methyl-8-azabicyclo [3.2.1]octane-2-carboxylic acid) from "Polfa" S.A.
- Codeine – drug Thiocodin
- Klorazepam – drug Clorannxen
- Medazepam – drug Rudotel
- Zolpidem – drug Zolpic.

Basic chemical reagents

Acetonitrile, 1-chlorobutane, dichloromethane and ethyl acetate pure for analyses (JT Company Baker).

Other auxiliary materials

Used in preparation of the samples for instrumental analysis were an IKA shaker, HAAKE cold trap, MLW JANETZKI centrifuge and an apparatus for the evaporation of the samples under nitrogen.

Blood Sample Preparation

To 1 ml of a blood sample, mefrusid and methyltestosterone were added as an internal standard, a 100 ug/ml concentration of each. For preparation of the samples, the method of double liquid-liquid extraction using 1-chlorobutane and a mixture of dichloromethane and ethyl acetate (70 : 30, v/v) were used. The samples, were mixed, shaken, centrifuged and refrigerated. Then, after the isolation of organic layers, both layers were evaporated under the stream of nitrogen. After evaporation of the eluates, the extracts were dissolved in an acetonitrile mixture: water (4 : 6, v/v), and the prepared sample was added to the column of the LC-MS/MS system.

The instrumental methods used

The liquid chromatography system conjugated with mass spectrometry (LC-MS/MS) was used, taking advantage of *Waters Alliance 2695XC LC/MS Micromass Quattro Micro API apparatus* of the tandem system of quadrupole mass analysers.

Chromatographic Separation

Chromatographic separation was carried out on a Waters Alliance 2695 liquid chromatograph column Restek Allure Biphenyl (100 x 2.1 mm, particle size 3 mm). LC conditions: flow through the column of

0.300 ml/min, column temperature 45°C, 10 ml dosing volume, 15 minutes analysis time. Chromatographic analysis was carried out in the system of gradient mixture of two mobile phases: phase A – water containing 0.5% acetic acid and the phase B – acetonitrile with 0.5% acetic acid. The compounds were separated using the following gradient (relative to the mobile phase A): 0 min 60%, 1 min 60%, 9 min 10%, 10 min 60%, 15 min 60%.

Marking

A Micromass Quattro Micro API mass spectrometer was used equipped with a quadrupole mass analyser

tandem system. The mass detector worked in electron ionization mode ESI + the mode conditions of monitoring MRM reactions and the scanning mode. The temperature of the ion source was 120°C and the desolvation temperature was 300°C. The isolation time of parent ions was 50 msec. The detector worked in accordance with the MS-MS option. Selected parameters of the analytical methods are presented in Table 3

Description of the research results

The presented method, using the LC-MS/MS system, was developed and used for screening analysis of the compounds shown in Tab. 3. The detection limit

Table 3

Selected analytical parameters of the compounds in the LC-MS/MS system

The tested compound	RT (min)	[M+H] ⁺	MRM m/z	The cone voltage (V)	The collisions energy (V)
Alprazolam	5	309.01	205.06	45	40
Bromazepam	2.63	315.9 315.9	182.1 209.07	45	30 25
Chlordiazepoxide	2.20	300.01	227.05	25	25
Clobazam	5.52	301.01	259	35	20
Clonazepam	4.36	315.98	269.99	45	25
Diazepam	6.44	285.07	154.02	45	25
Estazolam	4.41	295	267.03	45	25
Lorazepam	3.49	320.97	274.07	35	20
Lormetazepam	5.42	335.02	288.95	35	20
Nitrazepam	3.81	281.97	236.01	45	25
Temazepam	5.16	301.01	254.98	30	20
Oxazepam	3.30	287.01	240.97	30	20
Midazolam	3.18	326.04	290.99	45	25
Medzepam	3.90	271.07	90.95	35	30
Klorazepam	–	308.94	112.78 136.82	40	15
Zolpidem	2.44	308.98	236.12	40	35
Zaleplon	3.92	306.14	236.07	45	25
Zopiclone	–	389	245	30	25
Nordiazepam	4.48	271.07	140	45	30
MDMA	1.48	194.09	163.01	25	15
MDEA	1.63	208.15	163.04	25	15
MDA	1.38	180.1	163.01	25	15
Amphetamine	1.35	135.95	90.91 118.93	20	20 10
Methamphetamine	1.45	150.07	119.01	20	10
OH-Amphetamine	1.12	151.89	106.74	15	20
Cocaine	2.22	303.93	104.84	30	35
Benzoylecgonine	1.33	290.2	167.97	30	20
Morphine	1.10	286.07	153.01	40	45
Codeine	1.24	300.13	165.12	40	40
LSD	2.26	324.20	208.2 223.22	40	35 25
Methyltestosterone IS	6.77	303.3	97	45	27
Mefrusid IS	2.26	382.97	129.05	25	20

