UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF

Diagnostics and Public Health

DOCTORAL PROGRAM IN

Nanoscience and advanced technologies

XXXIV (34º) Cycle / 2018

Pharmacometabolomic approach in the investigation of the effects of physiologically active substances using *Danio rerio*

S.S.D MED / 43 MEDICINA LEGALE

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1. Introduction

1.1. Pharmacological investigations: classic models and tests

In the scientific field of drug research and development and organism chemical exposure studies, traditional methods of pharmacological research are used. They are usually based on phenotypic screening of a response to a compound to receive a specific or a predicted effect. The most common animal models and methods are applied to achieve those goals.

The use of animal models allows a comprehensive perspective on underlying pathological disease mechanisms via a variety of visualization approaches and in vivo tests [260]. In addition, animal genetic manipulations, as well as disease modeling, can provide an insight into new drug targets. For instance, genetic alterations were used to cause type 1 diabetes to investigate new therapeutic targets [243]. Currently, transgenic animal lines are used to study various pathologic processes, including immune, neurological and hematologic diseases and tumors [253]. Animal drug research also includes pharmacokinetic (ADME) and pharmacodynamic studies.

At present, animal models are casually involved in lead compounds tests and interrogation of specific chemical activity or toxicity of candidate drugs [136, 223, 315, 316]. These studies include biochemical (e.g., levels of glucose, amino acids, fatty acids, etc.), physiological and morphological (heartbeat frequency, ECG and MRT assays, embryotoxicity, fetotoxicity, teratogenic effect, etc.), behavioral tests (open field test, light/dark test, etc.) and, additionally, determination of effective and lethal doses (ED50 and LD50, respectively) [27, 51].

The use of animals for pharmacological studies is carried out by implementing animal research replacement, reduction, and refinement (3R) measures. According to these principles, researchers attempt to develop new methods and models.

1.2. Metabolomics as a research field: definition, analytical strategies and applications

1.2.1. Metabolomics in systems biology

Metabolomics is the high-throughput study of small endogenous organic molecules (<1500 Da) called metabolites in various biological samples [318]. Complete coverage of endogenous metabolites that can be identified in a cell, bodily fluid, or an entire organism is a metabolome [317]. Metabolome consists of a diverse number of chemical classes (e.g., lipids, amino acids, short-chain peptides, nucleic acids, sugars, alcohols, and organic acids) that are in the process of continuous synthesis and degradation [322]. DNA, RNA, and proteins trigger different pathways via changes in metabolites level. Also, metabolites themselves can play the roles of endpoints, describing an organism's phenotype at a specific time point [322].

Metabolomics studies are highly complex since they capture a wide variety of metabolites related to different pathways, compared to traditional analytical strategies focused on specific endogenous metabolite annotation and its derivatives in a single pathway study [8, 88]. Metabolome content is dependent on a multitude of internal factors such as the genome, physiological factors, age, sex, diet, or current state of gut microbiota and external factors such as environment [167]. Therefore, the metabolome can reflect any changes in the organism's metabolism.

1.2.2. Analytical strategies in metabolomics

Metabolomics studies can be carried out using different analytical platforms, including chromatography based on mass-spectrometry (e.g., high-performance liquid and gas chromatography-mass spectrometry, HPLC-MS and GC-MS) and nuclear magnetic resonance (NMR).

Mass-spectrometry (MS) is utterly different from NMR. In MS analysis, the sample undergoes ionization, then fragmentation and separation based on m/z. Annotation of mass-spectral characteristics (e.g., parent ion m/z and fragmentation spectra) to the compound libraries data can lead to identifying a molecular structure.

Additional chromatographic data such as retention time can also help to define an unknown molecule. Mass-spectrometry is a highly sensitive tool, resulting in even a small amount of a sample being enough to perform an analysis [12, 63, 238]. Since mass-spectrometry is a destructive assay method, analyzed chemicals can't be restored.

NMR detection involves determining the excess absorption or emission of electromagnetic energy of atomic nuclei of the sample. The perturbation of magnetic nuclear spins changes the resonance frequency, especially in 1H atoms, that creates characteristic NMR spectra that correlate with chemical structure level and location of 1H in the molecule. In contrast to mass-spectrometry, NMR analysis doesn't destroy the sample so that it can be analyzed again [319]. MS detection yielded much higher sensitivity than the NMR [321]. Modern NMR and MS analytical platforms can separate, detect, and definite hundreds and thousands of molecules through one analytical cycle [103, 320]. When the analysis is done, peak picking identifies the sample components, and then data analysis is performed.

Metabolomics studies can be categorized into four workflows: 1) untargeted or high-resolution metabolomic profiling; 2) targeted analysis; 3) fluxomics; 4) imaging metabolomics.

Untargeted metabolomic profiling provides broad metabolites coverage (e.g., ~10000 compounds for one analytic cycle). The untargeted metabolomics method is considered inappropriate for specific metabolite quantification; however, it can be used as a first screening approach for biomarker discovery or a hypothesis-generating strategy [28, 246, 323].

Targeted metabolomics studies are shaped towards identifying specific metabolites classes that are present in low concentrations (e.g., 10 to 500 for one analytic cycle). Targeted metabolomic profiling showed promising results in proof-of-concept studies and biomarker validation; also, it is most commonly used [200, 267].

Fluxomics (or fluxomics studies) is a kind of targeted metabolomic analysis that allows performing metabolites quantification via HPLC-MS and NMR methods

in real-time [170]. Fluxomics is used to investigate dynamic underlying metabolic processes in cells and tissues; hence it can provide a detailed understanding of biochemical pathways [44].

Imaging metabolomics is one of the workflows within metabolomics studies that include both in vivo and in vitro evaluation and imaging of endogenous metabolites in tissue samples by applying the NMR method, positron emission tomography (PET), and matrix-activated laser desorption/ionization (MALDI) [117, 296]. Imaging metabolomics is used to study the metabolism of a specific cell, tissue, or organ and to differentiate a patient's tissue in real-time when surgery is performed [130, 296, 301].

1.2.3. Metabolomics applications in systems biology studies

The main feature of metabolomic profile is its sensitivity to either exogenous exposure from the environment or endogenous characteristics of an organism itself (e.g., a physiological condition or a current state of intercellular interaction) [322]. The role of genotype is to predict an organism's possibilities. However, it's the role of the metabolome to reflect the organism's phenotype at the time of sampling [317]. Metabolomics gives an insight into the current state of an organism that can be integrated into several scientific fields, such as fundamental studies in physiology and pathophysiology, nutraceuticals, microbiology, plant studies, and, additionally, drug research and development [30, 140, 168, 305, 306, 318].

At first, metabolomics was used for diagnostic purposes to detect a disease or any other pathological state of an organ [66, 122, 183, 204]. Therefore it was considered an application of chemical analysis in clinical practice [322]. As the capabilities of analytical methods increased, the number of compounds that can be identified simultaneously enlarged. Since biochemical studies can identify a small number of chemicals, metabolomic profiling allows characterizing tens to hundreds of compounds for one assay. Metabolomic methods showed promising results in diagnostic and prognostic biomarker discovery and could provide mechanistic information on associated biochemical processes.

Metabolomic methods were used to investigate development mechanisms and consequences of chronic renal failure that is associated with the development of a large number of other chronic diseases, such as atherosclerosis, cardiovascular diseases, hypertension, stroke, infraction, cachexia, diabetes mellitus, tumor, dementia, gout and anemia [187]. The reason behind these conditions is urinary toxins, i.e., specific compounds that accumulate in blood and tissues due to chronic kidney disease [113, 187]. Several researchers described such endogenous substances, including acrolein, asymmetric dimethylarginine, creatinine, guanidino succinic acid, homocysteine, indoxyl sulfate, uric acid, urea, etc. as toxic chemicals that can cause cardiovascular [113, 288], bone [113] diseases, gout [298], cachexia [264], atherosclerosis [150, 284], stroke and infarction [50], dementia [50], diabetes mellitus [173], hypertension [202] and anemia [84].

Metabolomics application in normal physiology studies is commonly related to endogenous substances with regulatory and physiological activity. These substances have non-peptide structural characteristics, including amino acids, biogenic amines, eicosanoids, organic acids, steroids, and sugars [322].

Since the 80s, there have been studies on amino acids' role in human physiology. It has been proven that amino acids, especially branched-chain ones, play an essential role in insulin secretion [146, 185]; glutamine induces the secretion of glucagon-like peptide-1 [297]; arginine affects the secretion of both insulin and growth hormone [91, 104]. Amino acids derivatives also are important in the human organism. For example, thyroid hormones (thyroxine and triiodothyronine) are critical for carbohydrate, lipid, and protein metabolism [86]. Those findings influenced their role in insulin resistance and carbohydrate, lipid, and protein metabolism [137, 148, 177, 178].

Eicosanoids include prostaglandins, thromboxanes, leukotrienes, lipoxins, and resolvins. They are the polyunsaturated fatty acids metabolism product that can induce or inhibit inflammation and affect cell growth and blood pressure via changes in blood flow [272]. Due to the recent development of metabolomics methods, new

species of eicosanoids and oxylipins are being discovered, some of which carry previously unknown functions [115, 205].

Special attention is now given to organic acids. For instance, it has been established that lactic acid supports tumor growth and suppresses immune response [85]; succinic acid induces IL-1β and TNF-α secretion through specific SUCNR1 receptor activity that is primarily expressed in macrophages; hence it regulates the intensity of immune response [192, 208, 256]; citric acid is known to be responsible for bone structure formation involving osteoblasts [94]. Meanwhile, 2-ketocaproic and retinoic acids are known to induce insulin secretion and cell growth and development [115, 254]; more recently, new studies have unveiled other organic acids' functions.

Metabolomics also found its place in research on human gut microbiota biochemical processes. Since 2016, the complex of microorganisms that live in the digestive tracts of humans should be called microbiome [120]. In correlation with the fact that the human microbiome consists of many various molecules, science focus has shifted to the study of metabolomics of human microbiota. Currently, there were over 100 articles on metabolomic studies of the human microbiome that led to the discovery of 6700 unique metabolites from different chemical classes [166].

Gut microbiota metabolites (e.g., indole acids, tryptophan derivatives) play a considerable role as signal molecules that modulate or regulate several physiological processes. Their functional value was that predominant, so to describe their interaction with other organs, the new term "interaction axis" was implied. There are four "interaction axes" known: gut-brain axis, gut-liver axis, gut-lungs axis, and gut-kidneys axis [166]. It was found that changes in metabolites levels within these axes correlate with autism [118], schizophrenia [270], Parkinson's disease [77], and asthma [295]. It was also found that gut microbiota metabolites can regulate liver [128, 135] and kidney [181, 314] function.

As a result, metabolomics development influenced the successful application of its methods in drug discovery, pharmacological studies, and xenobiotic exposure studies.

1.2.4. The use of metabolomics analysis of biological samples in the investigation of pharmacological effects of physiologically active substances

Recently metabolomic analysis has been applied to study various chemical exposures on the human organism. It is also involved in drug development studies and research on its adverse effects. The application of metabolomics in the drug research pipeline developed a new workflow called pharmacometabolomics.

Pharmacometabolomics is a scientific field that studies drug or other substance mechanisms of action at a molecular level to predict organism's response to their exposure in correlation with patient's phenotype based on metabolomic profiles before and after the exposure [160]—in particular, measuring endogenous metabolites level after xenobiotic exposure provides detailed information on chemical's effect on specific metabolic pathways that can support traditional assays on pharmacodynamic liabilities to understand better main therapeutic and adverse impact of a drug [160, 162].

The pharmacometabolomic approach can be applied to every stage of drug development and candidate selection pipeline. Most of the most common diseases (e.g., cardiovascular diseases, diabetes mellitus, obesity, depression) are associated with metabolic alterations [209, 241]. Moreover, discovering new metabolic pathways results in the investigation of new drug targets.

Recent findings of trimethylamine synthesis from choline and carnitine by gut microbiota [171, 311] in correlation with the fact that trimethylamine is a precursor for trimethylamine oxide (a metabolite causing atherosclerotic lesions) led to the discovery of two potential drug targets against atherosclerosis – flavin-containing monooxygenase-3 and bacterial choline trimethylamine-lyase that are in charge for trimethylamine oxide synthesis [68]. Later, researchers Hazen et al. proposed a potential inhibitor for the enzymes mentioned above – 3,3-dimethyl-1-butanol naturally occurring in olive oil [311]. Then preclinical studies took place, and new enzyme inhibitors were discovered and later approved for medical use [229].

A prime example of pharmacometabolomics application for investigation of disease mechanisms and further target detection would be a recent invention of two

new drugs to treat relapsed acute myeloid leukemia – ivosidenib and enasidenib. Metabolomics application in relapsed myeloid leukemia studies can be found in Dang et al. [96] and Ward et al. [312]. Findings showed that mutated forms of isocitrate dehydrogenase 1 and 2 were present in patients who have that kind of leukemia. That enzyme turned α-ketoglutaric acid into α-hydroxyglutaric acid, which was elevated in patients [250]. Later it was proven that isocitrate dehydrogenase 1 and 2 inhibitors showed promising results in decreasing the level of α-hydroxyglutaric acid and tumor growth in vitro and in vivo studies [325]. Confirmation studies in vitro and in vivo [277] led to the development and later regulatory approval of enasidenib in the United States in 2017 [196, 219].

In terms of clinical studies, pharmacometabolomics can be used in drug metabolism discovery [9, 82, 308], measuring therapeutic response [10, 31, 95], rational dosing prediction [34, 35, 217, 234], and achieving optimal response for therapy [142]. Pharmacometabolomics is a promising tool for establishing a personalized approach to patient care and personalized medicine.

Personalized medicine aims to select a specific therapy considering the individual characteristics of every patient. A prime example of applying the pharmacometabolomic approach to manage disease would be therapy personalization in inborn errors of metabolism. Screening for metabolites related to inborn errors of metabolism is used not only to diagnose a disease but to choose personalized drug therapy and to monitor therapy response [124, 180]. In addition, there are other scientific approaches and initiatives developed for prospective longitudinal studies in large cohorts (over a few thousand participants) to determine their health status and control changes in metabolome to diagnose altered health conditions and evaluate therapy efficiency [83, 111].

Another pharmacometabolomic application in personalized medicine is therapeutic response monitoring. Response to therapy varies greatly depending on the organism's features, and the most common of them is the expression of different cytochrome P450 genes [1, 7, 26, 32, 302]. While pharmacogenomics shows promising results in personalized pharmacotherapy [33, 36],

pharmacometabolomics can provide data to support genomics [17, 18, 161]. Studies on drug metabolism showed that it depends not only on genetic features but also on ethnic origin, age, sex, weight, diet, etc. However, their impact is impossible to predict by genomics data [37, 116, 161]. Hence, Clayton et al. proposed a pharmacometabolomic approach to predict the metabolomic rate and rational dosing of a drug in individual recipients [87].

More recent articles on pharmacometabolomic application provide data to predict individual drug response. For instance, it has been observed that endogenous metabolites levels significantly correlate with therapeutic response to simvastatin [158, 159]. The pharmacometabolomic approach is a promising tool to select a drug therapy in the case of organ transplantation [258], treating Alzheimer's disease [236], personal statins dosing [175], and special drug groups, e.g., antidepressants [335] and other [105, 326]. For example, Kaddurah-Daouk et al. found changes in phosphatidylethanolamine and other lipids concentrations after olanzapine and risperidone 3-week therapy in schizophrenia patients [157]. As discovered by Krause et al. in a recent article, changes in kynurenine pathway metabolites of tryptophan metabolism correlate with therapy response on celecoxib in patients with the major depressive disorder [174].

Metabolomic analysis is also used to efficiently evaluate drug therapy to study the impact of neurotropic substances on endogenous metabolites levels. Kaddurah-Daouk et al. used citalopram to study a depressive state therapy and found that the serotonin level decreases while 5-hydroxytryptophan and 5-hydroxyindole acetic acid levels significantly increase in human plasma [57]. They also observed a change in catecholamines levels, oxidative stress metabolites, and gut microbiota metabolites levels [57]. Moreover, there was a correlation between the change and therapeutic success [255]. A change in indole acids metabolic profile in major depressive disorder patients who underwent ketamine therapy was described in Rotroff et al.

First attempts to benchmark the InnoMed PredTox Consortium performed data provided by traditional analytical methods to "omics" data. Their results

highlighted good translatability between metabolomics data and traditional methods data and its supportive role in existing drug toxicity knowledge [201]. Currently, some clinical studies imply the pharmacometabolomics approach. For example, Burt and Nandal's systematic research reported that 99 clinical drug trials performed in 2004-2015 used pharmacometabolomic analysis [71].

Studies in animal models provided data on abuse substances exposure on neurotransmitters levels. The use of methamphetamine increased dopamine, tryptophan, and phosphatidylcholine levels in the rat brain [203]. Heroin and morphine exposures caused serotonin synthesis disorder and harmed dopaminergic pathways [330, 333].

Pharmacometabolomics can also be integrated into preclinical trials in drug research and pharmacological and adverse effects study activities. Its use in the research on remoxipride (i.e., dopamine D2 receptor antagonist) revealed the correlation between amino acids and biogenic amines levels and pharmacological features on the drug itself; hence it allowed to expand drug-related toxicity knowledge [67].

Safety testing is essential in the drug development pipeline and post-marketing studies [309]. Investigating metabolic pathways related to drug-induced toxicity is a forward-looking tool that overcompensates specific target organ toxicity studies [131, 132] and general toxicity studies [56, 61].

1.3. Pharmacological effects assessments using a new animal model – *Danio rerio*

Research on new biological models to study the pharmacological impact of compounds is still in progress. Apart from the shift of the preclinical studies towards in vitro models, there is also a need for new animal models which allow better translatability, higher throughput, and cost-saving properties [247]. Recently, Danio rerio (or zebrafish) caught researchers' attention.

Danio rerio (zebrafish) is a small freshwater fish belonging to the minnow family Cyprinidae [169]. Danio rerio is used in developmental biology and

pathophysiology studies. Their physiological features were firstly described in 1995, and since then, they have been established as an alternative in vivo model in scientific research [13, 20, 169].

The main advantages of Danio rerio species compared to other models are:

i) their genome and physiology is highly conserved with humans (e.g., brain, intestinal, muscle structure, and immune system) [121, 127, 164, 165];

ii) conservation in pathogenesis (i.e., ~ 80% of genes related to diseases in humans are observed in zebrafish) [261];

iii) zebrafish reach maturity in 3-5 months, and then female zebrafish is capable of bearing ~200 eggs per spawning [65, 76];

iv) embryo development is quick, i.e., blastulation and gastrulation stages last five hours. An embryo is considered fully formed in 24h post-fertilization (hpf) and leaves its chorion and enters a free-feeding stage until 72 hpf [221];

v) zebrafish maintenance doesn't require significant expenses and free space [184].

Danio rerio application in developmental biology studies includes research on the nervous system and angiogenesis. Zebrafish embryos are transparent what allowed studies on gastrulation [249], angiogenesis [81, 149], blood flow [106, 176], and the development of peripheral and central nervous systems [52, 182, 329, 332] to be performed.

Most tissues and organs, except for lungs, prostate, and mammary glands, are present in Danio rerio and conserved with humans [240]. The availability of transgenic lines facilitates observing neurodegenerative disorders [2, 275], blood diseases [55], cardiovascular diseases [269], tumor [190], Parkinson's disease [263] and stress disorders [11, 80].

Many scientists worldwide have investigated the similarities between the fish species Danio rerio and humans. In 2013 Howe et al. sequenced the fish genome and concluded that 70% of its genome, including amino acids sequence coding genes for CYPP450 protein, is either conserved or homologous with humans [143]. Also,

protein composition studies of zebrafish blood plasma and organs revealed a high grade of similarity with mammals and humans, specifically [53, 186, 189].

Zebrafish brain structure was also studied and conserved with mammals [4, 16, 257]. There are medial, dorsal, and lateral pallial portions in the zebrafish brain that are relevant to the amygdala, cerebral cortex, and hippocampus in mammals, respectively [64, 213]. It has been proven that essential enzymes are critical to neurotransmitter synthesis and metabolism, such as tyrosine hydroxylase [75], monoamine oxidase [48], catechol-O-methyltransferase [228], and choline acetyltransferase [214], are also present in zebrafish.

Zebrafish neurotransmitter systems are also conserved with humans. GABAergic, dopaminergic, adrenergic, serotoninergic, cholinergic systems and glycine, glutamate, and aspartate binding sites are present in Danio rerio [141, 228].

Danio rerio species fish is a suitable model for uncovering pharmaceutical substances, cosmetics, nanoparticles, and pesticides [14, 294]. A number of toxicological studies using zebrafish grow exponentially due to the low amount of substance needed for an assay [21, 220], high reproduction rate [300], and good data translatability to mammal models [134]. The most common testing performed in zebrafish is sublethal dose determination (LD50) to study acute toxicity. In Russia, acute toxicity testing in Danio rerio species fish is described in GOST 33774-2016 "Analytical methods for substances that are hazardous to the environment. Acute toxicity in fish embryos". Chemical-induced lethality is measured every 24h for 96h. Lethality is based on the abundance of coagulated embryos, a lack of somites, tail detachment, or a heartbeat and inspected visually [273]. While zebrafish embryos are not considered laboratory animals until six days post-fertilization (dpf), acute toxicity tests in fish embryos are seen as an alternative to adult fish tests since the latter requires an ethical committee approval according to international laws [283]. The use of zebrafish embryos reduced the number of animal models involved in lethality studies and potentially replaced traditional test models.

Fish species Danio rerio is also used to assess morphological changes caused by chemical exposure. Morphological changes include developmental heart and

swim bladder disorders, body structure (e.g., somites, tail or fins), nervous system (e.g., brain, notochord, or eyes) disturbances. These effects can be measured via visual inspection or light microscope [230] and accessible to be differentiated compared to controls. This kind of study is considered a substitute for traditional mammal model testing [226, 313].

Spontaneous and drug-stimulated locomotor activity studies in Danio rerio can reveal chemicals exposure on the central nervous system [19, 38, 179]. Zebrafish have a fully functioning blood-brain barrier [69, 163]; hence behavioral tests in it are available to perform to study presumably neurotoxic substances. For example, spontaneous movements (e.g., speed, longitude, and length while swimming) significantly decrease due to pesticide [156] and industrial chemicals [304] exposure in zebrafish. A change in behavioral activity as a response to visual stimulations (e.g., dark-light task and light stimulus test) can be applied to study pharmaceutical substances [195] and neurotoxins [172] exposure on CNS.

Promising results of traditional methods in investigating substances exposure in Danio rerio species prompted the development of a metabolomic and pharmacometabolomic approach for their implication in chemicals study in zebrafish. The most prominent example of metabolomic approach application is a study of polystyrene microplastic impact on zebrafish. These compounds caused changes in amino acids, including proline, leucine, lysine, threonine, and lipid (linolenic acid and palmitic acid) levels that indicate liver dysfunction [239].

Pharmacometabolomic studies in zebrafish showed promising results in the annotation of pesticides and insecticides as environmental pollutants and hazardous molecules for humans. Studies on bisphenol A [222], chlorpyrifos [144], fipronil [324], flutolanil [291], and isocarbophos [155] showed their significant impact on zebrafish metabolomic profile, proving their cardio-, liver-, brain- and muscle toxic attributes.

Studies of pharmacometabolomic changes in exposure to pharmaceutical substances on fish of Danio rerio species are just beginning to gain popularity. Using the pharmacometabolomic approach, it has been proven that fungicides,

diniconazole, and difenoconazole, induce serious lipids, amino acids, sugars, and nucleotides metabolism disturbances [291, 309]. Antimicrobial agent triclosan caused urea, citric acid, galactose, glucose, stearic acid, proline, phenylalanine, and glutamine changes [111]. Exposure to citalopram, fluoxetine, amitriptyline changes levels of glutamate, tryptophan, acetylcholine, and serotonin in zebrafish brain [97].

Aims and scope of the study

To summarize, it can be concluded that the use of the pharmacometabolomic approach in the investigation of pharmacological effects of physiologically active drugs is a field of great scientific interest. The use of zebrafish as an alternative animal model for this type of the studies may also be very promising.

There are studies in the field of the investigation of the effects of different drugs using zebrafish as a model organism. But there is a lack of studies in the field of pharmacometabolomic investigations using *Danio rerio*. Also, these sporadic studies do not follow the same methodology to form a unified approach in these investigations.

In this regard, an urgent task is to develop the methodical and methodological basis of the pharmacometabolomics approach in the investigations of the pharmacological effects of physiologically active drugs using zebrafish as a model organism.

The aims of this study were:

1. To identify the most relevant metabolic pathways for investigating the pharmacological effects of physiologically active drugs of neurotropic action based on untargeted metabolomics profiling.

2. To develop and validate analytical methods of the quantitative determination of endogenous metabolites of specific biochemical pathways of neurotransmitters metabolism (to develop a targeted metabolic panel).

3. To determine specific quantitative characteristics of the metabolomics profile of zebrafish larvae after the exposure to a pharmacological substance of neurotropic action with a well-studied model of action using targeted metabolomics.

4. To determine specific quantitative characteristics of the metabolomics profile of zebrafish larvae after exposure to a novel psychoactive substance of the cannabimimetic family.

5. To investigate the behavioral changes caused by the novel psychoactive substance to find a correlation between metabolomics and behavioral changes.

2. Materials and methods

2.1. Chemicals and equipment

2.1.1. Pharmacologically active substances

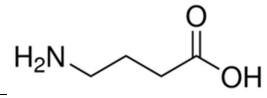
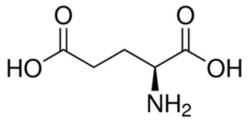
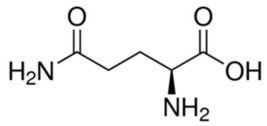
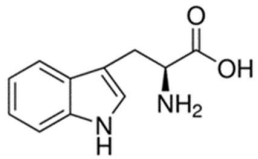
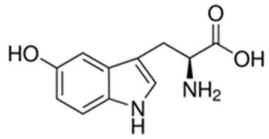
Diazepam (7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepine-2-one) was purchased from Sigma-Aldrich (Darmstadt, Germany). Diazepam is a pharmacologically active substance with a well-defined mechanism of action. Diazepam exposure leads to the allosteric modification of the GABAA receptors, increasing the affinity of the receptors to GABA [5]. Diazepam is used as a "golden standard" in assessing the pharmacological investigations of the anxiolytic action of lead compounds [41, 42]. There are scientific studies that describe the action of diazepam on the different neurotransmitters systems, including the GABAergic system [72, 244], serotonergic system [62, 192, 236], dopaminergic/adrenergic system [273, 281], and cholinergic system [93, 231]. According to this data, diazepam was chosen as a primary compound in investigating the effects of pharmacologically active substances on neurotransmitters metabolomics using zebrafish larvae.

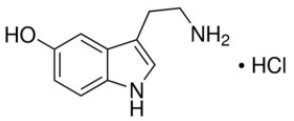
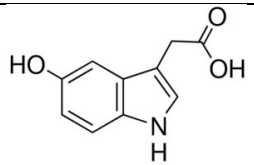
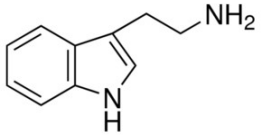
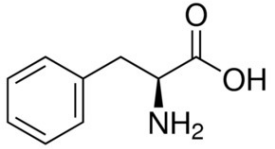
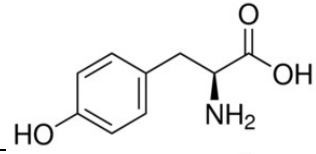
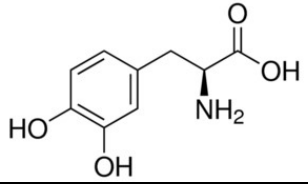
5F-APINAC (adamantane-1-yl-1-(5-fluoropentyl)-1H-indazole-3-carboxylate) was obtained from Cayman Chemicals (Michigan, USA). According to its' chemical structure, 5F-APINAC is a synthetic cannabinoid – a class of pharmacologically active substances whose main mode of action is realized through the affinity to the type 1 and 2 cannabinoid receptors in the central nervous system [49, 150]. The mechanistic reason for their action is the depolarization-induced suppression of inhibition and excitation of neurons, which is realized through the lowering of the presynaptic neurons firing rate [79, 286]. Although there are studies in the field of the effects of cannabimimetics on the different neurotransmitter systems, there were no complex metabolomics studies conducted [58, 98, 100, 107, 118]. 5F-APINAC was chosen as a pharmacologically active substance with a relatively unknown mode of action on the neurotransmitter systems.

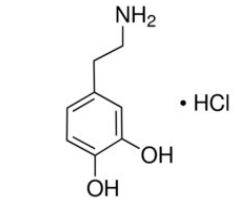
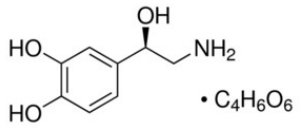
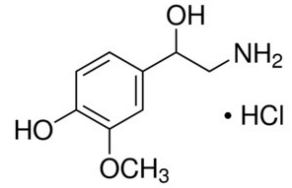
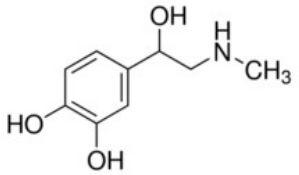
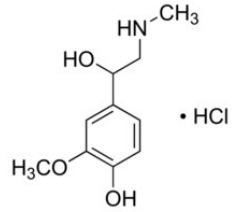
2.1.2. Standard substances

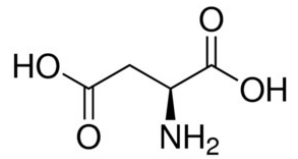
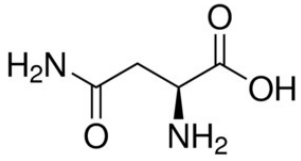
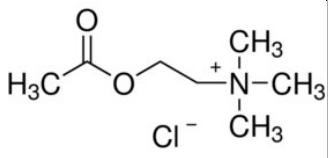
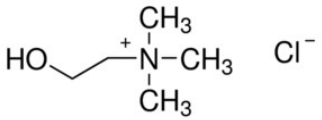
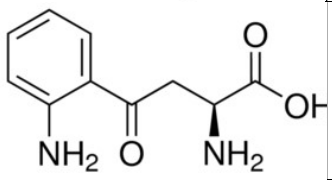
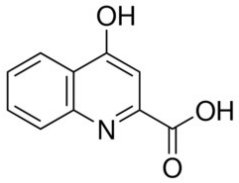
The standard substances of GABAergic, serotonergic, dopaminergic/adrenergic, cholinergic, aspartic acid systems, kynurenine and indole pathways, and other metabolites, connected to neurotransmission, were purchased from Sigma-Aldrich (Darmstadt, Germany) and Honeywell Fluka (Leicestershire, UK). Isotopically-labeled standards were purchased from Sigma-Aldrich and Toronto Research Chemicals (Toronto, Canada). The purity of all standards was >99%. The list of the compounds is presented in Table 1.