is 100 pg/ml for the compounds from the group of benzodiazepines, 500 pg/ml for amphetamines, cocaine and codeine, 2 ng/ml for morphine, and 5 ng/ml for benzoylecgonine (Fig. 2 The chromatogram of the control sample; see Polish version) shows the example chromatogram of the control sample for the compounds tested in this method.

In the years 2011 and 2012, the Department of Anti-Doping Research Institute of Sport tested 264 blood samples taken from drivers. The samples, due to using the described above method, showed the presence of compounds of the amphetamine group (amphetamine, methamphetamine, MDA, MDMA, hydroxyamphetamine), opiates (morphine, codeine) and cocaine and benzoylecgonine (Fig. 3 Positive test cases of the analysed blood samples of drivers in the ZBA in 2011–2012 (n = 264); see Polish version).

Marked concentration of amphetamine in the test samples ranged 2.7–4300 ng/ml (Fig. 4 Cases of blood samples analysed in the ZBA amphetamine in 2011–2012; see Polish version).

The largest number of cases of the detection of amphetamine, was concerned with samples in which the concentration of this compound exceeded the value of 100 ng/ml.

The reverse situation occurred in the case of samples with cocaine – in two cases, the drug was detected at concentrations above 100 ng/ml (158 ng/ml and 2.8 µg/ml) (Fig. 5 Cases of blood samples analysed in the ZBA in 2011–2012 in which cocaine was detected; see Polish version).

In the tested samples was also found the presence of drugs from the group of benzodiazepine derivatives, as shown in Fig. 6 (Positive samples containing substances from the group of benzodiazepines analysed in the ZBA in 2011–2012; see Polish version).

The most frequently detected substance from the group of benzodiazepines was nordazepam. During the described period, there were 23 cases showing the presence of this substance. In most cases the concentration of nordazepam was within the concentration range from 2–60 ng/ml. In two cases, the marked concentration was greater than 100 ng/ml, and amounted to 137 ng/ml and 438 ng/ml.

For detecting the presence of Δ9-THC, a particular analytical method was applied, taking advantage of the GC-MS/MS system. During this period, there were 42 cases of the presence of Δ9-THC and 110 cases of the presence of THC-COOH.

Fig. 7 (Chromatogram of a blood sample containing psychoactive substances; see Polish version) shows a case of practice, in which a sample of blood test revealed the presence of the following compounds:

- cocaine in concentration of 2 ng/ml,
- benzoylecgonine in concentration of 27 ng/ml,
- zolpidem in concentration of 63 ng/ml,
- midazolam in concentration of 111 ng/ml,
- lorazepam in concentration of 402 ng/ml.

For comparison, the chromatogram of the control sample, wherein the concentrations of the standard substances were at the level of 100 ng/ml (Fig. 8 Chromatograms of the control sample; see Polish version).

Summary

On the basis of the carried-out research, the developed method of identification of psychoactive substances in the blood samples was validated and its usefulness was confirmed in the routine analyses of samples taken from drivers. In the years 2011–2012, the majority of detected compounds in the population of being tested drivers were substances belonging to the group of amphetamine and its derivatives, and the group of benzodiazepine derivatives. It is worth noting that in the case of interpreting the obtained results of the analytical research when issuing opinions for the judicial purposes, the most common problem is to determine whether the substance detected in the blood could affect the driver, to what extent and whether it is possible to determine the time of its ingestion, and reference of this result to the *state after use* and *under the influence* of psychotropic substances. Therefore, when expressing opinions, it is necessary to have knowledge on metabolism of the tested substances in the body, the dose, the concentration depending on the route of administration as well as knowledge of the drugs available on the market which can be metabolised into the controlled substances. Also, the important thing is to have the knowledge about the elapsed time from the event to the taking of the sample, and how long and under what conditions the material was stored for research.

Source

Figs. 1–8: authors

Tabs. 1–3: own elaboration

Translation: Rafał Wierchosławski