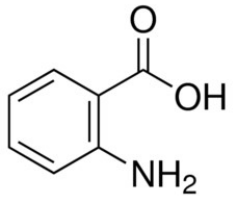
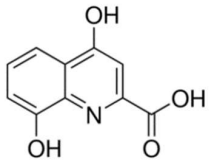
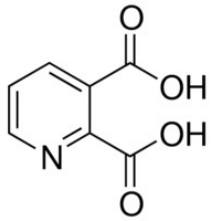
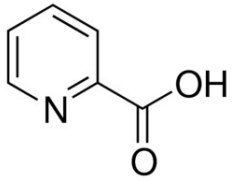
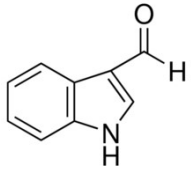
Table 1 – Analytical standards

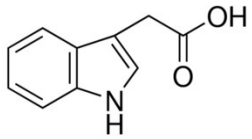
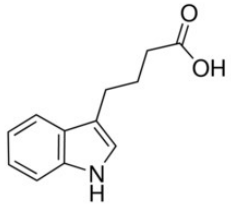
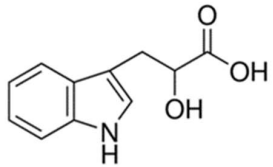
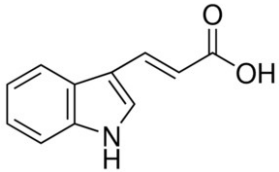
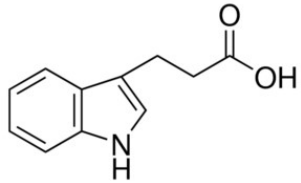
Neurotransmitter system or metabolic pathway	Metabolite	Role	Formula	Molar mass, g/mol	Cas№
GABAergic system	Gamma-aminobutyric acid	Neurotransmitter		103,12	56-12-2
	Glutamic acid	Neurotransmitter, precursor		147,13	56-86-0
	Glutamine	Precursor		146,12	56-85-9
Serotonergic system	Tryptophan	Precursor		204,23	73-22-3
	5-hydroxytryptophan	Precursor		220,22	4350-09-8

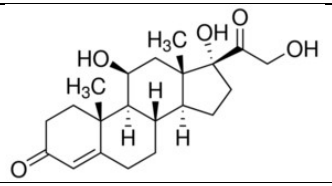
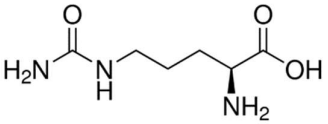
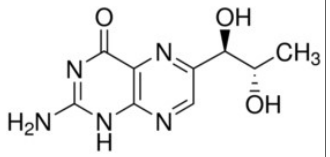
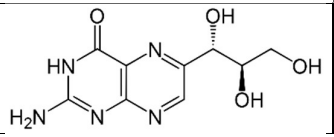
	Serotonin	Neurotransmitter		212,68 (HCl)	153-98-0
	5-hydroxyindole acetic acid	Metabolite		191,18	54-16-0
	Tryptamine	Alternative metabolite of tryptophan conversion		160,22	61-54-1
Dopaminergic/ adrenergic system	Phenylalanine	Precursor		165,19	63-91-2
	Tyrosine	Precursor		181,19	60-18-4
	L-DOPA	Precursor		197,19	59-92-7

	Dopamine	Neurotransmitter, precursor		189,64 (HCl)	62-31-7
	Norepinephrine	Neurotransmitter, precursor		319,26 (tartrate)	3414-63-9
	Normetanephrine	Metabolite		219,67 (HCl)	1011-74-1
	Epinephrine	Neurotransmitter		219,67 (HCl)	329-63-5
	Metanephrine	Metabolite		233,69 (HCl)	881-95-8

Aspartic acid system	Aspartic acid	Neurotransmitter		133,10	56-84-8
	Asparagine	Precursor		132,12	70-47-3
Cholinergic system	Acetylcholine	Neurotransmitter		181,66 (Cl^-)	60-31-1
	Choline	Precursor		139,62 (Cl^-)	67-48-1
Kynurenine pathway	Kynurenine	Tryptophan catabolism		208,21	2922-83-0
	Kynurenic acid	Tryptophan catabolism		189,17	492-27-3

	Anthranilic acid	Tryptophan catabolism		137,14	118-92-3
	Xanthurenic acid	Tryptophan catabolism		205,17	59-00-7
	Quinolinic acid	Tryptophan catabolism		167,12	89-00-9
	Picolinic acid	Tryptophan catabolism		123,11	98-98-6
Indole pathway	Indole-3-carboxaldehyde	Tryptophan catabolism		145,16	487-89-8

	Indole-3-acetic acid	Tryptophan catabolism		175,18	87-51-4
	Indole-3-butyric acid	Tryptophan catabolism		203,24	133-32-4
	Indole-3-lactic acid	Tryptophan catabolism		205,21	832-97-3
	Indole-3-acrylic acid	Tryptophan catabolism		187,19	1204-06-4
	Indole-3-propionic acid	Tryptophan catabolism		189,21	830-96-6

Others	Cortisol	Stress biomarker		362,46	50-23-7
	Citrulline	Glutamine metabolite		175,19	372-75-8
	Biopterin	Cofactor of enzymes responsible for monoamines biosynthesis		237,22	22150-76-1
	Neopterin	Metabolites of extensive biopterin elimination		253,21	2009-64-5

2.1.3. Reagents

The following reagents were used in the study: dimethyl sulfoxide (DMSO) (ACS reagent, >99.9%, Merck, Germany), sodium chloride (pure, Prime Chemicals Group, Russia), potassium chloride (pure, Prime Chemicals Group, Russia), calcium chloride*2H2O (pure, Prime Chemicals Group, Russia), magnesium sulfate*7H2O (pure, Prime Chemicals Group, Russia), methylene blue (>97%, Prime Chemicals Group, Russia), ethanol (puriss, Merck, Germany), sodium metabisulfite (puriss, Merck, Germany), methanol (puriss, Chimmed, Russia), ascorbic acid (99%, Merck, Germany), acetonitrile (HPLC, Fisher Chemical, USA), formic acid (99%+, Optima LC-MS grade, Fisher Chemical, USA), methoxyamine hydrochloride (98%, Sigma-Aldrich, Germany), pyridine anhydrous (99.8%, Sigma-Aldrich, Germany), MSTFA (for GC, Sigma-Aldrich, Germany), toluene anhydrous (99.8%, Sigma-Aldrich, Germany). Water was obtained using the Millipore Direct Q3-UV system (Merck-Millipore, USA).

2.1.4. Equipment

The following equipment were used in the study: Agilent 1290 II Infinity high-performance liquid chromatographer coupled to triple quadrupole mass-spectrometer Agilent 6470 (Agilent Technologies, USE); Discovery HS F5-3 PFP (150 mm, 2.1 mm, 3 um) analytical column for liquid chromatography (Supelco, Germany); Agilent 7820A gas chromatographer coupled to mass-selective detector MSD 5977 (Agilent Technologies, USA); Agilent HP-5MS (30 m, 0,25 mm, 0,25 mm) analytical column for gas chromatography (Agilent Technologies, USA); Adventurer AR 1530 analytical scales (Ohaus Corporation, Switzerland); coolable centrifuge 5418R (Eppendorf AG, Germany); Genius 3 vortex shaker (BioSan, Latvia); MiVac Quattro Concentrator heated vacuum concentrator (Fisher Scientific, USA); DT 255 H ultrasonic bath (Bandelin, Germany); adjustable volume Eppendorf pipets (Eppendorf AG, Germany); Millipore Direct Q3-UV water purification system (Merck-Millipore, USA); Leica DM2000 light microscope

(Leica Microsystems, Germany); high-performance video tracking system (DanioVision, Noldus, Netherlands); 6-, 12-, and 96-well plates (Corning, USA).

2.2. Animals

Zebrafish were housed according to the GOST 33219-2014 "Guidelines for accommodation and care of laboratory animals. Species-specific provisions for fish, amphibians, and reptiles" and ETS 123 "European convention for the protection of vertebrate animals used for experimental and other scientific purposes" [23, 70].

Wild-type adult zebrafish for obtaining eggs were purchased from a local pet shop (Moscow, Russia). Males and females were housed separately to prevent uncontrollable breeding. The fish were housed in the water tanks with the density of 1 fish per 2 liters of water; the water in the tank was constantly changing with a rate of 4-6 tank volumes per hour. The light/dark cycle was set at 14/10 hours, respectively. The temperature of the water and air was constant (26±0,5°C and 25°C, respectively) [23]. Fish were fed twice a day with dry food (TetraMin, Tetra GmbH, Германия) and thrice a week with a portion of live food (Artemia salina nauplii).

To obtain fertilized eggs, the following scheme of the shoal breeding was used: males and females in a ratio of 2:1 were transferred into the breeding tank; the bottom of the tank was covered with a net frame; the tank was filled with E3 medium in such a way that the water level was 4-5 cm above the frame (the placement of the net frame, as well as low water level, prevents the eggs from being eaten by the parents). The temperature of the water was set at 28±0,5°C (2°C higher than the one for housing) in order to initiate the maturation of the eggs in females. The spawning takes place in the early morning and lasts for approximately 30 min. After the spawning is finished, the parent flock is transferred back into the aquaria connected to the filtration system; after that, the net frame was pulled out from the breeding tank, the water containing eggs were filtered through mesh net; then, the eggs were washed twice using E3, the unfertilized eggs were separated; the fertilized eggs were then held for one day at a temperature of 28±0,5°C to separate the unfertilized eggs

again; the viability of the eggs was assessed visually using Leica DM2000 light microscope.

2.3. Preparation of the working solutions and buffers

2.3.1. Preparation of the solutions of the pharmacologically active substances

Stock solutions of diazepam and 5F-APINAC were prepared by dissolving a precisely weighted standard substance in ethanol or DMSO of HPLC. Working solutions were made utilizing dilution. The diluent was medium for zebrafish larvae (E3) in order to have a correct ion balance in the working solution. The concentration of the carrier solvent was held at 0.1%. The working solutions were stored in the fridge for longer than one day.

2.3.2. Preparation of the working solution of zebrafish embryo medium (E3)

To maintain the normal functioning and viability of the embryos, they should be kept in a saline solution with well-defined ions concentrations. For this purpose, a series of the E3 medium stock solution was made: the starting 50x E3 solution consisted of 14.6 g of NaCl, 0.63 g of KCl, 2.43 g of $CaCl_2*2H_2O$, and 4.07 g of $MgSO_4*7H_2O$per 1 liter of deionized water. After that, the solution is diluted to obtain 1x E3 medium, and 5 l of this solution is spiked with 100 ul of 1% aquatic solution of methylene blue. The stock and working E3 solutions were stored in the fridge for longer than a week.

2.4. Concentration ranges for diazepam and 5F-APINAC assessments

Experimental design

All of the experiments were conducted in correspondence with the GOST 33774-2016 "Testing of chemicals of an environmental hazard. Fish embryo acute toxicity" and ETS 123 "European convention for the protection of vertebrate animals

used for experimental and other scientific purposes". All of the experiments were approved by the Local Ethical Committee of the Sechenov University.

Evaluation of the diazepam solutions concentration range

The doses of exposure concentrations were exponentially increased, starting from 0.8 μg/L to 160 μg/L. A 0.8 μg/L concentration has been reported to be the highest environmental concentration of diazepam, while the maximum dose of 160 μg/L has been shown to cause behavioral effects in 100% of zebrafish larvae [232].

Evaluation of the 5F-APINAC concentration range (determination of LC_{50} of 5F-APINAC)

To evaluate the concentration range of 5F-APINAC, the investigation of its' half-lethal concentration (LC_{50}) was conducted; the concentration range was also dependent on the data about the toxicity of APINAC – defluorinated analog of 5F-APINAC [23, 39, 210].

The assessment was carried out right after the spawning and lasted for 96 h; in the experiment, 40 zebrafish eggs were transferred in the 12-well plates. The use of the double amount of the eggs is explained by the unavailability of determination of fertilized eggs before 6 h post-fertilization. The unfertilized eggs separated, and 20 eggs per concentration were used in the experiment. The experiment was conducted using the following groups: 5 experimental groups that were exposed to 10, 20, 40, 80, and 100 uM solutions of 5F-APINAC in 1% DMSO in E3 medium; the carrier solvent control group – 20 zebrafish eggs in the 1% solution of DMSO in water; positive control group – 4 ug/l solutions of 3,4-dichloroaniline in 1% DMSO in water; negative control group, that were contained in E3 medium. The viability assessment included the fixation of the following parameters: coagulation, the absence of somite formation, the lack of heartbeat, and the absence of tail detachment. If one of the parameters were present, the embryo was considered dead. The observations were made after each 24 h. The calculation of LC_{50} was created using the probit model.

2.5. Untargeted metabolomic profiling

Experimental design

In this assessment, six days post-fertilization (dpf) larvae were used. The experiment was done in six replicates. In each experiment, 20 embryos were transferred into each well of the 6-well plates, each plate containing 5 ml of E3 medium. The experimental groups were exposed to diazepam solution with a concentration of 160 ug/l for 2.5 h.

The scheme of the experimental design is presented in the Figure 1.

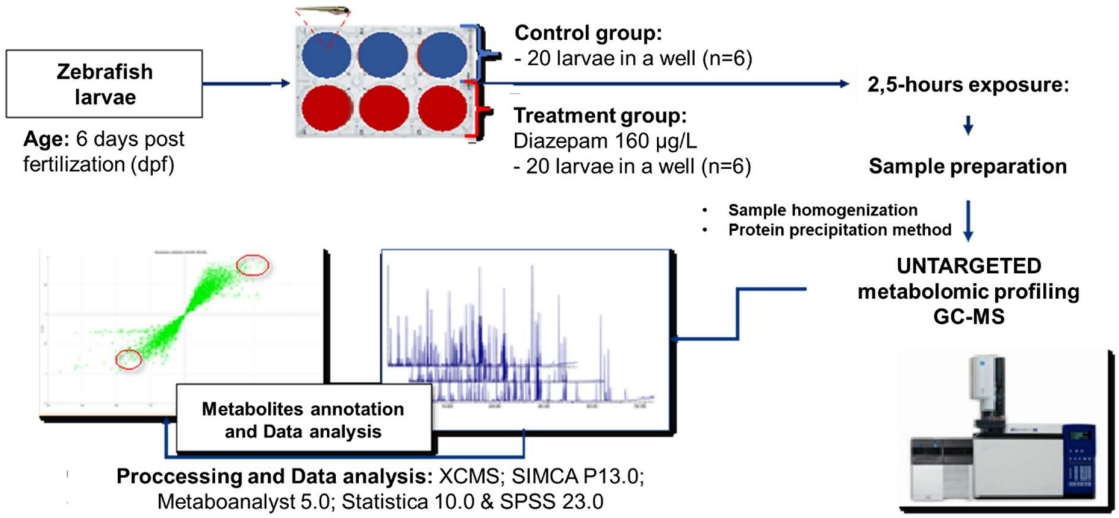


Figure 1 – Design of the experiment of the exposure of diazepam on the zebrafish larvae for untargeted metabolomics

Sample preparation

The following sample preparation method was used: zebrafish larvae were transferred into 1.5 ml microtubes. The aqueous phase was removed, and 10 µL of cold 13 mM aqueous solution of sodium metabisulfite was added to the sample to prevent metabolite oxidation and to euthanize the fish. Then, 10 uL of the internal standard mixture was added, and the samples were vortexed. After, 450 µL of cold methanol was added, vortexed for 20 s. Subsequently, the mixture was sonicated in an ice bath for 15 minutes and centrifuged for 5 minutes at 16.900 rcf at 4°C.

Afterward, 200 μL of the supernatant was transferred to a new microtube and evaporated to dryness.

After that, 20 uL of 40 mg/ml solution of methoxyamine hydrochloride in pyridine was added, the samples were vortexed and heated for one h at a temperature of 60°C. Then, 70 uL of toluene and 30 uL of MSTFA were added, the samples were shaken and heated for 50 min at a temperature of 70°C. After cooling down, the samples were transferred into GC vials, and 1 uL of the samples were injected into the system.

GC-MS analysis

The instrumental analysis was performed using an Agilent 7820A (Agilent Technologies, USA) coupled to Agilent 5850 single quadrupole mass spectrometer (Agilent Technologies, USA). The chromatographic separation was achieved using Agilent HP-5MS column; the column parameters were: length – 30 m, internal diameter – 250 um, solid-phase particles size – 0.25 um; chromatographic conditions were: injection in the split mode (1:5), helium as a carrier gas was used at a constant flow rate of 1 ml/min; the interface temperature – 290°C. The oven temperature was ramped from 70°C with 2 min hold to 310°C with 5°C/min. The transfer line temperature was 280°C, and the electron ionization voltage was 70 eV. The identification of metabolites was made using Human Metabolome Database and METLIN Metabolite through NIST mass-spectral library.

Quality control

Quality control samples (QC) were used to confirm the sample preparation and instrumental analysis validity. The preparation of the QC samples consists of mixing the aliquots of the similar volumes of each zebrafish sample. Sample preparation of the QC is similar to this for a regular sample. Besides that, to exclude carryover and batch effect, the obtained experimental samples were analyzed in a random order, while QC samples were analyzed before, in the middle, and at the end of the GC-MS assessment.

Untargeted data preprocessing

To complete data preprocessing, the chromatograms were converted into the appropriate format using Proteowizard software (ProteoWizard Software Foundation, CA, USA). These chromatograms were processed with XCMS software (https://xcmsonline.scripps.edu/, США) to obtain a database consisting of the mass fragments of the compounds, their retention time and intensity. The data were checked for the presence of blank cells; if the metabolite had more than 20% of the data missing, it was excluded from further analysis. Deconvolution and smoothing the peaks were made using the following parameters: slope – 2000 l/min, intensity threshold – 20000, mass tolerance – 0.7 Da, retention time delta – 0.2 min. Each feature in the obtained matrix had its specific m/z, retention time, and intensity in each of the samples. The features that presented more than 30% of the relative intensity deviation in the QC samples were also excluded from the analysis. The resulting matrix was converted into an appropriate electronic table for further statistical analysis.

Samples chromatograms were also processed using AMDIS software; this assessment aimed to juxtapose the unknown features with mass-spectral libraries to identify metabolites' names. After identifying the metabolites, features were matched with those obtained from XCMS. Thus, the untargeted dataset containing the names of the metabolites and their intensities in the samples was obtained.

2.6. Targeted metabolomic profiling

Method validation

The validation of the developed HPLC-MS/MS method was made according to FDA and ICH guidelines on the validation of bioanalytical methods [27, 109, 146]. In the validation process, it was proven that the developed method is capable of measuring the concentrations of target endogenous metabolites of interest. For each of the metabolites, the acceptance criteria were measured. The method's suitability was evaluated using such parameters as selectivity, linearity, calibration curve, lover limit of quantification (LLOQ), carryover, within- and between-run precision and accuracy, extraction coefficient (recovery), matrix effect, and stability.

Experimental designs

During targeted metabolomics study, the profiles of GABAergic, serotonergic, dopaminergic/adrenergic, cholinergic, aspartic acid systems, kynurenine, indole pathways of tryptophan metabolism were investigated to evaluate the effect of diazepam of 5F-APINAC on these neurotransmitter systems in zebrafish larvae.

Short- and long-term exposure to diazepam

Two independent metabolomics studies were performed on zebrafish (short- and long-term exposure). In the two experiments, zebrafish embryos were transferred into 12-well plates (20 embryos per well, each containing 5 mL E3 medium without methylene blue). Experiment I (2.5 h exposure) was designed to assess the influence of the drug 2.5 h after administration on the sixth dpf (at 11 am). In this experiment, four intervention groups at concentration doses equal to 0.8, 1.6, 16, and 160 μg/L, and a vehicle control group (0.1% ethanol in E3 medium) were used. Experiment II (96 h exposure) was similar to experiment I, the only difference being that diazepam started to be administered from the second until the sixth dpf.

The scheme of the experimental design is presented in the Figure 2.

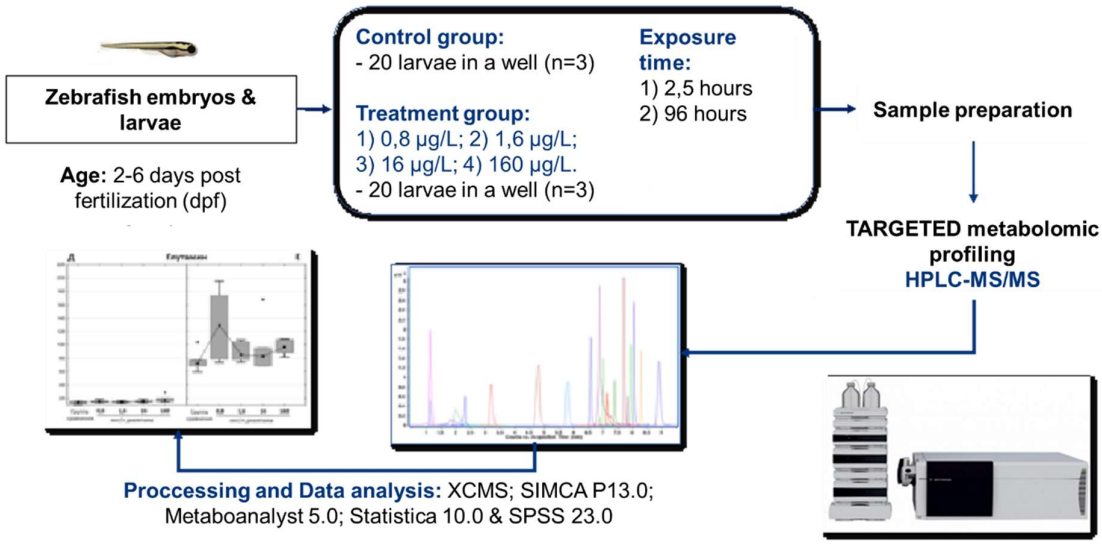


Figure 2 – Design of the experiment of the exposure of diazepam on the zebrafish larvae for targeted metabolomics

Short- and long-term exposure of 5F-APINAC

Two independent studies were performed on zebrafish larvae/eggs (acute and chronic exposure). In the two experiments, zebrafish embryos were transferred into 12-well plates (20 embryos per well, each containing 5 ml E3 medium without methylene blue). Experiment I (acute exposure) was designed to assess the influence of the drug 4 h after administration on the sixth day postfertilization (dpf) at 11 am. In this experiment, five intervention groups at concentration doses equal to 0.001, 0.01, 0.1, 1.0, and 10 µM, and a vehicle control group (0.1% DMSO in E3 medium) were used. Experiment II (chronic exposure) was similar to experiment I, the only difference being that 5F-APINAC started to be administered from the second until the sixth dpf.

The scheme of the experimental design is presented in the Figure 3.

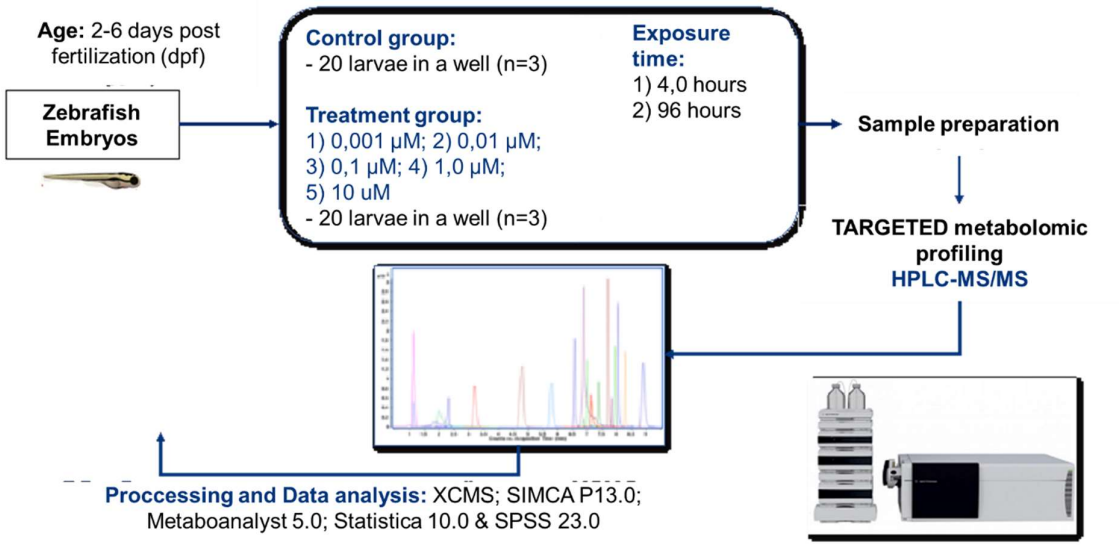


Figure 3 – Design of the experiment of the exposure of 5F-APINAC on the zebrafish larvae for targeted metabolomics

Sample preparation

Larvae were transferred into 1.5 ml microtubes. The aqueous phase was removed, and 10 µl of 13 mM cold aqueous solution of sodium metabisulfite was added to the sample to prevent metabolite oxidation. Then, 10 µl of the internal standard solution were added, and the sample was vortexed for 10 s. Next, 450 µl of

cold methanol was added, vortexed for 20 s. Then the mixture was sonicated in an ice bath for 15 min and centrifuged for 5 min at 16.900*g at 4 ◦C. Subsequently, 200 µl of the supernatant were transferred to a new microtube and evaporated to dryness. The dried sample was reconstituted in 50 µl of 0.02% solution of ascorbic acid in 1:1 (v/v) water:methanol mixture and 2 µl of the solution were injected into the UPLC-MS/MS system.

HPLC-MS/MS analysis

Chromatographic separation was conducted using the Agilent 1290 Infinity II UPLC system coupled to the Agilent 6470 Triple Quad tandem mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, Santa Clara, CA, USA). The liquid chromatograph was equipped with a Discovery HS F5-3 PFP column (150 mm × 2.1 mm × 3 µm) (Supelco, Inc., Darmstadt, Germany). The column oven temperature was set at 45 °C. Gradient elution was used (eluent solution A - 0.1% formic acid in water, eluent solution B - 0.1% formic acid in acetonitrile), as described in Table 2.

Table 2 – HPLC gradient conditions

Time, min	**Flow, ml/min**	**% mobile phase**	
		A	**B**
0	0,4	90	10
4	0,4	90	10
9	0,4	10	90
10	0,4	10	90
10,10	0,4	90	10
12	0,4	90	10

Metabolite determination was performed in positive ionization mode with multiple reaction monitoring (MRM). Mass-spectrometer parameters were: capillary voltage – 1.5 kV, source temperature - 150 °C, evaporation gas – nitrogen, gas temperature – 300 °C, gas flow – 3 l/min. MRM parameters and retention times for all metabolites were optimized separately.

2.7. Behavioral studies

Experimental design

Zebrafish larvae were exposed to a wide range of 5F-APINAC concentrations (0.001-10 uM). The control group was exposed to the carrier solvent (0.1% DMSO in E3). Zebrafish larvae at the age of 5 dpf were set into the wells of 96-well plate (1 larva per well). The exposure began at the sixth dpf at 11 am [197].

The locomotor activity of zebrafish was registered for full two days (48 h). This assessment allows investigating the acute response to 5F-APINAC and the long-term exposure on the locomotor activity of zebrafish larvae. The activity was recorded at a frame rate of 25 fps under a constant infrared light using the high-throughput video tracking system DanioVision (Noldus, Netherlands). The activity of each fish was calculated as the total distance traveled by a fish for 10 min. The scheme of the experimental design is presented in the Figure 4.

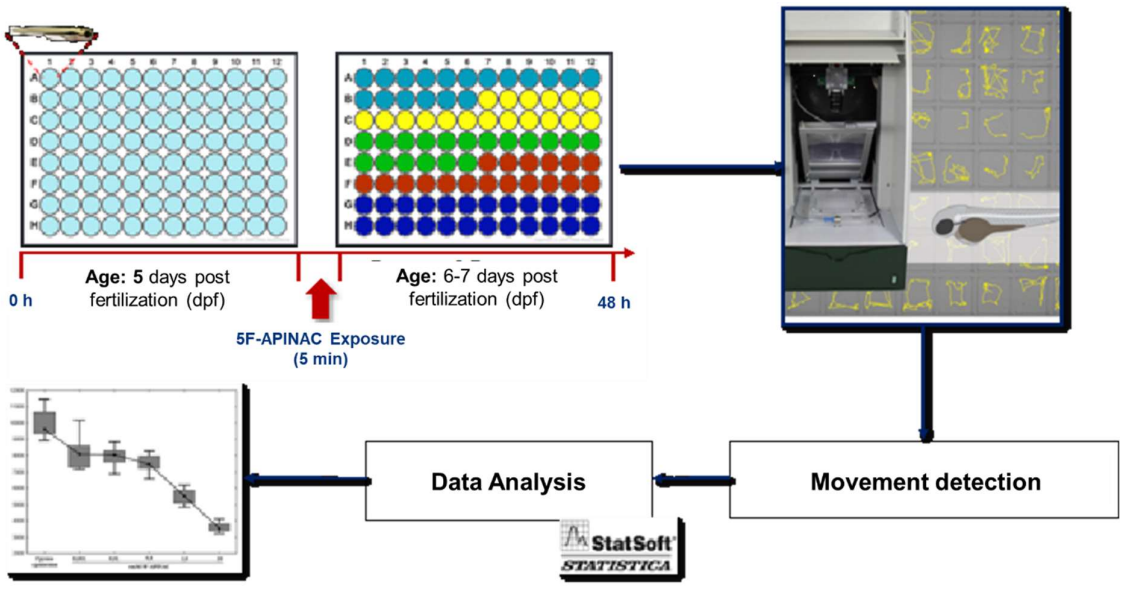


Figure 4 – Design of the experiment of the exposure of 5F-APINAC on the zebrafish larvae for behavioral studies

2.8. Statistical analysis

Statistical analysis of the data from the behavioral study, correlation search and LC_{50} determination

Statistical analysis of the behavioral data was conducted using a one-way ANOVA with Tukey posthoc assessment. The correlation coefficients between behavioral and metabolomics data were calculated using nonparametric Spearman's ranking. The analyses were carried out using Statistica 10.0 software (Statsoft, USA). LC_{50} was calculated with the probit method regression model. This analysis was carried out using SPSS 23.0 (SPSS Inc., USA).

Untargeted metabolomics profiling

The data obtained from the untargeted profiling was log-normalized, mean-centered, and Pareto scaled. Then, it was uploaded into the SIMCA-P 13.0 (Umetrics, Sweden) for further search for significant metabolites. Variance importance in the projection (VIP, VIP-score) represents the value of the feature in the discriminant analysis as a correlation factor. This analysis coupled with S-plot helps to evaluate the most significant metabolites in the dataset. Features with a VIP score of more than one were considered to have discriminative power across groups.

To evaluate the trending of concentrations between the two groups and also to exclude the outliers, principal component analysis was made. Further, orthogonal projection to latent structures-discriminant analysis was made to evaluate the features with the most statistical potency, and an S-plot was made using these metabolites. For the potentially significant features, depending on the normality of the data, the Student t-test or Mann-Whitney U-test were made.

After that, the Metaboanalyst 4.0 online platform was used to obtain the data about the metabolic pathway enrichment analysis. This analysis allows us to identify the metabolic pathways most affected by the substance exposure to form a targeted metabolomic panel of metabolites.

Targeted pharmacometabolomic profiling

Data were inspected to detect extreme values. Extreme values were defined as concentrations higher than the 75th percentile +3 interquartile ranges (IQR).

These values were replaced by the corresponding concentrations for the 75th percentile +1.5 IQR (winsorization). The normality of the data was evaluated using the Shapiro-Wilk test. Most of the data was non-normally distributed. The nonparametric Kruskal-Wallis one-way ANOVA test was used to compare central tendencies among groups exposed to different diazepam and 5F-APINAC concentrations, followed by Dunn's post hoc correction. Statistical analyses were performed using the STATISTICA software version 10.0 (data analysis software system, Tulsa, Okla, USA).

3. Results

3.1. Untargeted metabolomics profiling of zebrafish larvae after diazepam exposure

GC-MS untargeted metabolomics profiling was made to identify the panel of endogenous metabolites necessary to investigate the pharmacological effects of physiologically active drugs of neurotropic action using Danio rerio as a model organism. To conduct the untargeted profiling, zebrafish exposed to 160 ug/L of diazepam and a control group were used. One thousand three hundred fifty-six features were identified during the untargeted approach. Principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were used to detect significant metabolites.

PCA showed that there were metabolites significantly changed in the exposure group versus the control group (Figure 5A). According to OPLS-DA data (Figure 5B), the explained variation (R2) was 0.91 what indicates that there are components capable of discriminating the groups in the model. The test-model variability (Q2) was 0.63, which tells about the high predictive power of the model. Generally, the analyses showed good discrimination of the groups, which means suitable method reproducibility.

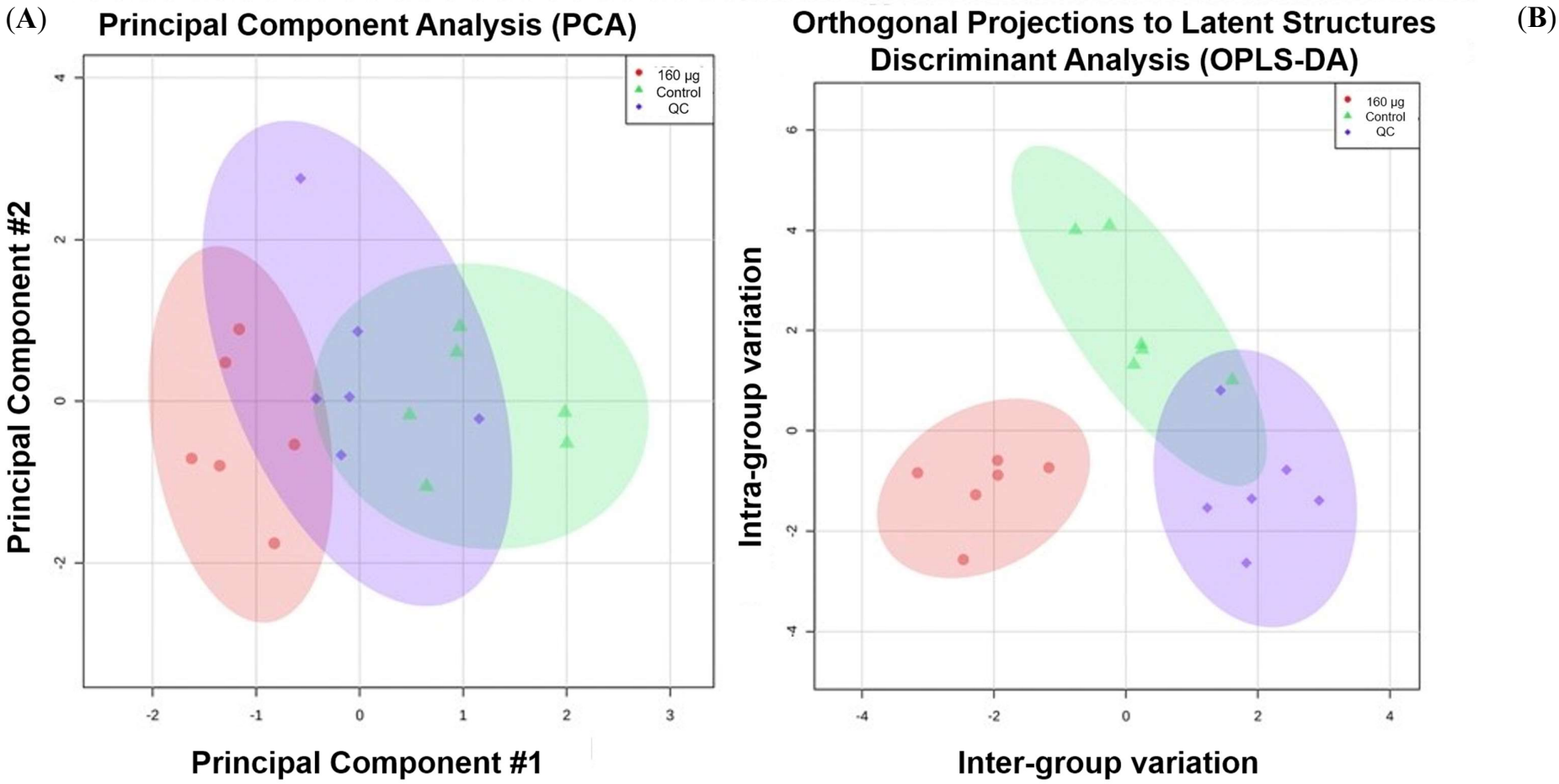


Figure 5 – Principal component analysis of the investigated groups (A). Orthogonal projections to latent structures-discriminant analysis (B).

S-plot had shown statistically significant metabolites that could be used to discriminate between exposure and control groups (Figure 6).

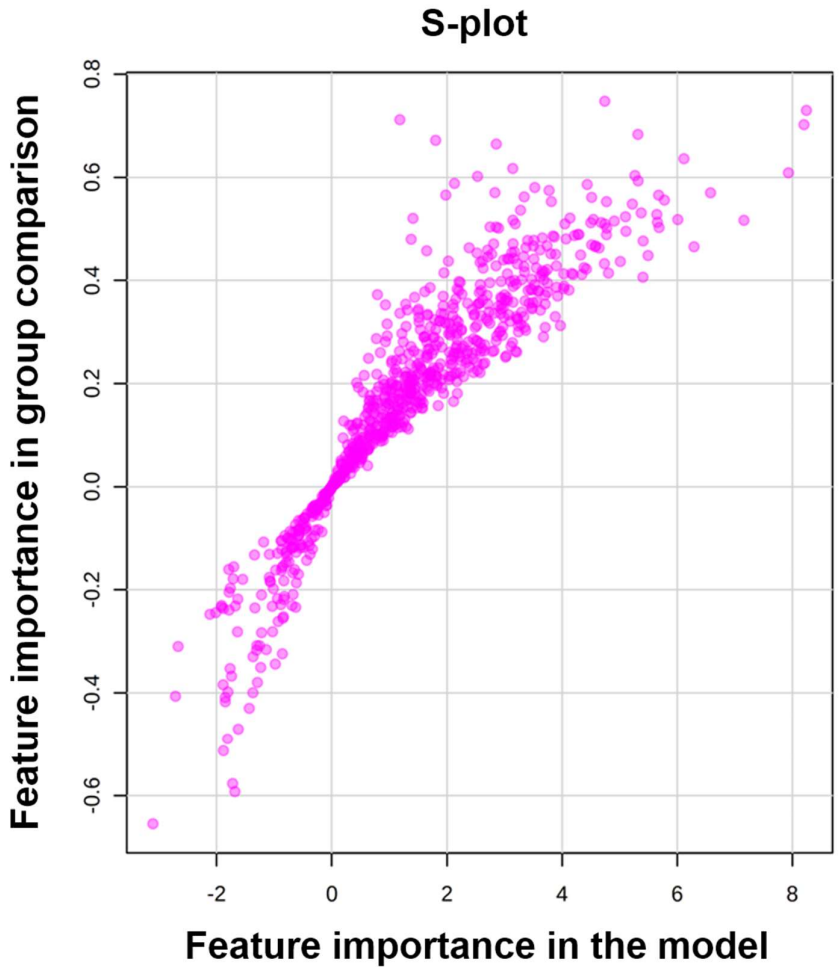


Figure 6 – S-plot, based on the OPLS-DA model from untargeted metabolomic data.

Based on the acquired data, statistically significant metabolites were identified. Twenty-five metabolic pathways associated with diazepam exposure were identified (Figure 7).

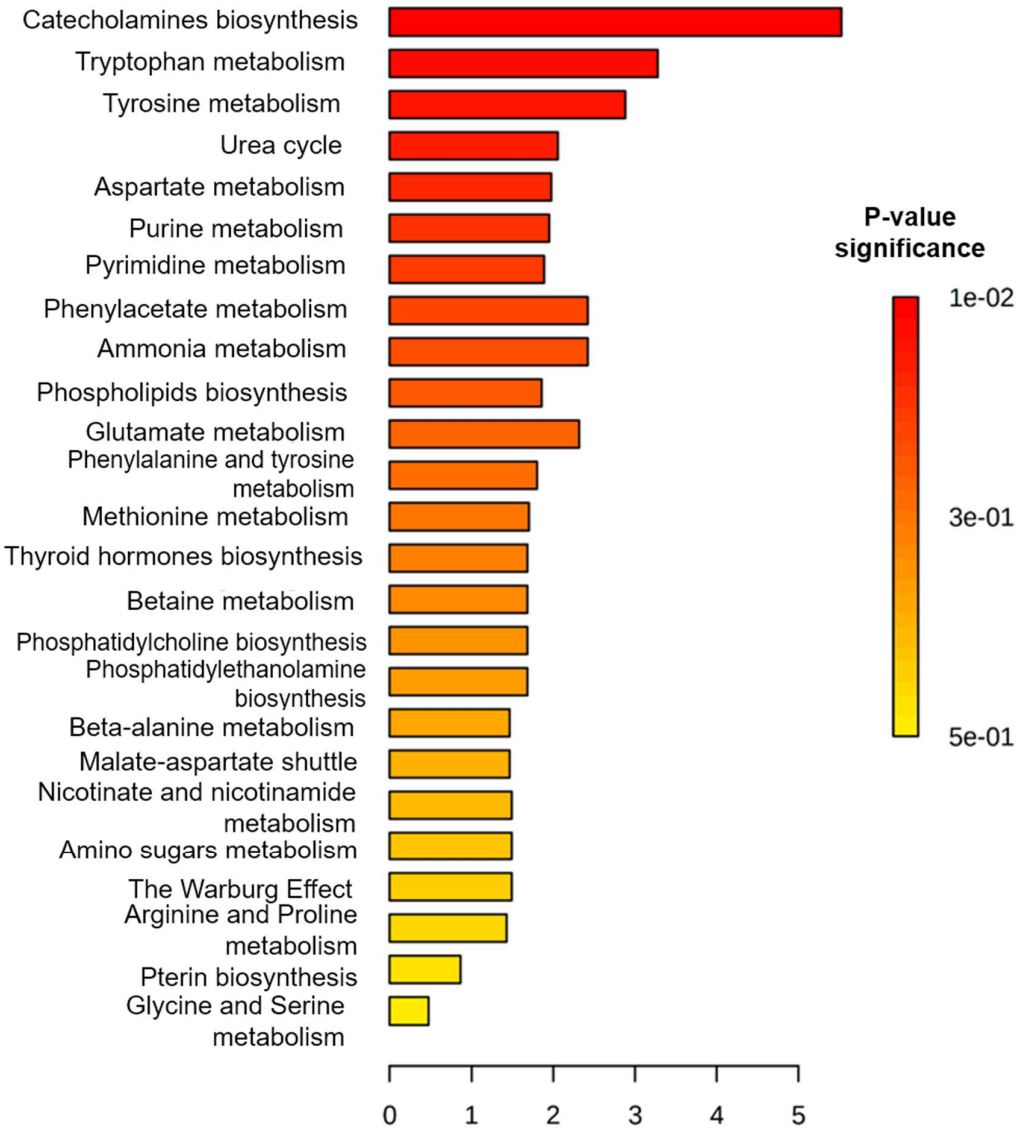


Figure 7 – Metabolomic set enrichment analysis.

The acquired data had shown that diazepam exposure led to alterations in the catecholamine biosynthesis, tryptophan metabolism, and tyrosine metabolism biochemical pathways (Table 3). Based on the results, it can be postulated that diazepam exposure led to the alterations in the concentrations of metabolites related to neurotransmission.

Table 3 – Metabolic pathways associated with the diazepam action on the zebrafish larvae

Metabolic pathway	*p*-value*	Metabolites	Direction
Catecholamine biosynthesis	0,013	Dopamine	↓
		Norepinephrine	↑
		Tyrosine	↑
		L-DOPA	↓
Tryptophan metabolism	0,081	Tryptophan	↑
		Serotonin	↑
		Kynurenine	↓
Tyrosine metabolism	0,097	Tyrosine	↑
		Homogentisic acid	
		L-aspartic acid	
Urea cycle	0,202	Arginine	↑
		Citrulline	
Aspartate metabolism	0,217	L-Aspartic acid	↑
		L-glutamic acid	↑
Purine metabolism	0,235	Guanine	↓
		Inosine	
Pyrimidine metabolism	0,246	Uridine	↓
		Citidine	
Phenylacetate metabolism	0,251	Phenylacetic acid	↓
		L-glutamic acid	↑
Ammonia metabolism	0,269	L-serine	↑
		L-aspartic acid	
		Oxoglutaric acid	↑
		Glycine	↓
Phospholipid biosynthesis	0,272	Palmitic acid	↑
Glutamate metabolism	0,273	GABA	↓
		L-cysteine	↑
		L-Aspartic acid	↑
Phenylalanine and tyrosine metabolism	0,280	Tyrosine	↑
		Phenylalanine	
		Oxoglutaric acid	
Methionine metabolism	0,281	L-methionine	↓
Thyroid hormones synthesis	0,296	Tyrosine	↑
Betaine metabolism	0,306	Adenosine	↑
		L-methionine	↓
Phosphatidylcholine biosynthesis	0,315	Cytidine	↓
		Ethanolamine	↑
Phosphatidylethanolamine biosynthesis	0,315	Cytidine	↓
		Ethanolamine	↑
Beta-alanine metabolism	0,324	Alanine	↑
Malate-aspartate shuttle	0,335	Malic acid	

Metabolic pathway	*p*-value*	Metabolites	Direction
		Aspartic acid	↓
Nicotine and nicotinamide metabolism	0,384	L-glutamic acid	↑
		Nicotinamide	
Amino sugars metabolism	0,421	D-fructose	↓
Warburg effect	0,457	Succinic acid	↑
Arginine and proline metabolism	0,469	Arginine	↑
Pterin biosynthesis	0,478	Neopterin	↑
Glycine and serine metabolism	0,512	Glycine	↓

** P-values represent the strength of the metabolite's contributions into the pathway. P-values were calculated based on the number of metabolites that are engaged in the certain pathway.*

Thus, to investigate the pharmacological effects of the neurotropic drugs, based on the untargeted metabolomics profile, the targeted metabolic panel was made. The panel included the metabolites of the dopaminergic/adrenergic system and serotonergic system. Also, the metabolites of the main neurotransmission systems (GABAergic, cholinergic, aspartic acid) were included in the study [140]. Also, the metabolites of the concomitant pathways were included in the study. Such pathways were: kynurenine pathway, which is the primary biochemical pathway of tryptophan catabolism [265]; indole (microbiota) pathway of tryptophan conversion through microbial reactions [144]; biopterin and neopterin, which are the metabolites of cofactors of the enzymes related to monoamines synthesis [138]; cortisol as one of the principal stress biomarkers [292].

3.2. Development and validation of the targeted HPLC-MS/MS method for the determination of the neurotransmitters and their metabolites in zebrafish larvae

During the validation process, the applicability of the developed method was proven. The results of the method validation are represented below.

3.2.1. Mass-spectrometry parameters and selectivity

By analyzing the mass-spectrometry data of the individual solutions of the studied compounds and internal standards, MS/MS parameters of the compounds

were optimized. The parameters of ionization, the retention time were also optimized. The results are shown in Tables 4 and 5.

Table 4 – HPLC-MS/MS MRM parameters for targeted metabolites

Metabolite	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collusion energy (V)	Retention time (min)	Polarity
GABA	104	87	90	10	1,2	Positive
		69	90	15		
5-hydroxytryptophan	221	162	90	20	3,8	Positive
		160	90	20		
Acetylcholine	146	87	90	15	3,8	Positive
		60	90	10		
		43	90	30		
Anthranilic acid	138	120,1	95	10	6,2	Positive
		92	95	20		
Asparagine	133	87	100	10	1,0	Positive
		74	100	20		
Aspartic acid	134	88	100	10	1,0	Positive
		74	100	15		
Biopterin	238	194	90	20	1,9	Positive
		178	90	20		
Choline	104	60	90	20	1,2	Positive
Citrulline	176	113,1	90	20	1,1	Positive
		70,1	90	30		
Cortisol	363	327	120	10	7,3	Positive
		121	120	20	7,3	
Dopamine	154	91	90	30	1,7	Positive
		137	90	10		
	137	91	90	10		

Metabolite	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collusion energy (V)	Retention time (min)	Polarity
Epinephrine	184	166	90	5	1,9	Positive
		151	90	25		
	166	151	110	20		
Glutamic acid	148	84	100	15	1,0	Positive
		56	100	30		
Glutamine	147	130	100	10	2,0	Positive
		84	100	15		
5-hydroxyindole acetic acid	192	146	100	20	5,9	Positive
Indole-3-acetic acid	176	130	95	20	7,1	Positive
Indole-3-acrylic acid	188	170	100	20	7,4	Positive
		115	100	25		
Indole-3-butyric acid	204	144	100	25	7,7	Positive
		130	100	25		
Indole-3-carboxaldehyde	146	118	95	15	7,2	Positive
		91	95	30		
Indole-3-lactic acid	206	118	95	22	6,8	Positive
Indole-3-propionic acid	190	130	95	20	7,5	Positive
Kynurenic acid	190	144	95	20	7,8	Positive
Kynurenine	209	146	95	30	3,3	Positive
		94	95	10		
L-DOPA	198	181	100	10	1,9	Positive
		152	100	10		
Metanephrine	198	180	90	5	3,5	Positive
		165	90	20		
		148	90	25		

Metabolite	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collusion energy (V)	Retention time (min)	Polarity
Neopterin	254	206,1	100	20	1,3	Positive
		190,1	100	20		
Picolinic acid	124	78,1	90	20	1,7	Positive
		53,1	90	20		
Norepinephrine	170	152	90	5	1,4	Positive
	152	107	110	20		
Normetanephrine	166	134	100	15	2,3	Positive
Phenylalanine	166	120	100	15	3,8	Positive
Quinolinic acid	168	106,1	95	15	3,2	Positive
		78,1	95	30		
Serotonin	160	132	120	20	3,5	Positive
		105	120	30		
Tryptamine	161	144,1	95	10	6,3	Positive
		115,1	95	30		
Tryptophan	205	188	100	10	5,2	Negative
		146	100	20		
Tyrosine	182	165	90	5	2,5	Positive
		136	90	10		
Xanthurenic acid	206	160	95	20	7,6	Positive
		132,1	95	30		

Table 5 – HPLC-MS/MS MRM parameters for internal standards

Standard	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (V)	Retention time (min)	Polarity	Concentration in the sample (ng/ml)
GABA-d6	110	93	90	10	1,2	Positive	200
		73	90	15			
Dopamine-d4	158	123	90	10	1,7	Positive	20
		95	90	30			
5-hydroxyindole acetic acid-d5	197	150	100	20	5,9	Positive	20
Indole-3-acetic acid-d4	180,1	133	95	20	7,1	Positive	20
Indole-3-butyric acid-d4	208	132	100	25	7,7	Positive	200
Indole-3-carboxaldehyde-$_{13}$C8	154	126	95	30	7,2	Positive	20
		98	95	30			
Indole-3-lactic acid-d5	211	122	95	22	6,8	Positive	200
Indole-3-propionic acid-d2	192	130	95	20	7,5	Positive	200
Kynurenic acid-d5	195	149	95	30	7,8	Positive	20
Kynurenine-d4	213	98	95	10	3,3	Positive	200
Tryptophan-d5	208	164	100	15	5,2	Negative	20000
		120	100	15			
Normetanephrine-d3	187	137	100	15	2,3	Positive	20
Quinolinic acid-d3	171	109	95	15	3,2	Positive	200

Standard	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (V)	Retention time (min)	Polarity	Concentration in the sample (ng/ml)
		81	95	30			
Serotonin-d4	181	164	90	7	3,5	Positive	200
		136	120	20			
Xanthurenic acid-d4	210	164	95	20	7,6	Positive	20
		136	95	30			
Neopterin-$_{13}$C5	259,1	211	100	20	1,3	Positive	20
Cortisol-d4	367	331	120	10	7,3	Positive	20

The selectivity of the developed method was assessed by analyzing the interference strength in the blank samples compared to quality control (QC) samples. Six blank samples were used (blanks were 5% solution of bovine serum albumin in 0.9% NaCl).

The method's selectivity was acceptable when the investigated blank samples have not had the peaks that interfere with the retention time of the peaks corresponding to the targeted metabolites. The selectivity was fine if the level of interference in the blank sample was less than 20% of the LLOQ.

For all of the investigated analytes, the selectivity assay was acceptable. The chromatograms of the blank samples, QC chromatograms, and internal standard chromatograms are represented in Figure 8.

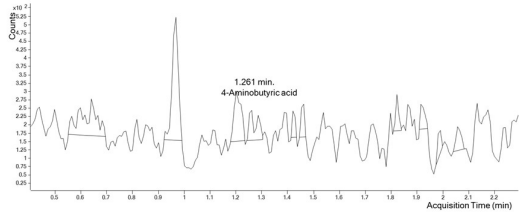
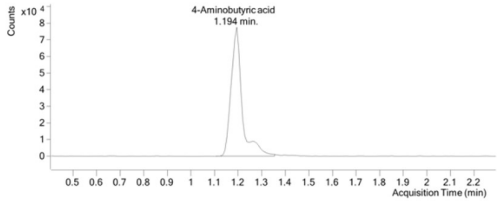
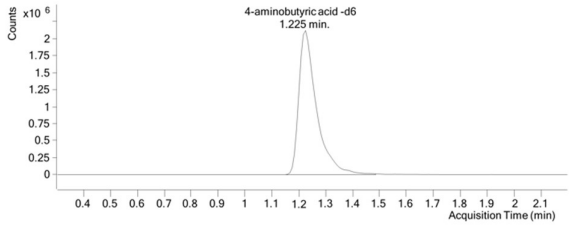
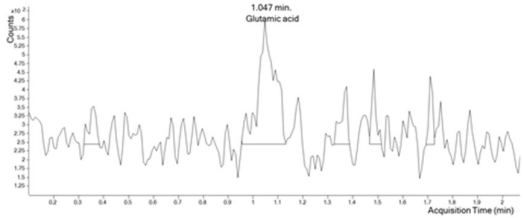
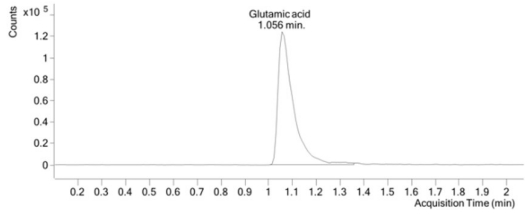
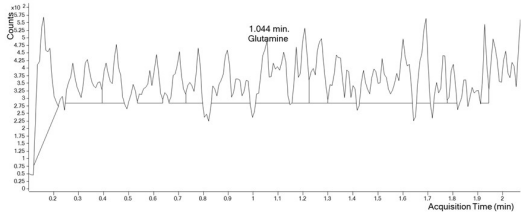
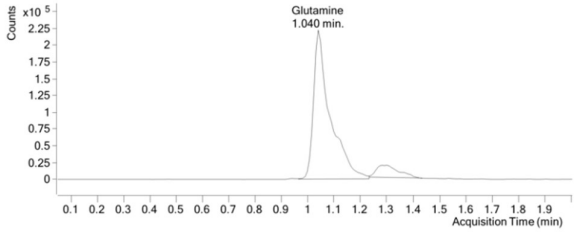
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
GABA (*m/z* 104 → *m/z* 87) 	GABA (*m/z* 104 → *m/z* 87) 	GABA-d6 (*m/z* 110 → *m/z* 93) 
Glutamic acid (*m/z* 148 → *m/z* 130) 	Glutamic acid (*m/z* 148 → *m/z* 130) 	
Glutamine (*m/z* 147 → *m/z* 130) 	Glutamine (*m/z* 147 → *m/z* 130) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards

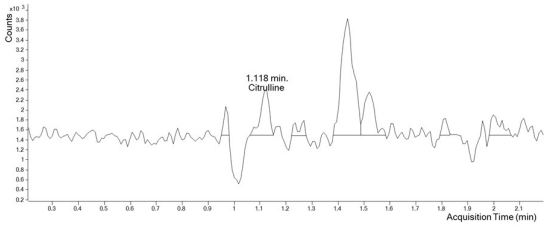
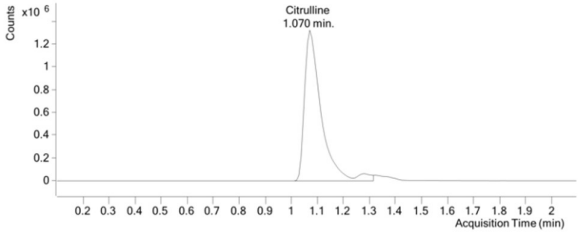
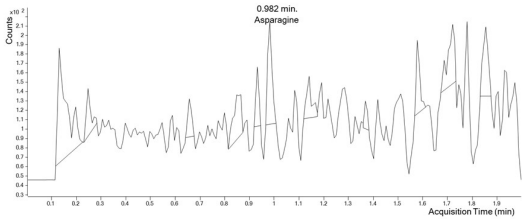
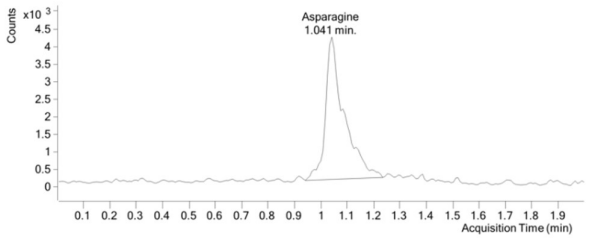
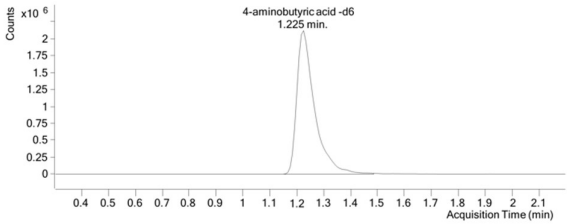
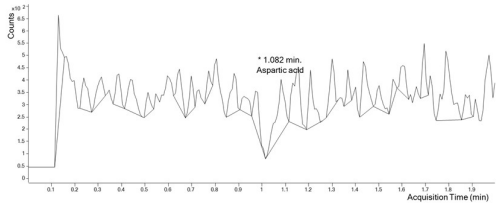
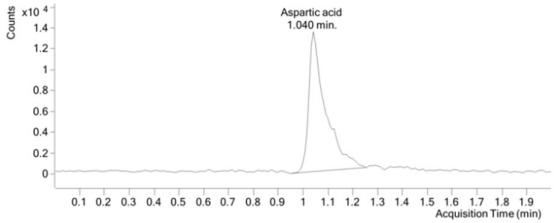
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Citrulline (*m/z* 176 → *m/z* 113,1) 	Citrulline (*m/z* 176 → *m/z* 113,1) 	
Asparagine (*m/z* 133 → *m/z* 87) 	Asparagine (*m/z* 133 → *m/z* 87) 	GABA-d6 (*m/z* 110 → *m/z* 93) 
Aspartic acid (*m/z* 134 → *m/z* 88) 	Aspartic acid (*m/z* 134 → *m/z* 88) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

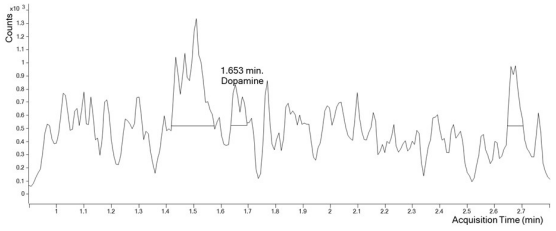
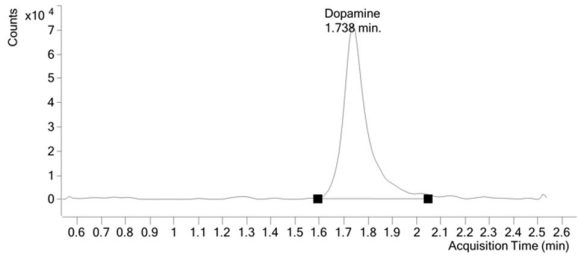
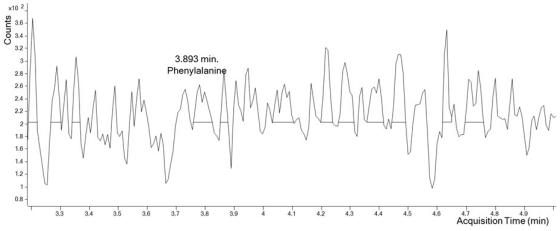
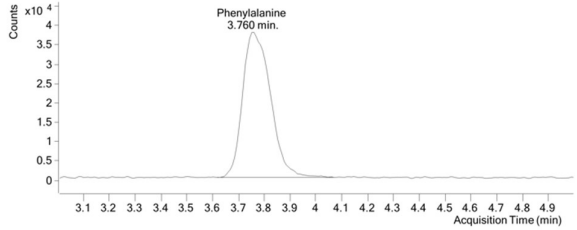
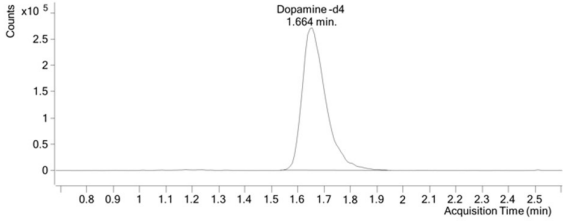
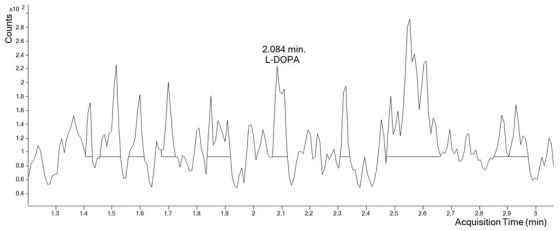
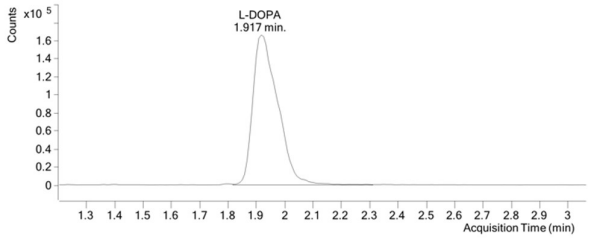
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Dopamine (*m/z* 154 → *m/z* 91) 	Dopamine (*m/z* 154 → *m/z* 91) 	
Phenylalanine (*m/z* 166 → *m/z* 120) 	Phenylalanine (*m/z* 166 → *m/z* 120) 	Dopamine-d4 (*m/z* 158 → *m/z* 123) 
L-DOPA (*m/z* 198 → *m/z* 181) 	L-DOPA (*m/z* 198 → *m/z* 181) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

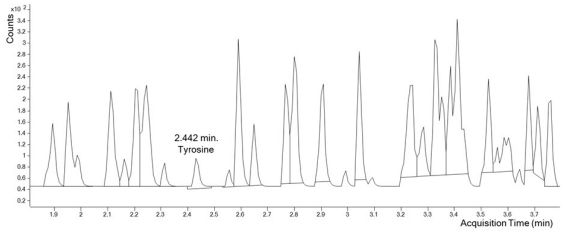
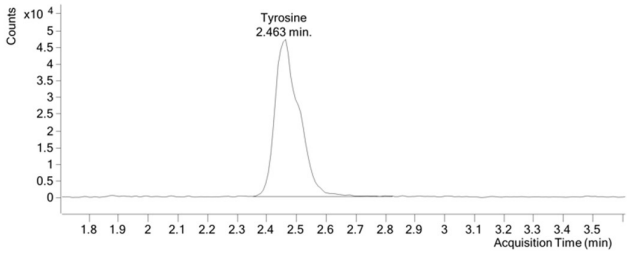
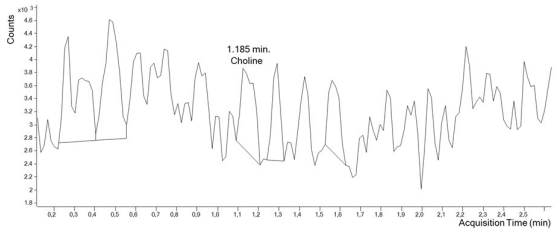
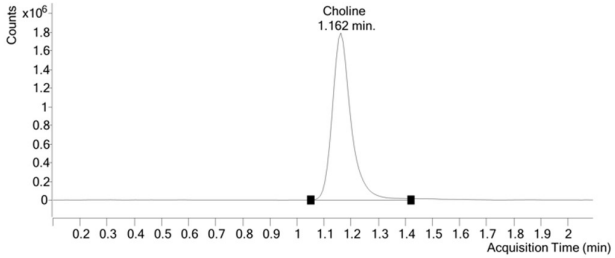
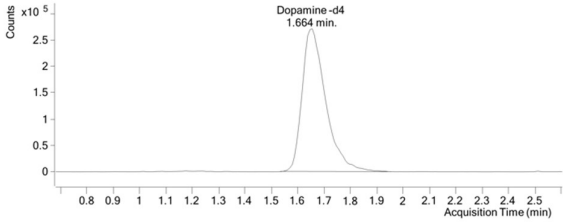
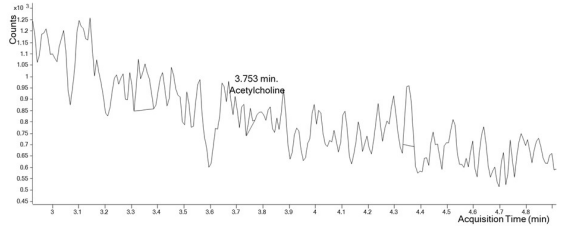
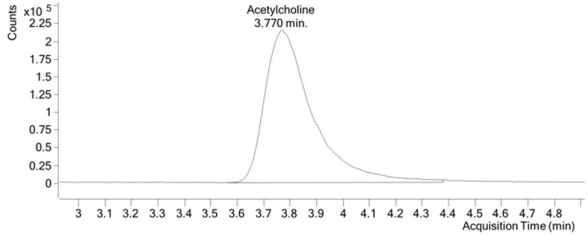
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Tyrosine (*m/z* 182 → *m/z* 165) 	Tyrosine (*m/z* 182 → *m/z* 165) 	
Choline (*m/z* 104 → *m/z* 60) 	Choline (*m/z* 104 → *m/z* 60) 	Dopamine-d4 (*m/z* 158 → *m/z* 123) 
Acetylcholine (*m/z* 146 → *m/z* 87) 	Acetylcholine (*m/z* 146 → *m/z* 87) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

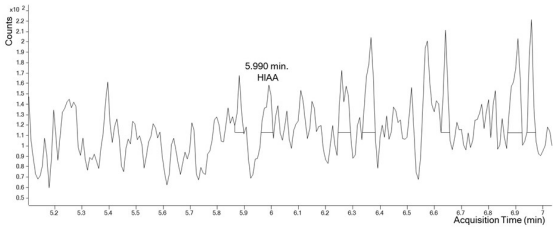
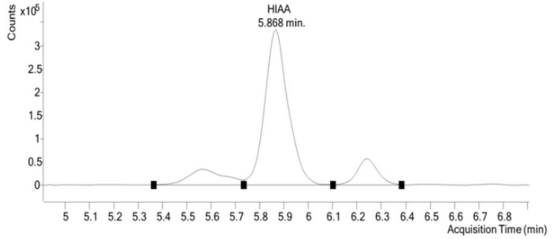
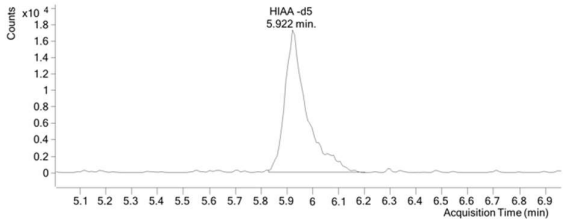
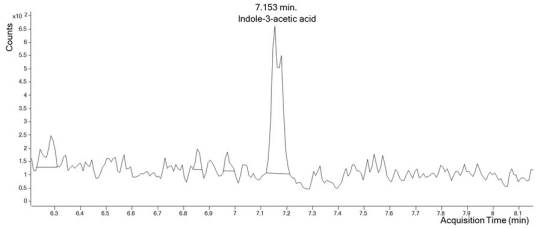
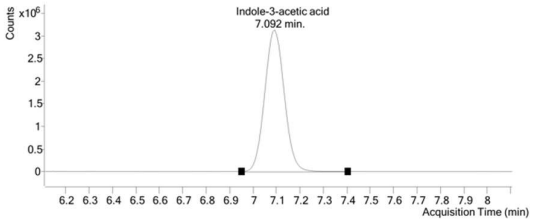
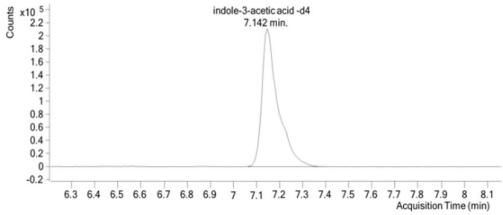
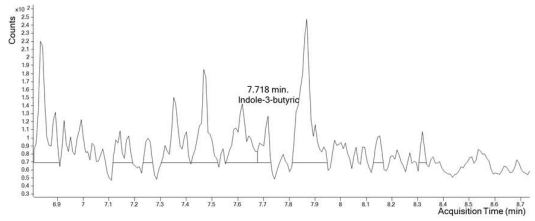
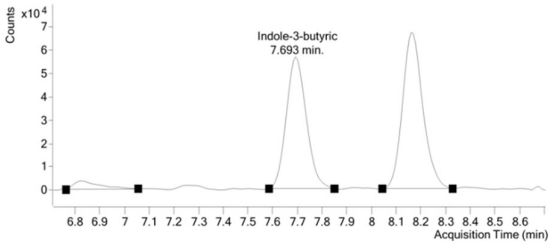
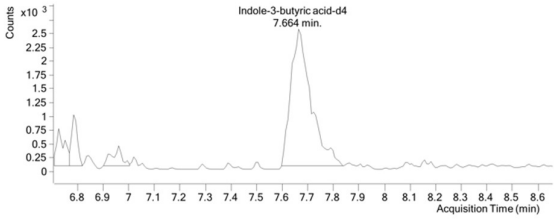
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
5-hydroxyindole acetic acid (*m/z* 192 → *m/z* 146) 	5-hydroxyindole acetic acid (*m/z* 192 → *m/z* 146) 	5-hydroxyindole acetic acid-d5 (*m/z* 197 → *m/z* 150) 
Indole-3-acetic acid (*m/z* 176 → *m/z* 130) 	Indole-3-acetic acid (*m/z* 176 → *m/z* 130) 	Indole-3-acetic acid-d4 (*m/z* 180,1 → *m/z* 133) 
Indole-3-butyric acid (*m/z* 204 → *m/z* 144) 	Indole-3-butyric acid (*m/z* 204 → *m/z* 144) 	Indole-3-butyric acid-d4 (*m/z* 208 → *m/z* 132) 

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

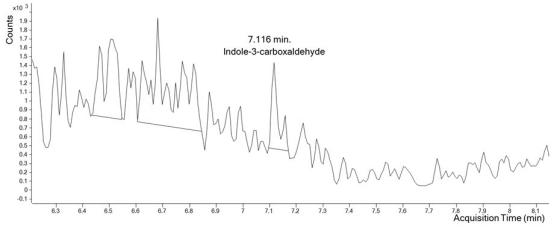
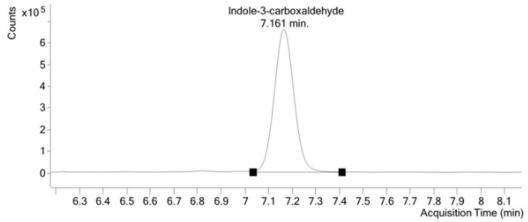
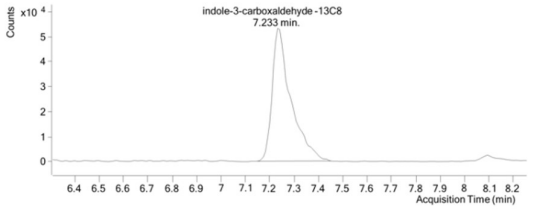
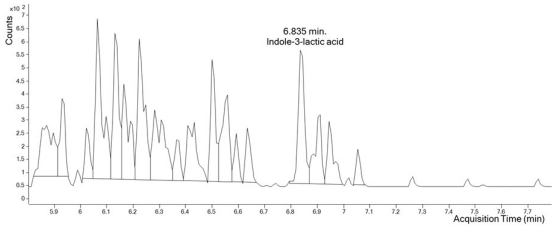
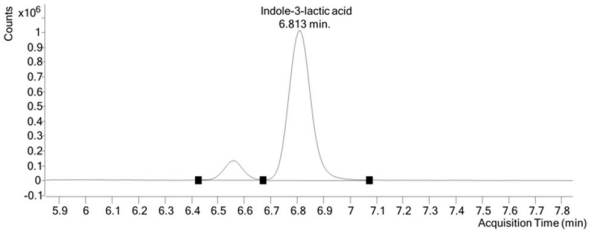
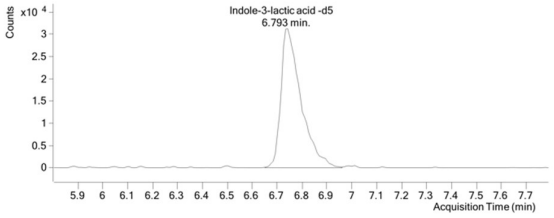
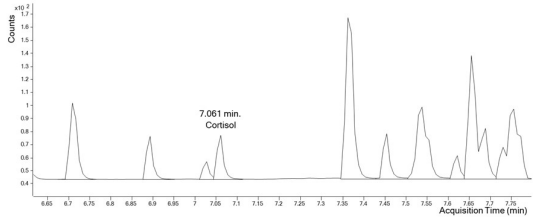
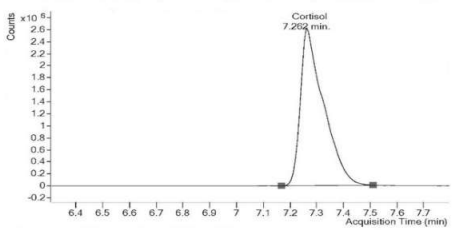
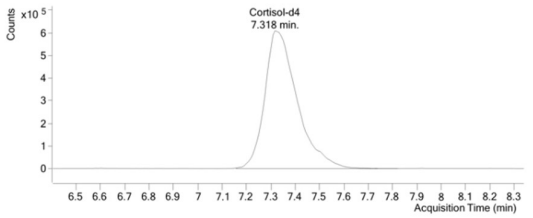
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Indole-3-carboxaldehyde (*m/z* 146 → *m/z* 118) 	Indole-3-carboxaldehyde (*m/z* 146 → *m/z* 118) 	Indole-3-carboxaldehyde-$_{13}$C8 (*m/z* 154 → *m/z* 126) 
Indole-3-lactic acid (*m/z* 206 → *m/z* 118) 	Indole-3-lactic acid (*m/z* 206 → *m/z* 118) 	Indole-3-lactic acid-d5 (*m/z* 211 → *m/z* 122) 
Cortisol (*m/z* 363 → *m/z* 327) 	Cortisol (*m/z* 363 → *m/z* 327) 	Cortisol-d4 (*m/z* 367 → *m/z* 331) 

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

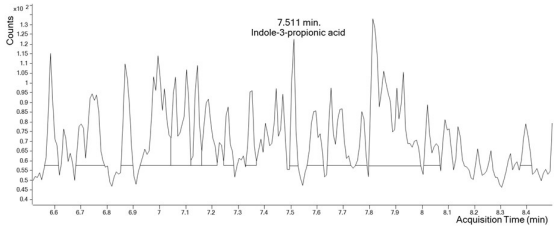
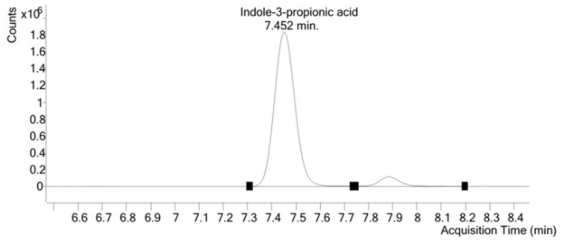
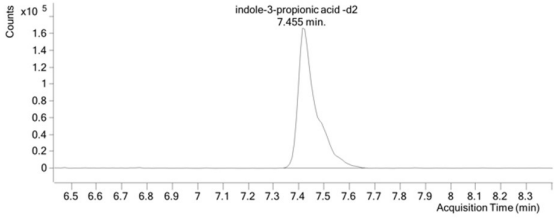
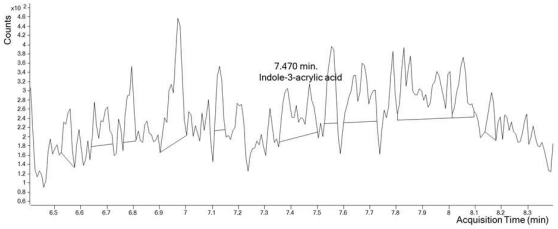
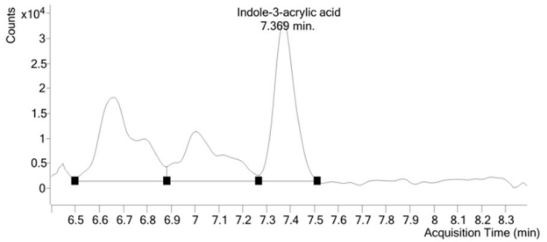
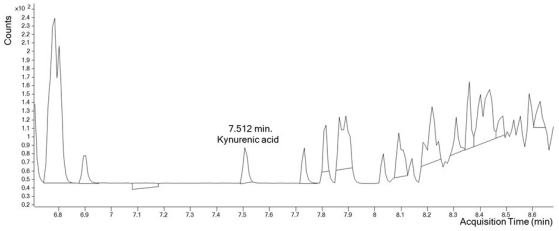
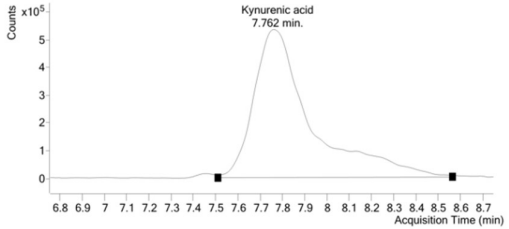
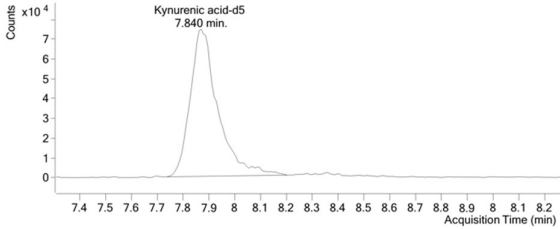
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Indole-3-propionic acid (*m/z* 190 → *m/z* 130) 	Indole-3-propionic acid (*m/z* 190 → *m/z* 130) 	Indole-3-propionic acid-d2 (*m/z* 192 → *m/z* 130) 
Indole-3-acrylic acid (*m/z* 188 → *m/z* 170) 	Indole-3-acrylic acid (*m/z* 188 → *m/z* 170) 	
Kynurenic acid (*m/z* 190 → *m/z* 144) 	Kynurenic acid (*m/z* 190 → *m/z* 144) 	Kynurenic acid-d5 (*m/z* 195 → *m/z* 149) 

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

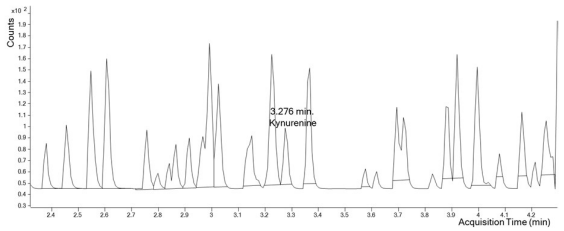
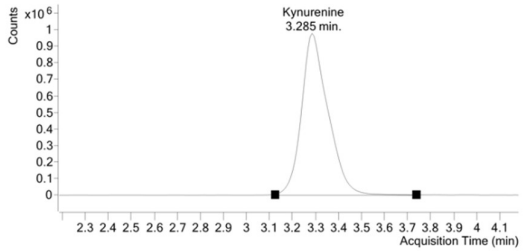
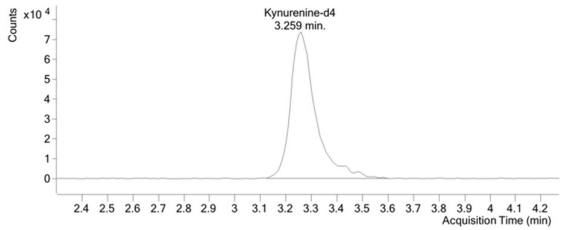
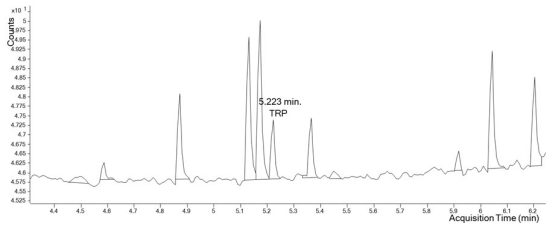
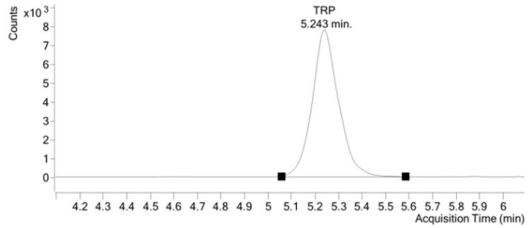
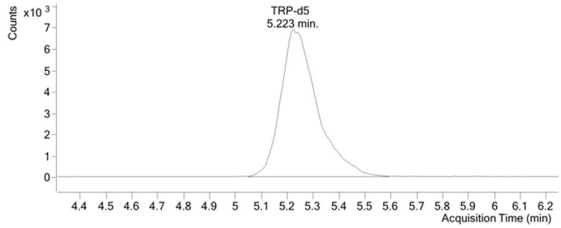
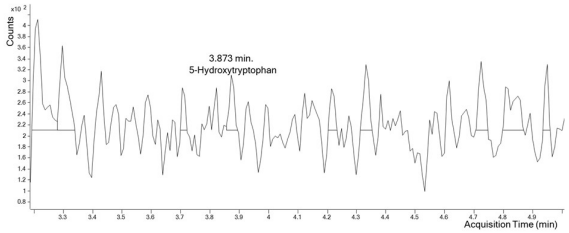
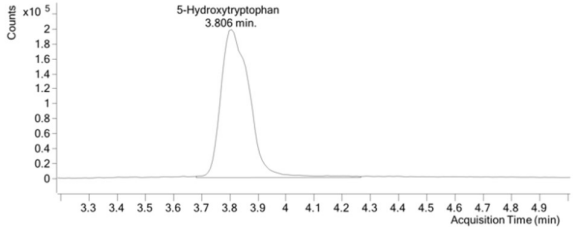
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Kynurenine (*m/z* 209 → *m/z* 146) 	Kynurenine (*m/z* 209 → *m/z* 146) 	Kynurenine-d4 (*m/z* 213 → *m/z* 98) 
Tryptophan (*m/z* 205 → *m/z* 188) 	Tryptophan (*m/z* 205 → *m/z* 188) 	Tryptophan-d5 (*m/z* 208 → *m/z* 164) 
5-hydroxytryptophan (*m/z* 221 → *m/z* 162) 	5-hydroxytryptophan (*m/z* 221 → *m/z* 162) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

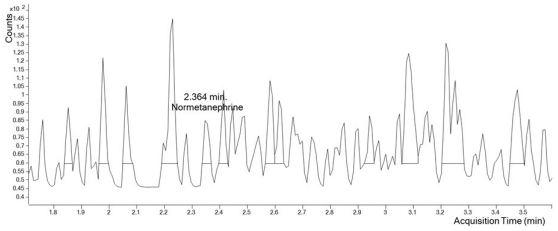
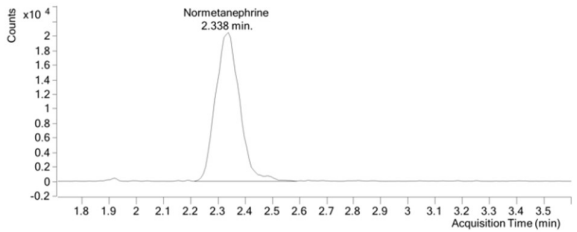
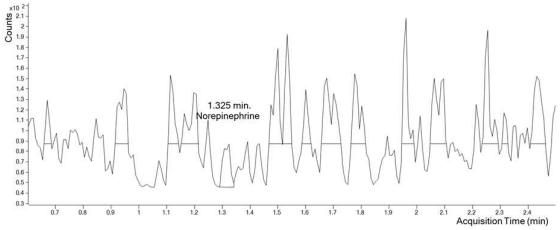
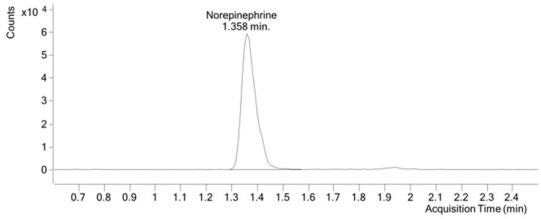
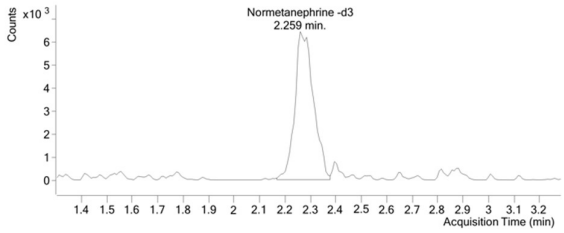
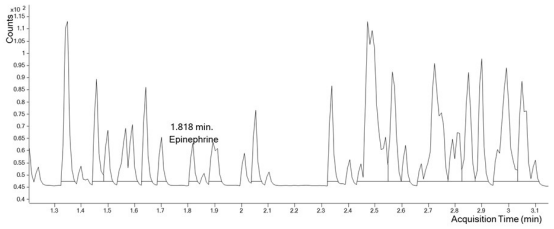
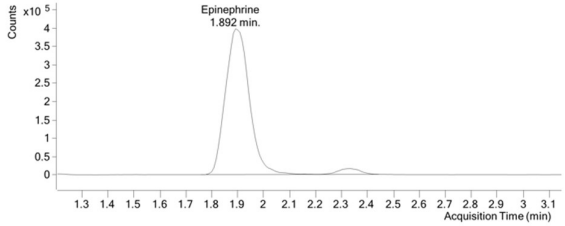
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Normetanephrine (*m/z* 166 → *m/z* 134) 	Normetanephrine (*m/z* 166 → *m/z* 134) 	
Norepinephrine (*m/z* 170 → *m/z* 152) 	Norepinephrine (*m/z* 170 → *m/z* 152) 	Normetanephrine-d3 (*m/z* 187 → *m/z* 137) 
Epinephrine (*m/z* 184 → *m/z* 166) 	Epinephrine (*m/z* 184 → *m/z* 166) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

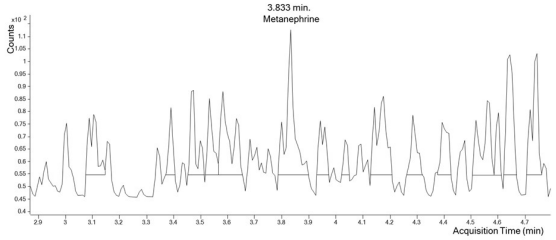
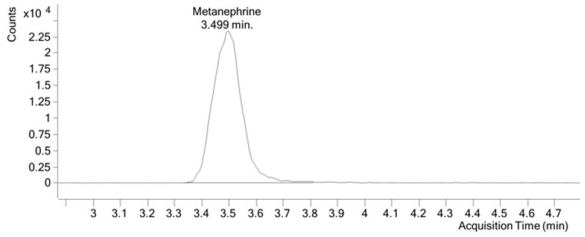
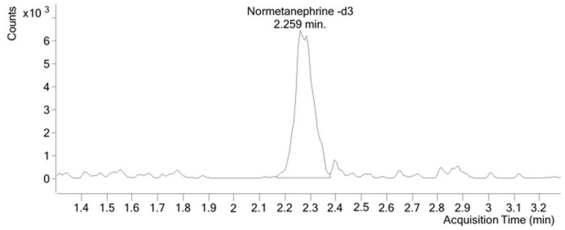
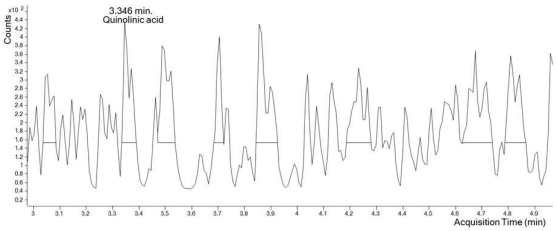
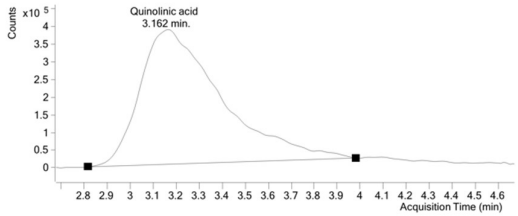
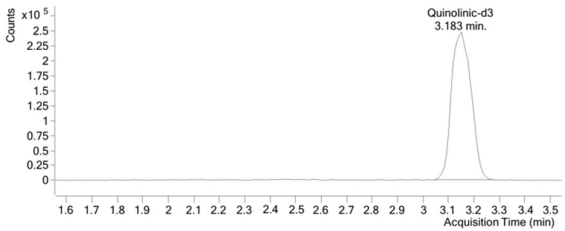
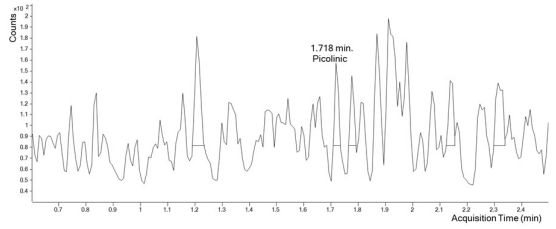
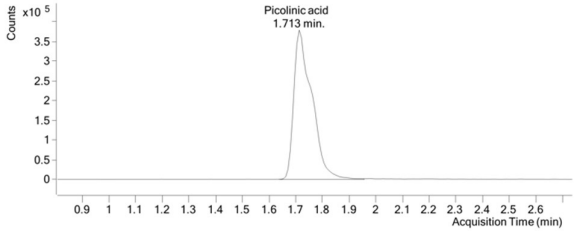
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Metanephrine (*m/z* 198 → *m/z* 180) 	Metanephrine (*m/z* 198 → *m/z* 180) 	Normetanephrine-d3 (*m/z* 187 → *m/z* 137) 
Quinolinic acid (*m/z* 168 → *m/z* 106,1) 	Quinolinic acid (*m/z* 168 → *m/z* 106,1) 	Quinolinic acid-d3 (*m/z* 171 → *m/z* 109) 
Picolinic acid (*m/z* 124 → *m/z* 78,1) 	Picolinic acid (*m/z* 124 → *m/z* 78,1) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

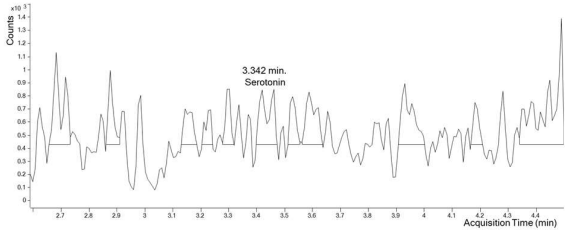
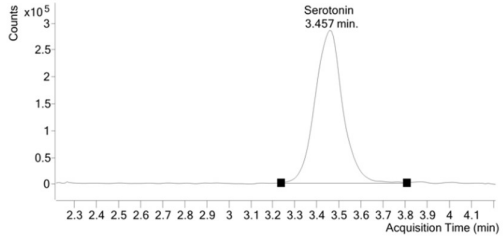
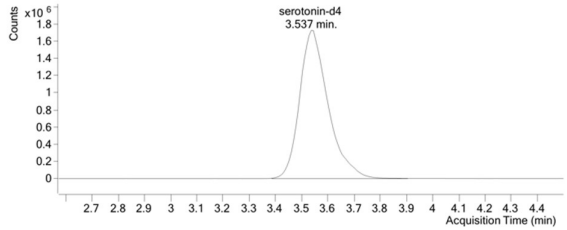
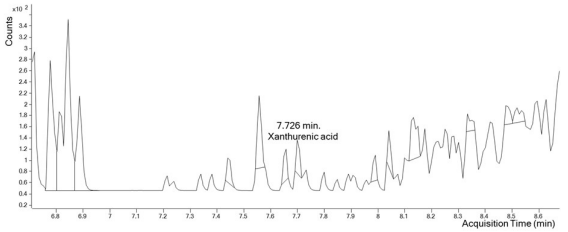
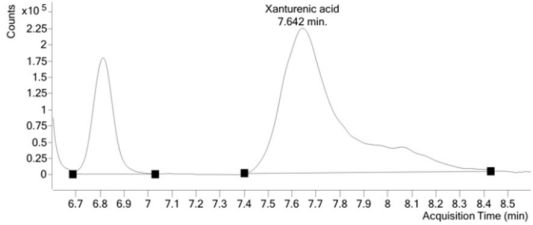
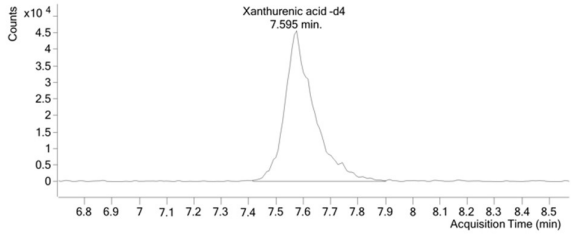
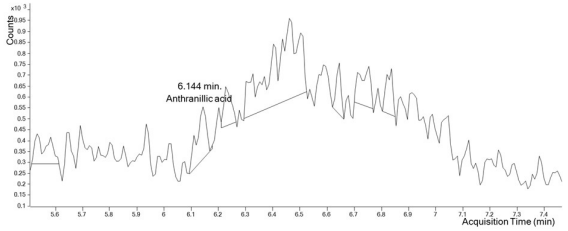
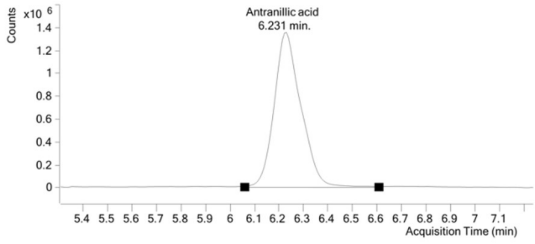
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Serotonin (*m/z* 160 → *m/z* 132) 	Serotonin (*m/z* 160 → *m/z* 132) 	Serotonin-d4 (*m/z* 181 → *m/z* 164) 
Xanthurenic acid (*m/z* 206 → *m/z* 160) 	Xanthurenic acid (*m/z* 206 → *m/z* 160) 	Xanthurenic acid-d4 (*m/z* 210 → *m/z* 164) 
Anthranilic acid (*m/z* 138 → *m/z* 120,1) 	Anthranilic acid (*m/z* 138 → *m/z* 120,1) 	

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

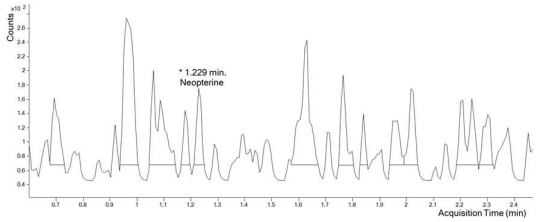
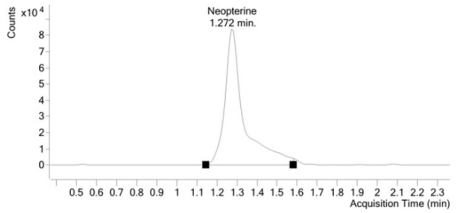
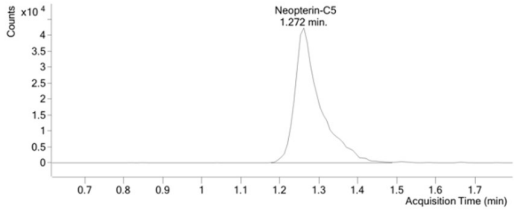
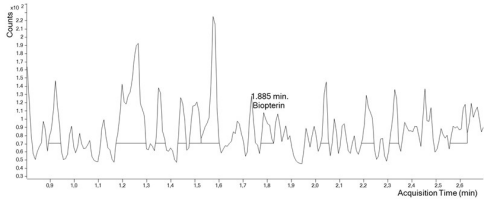
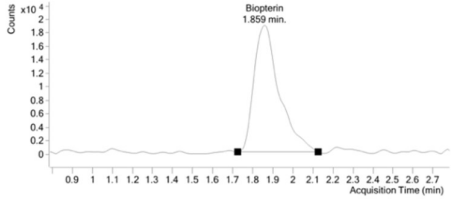
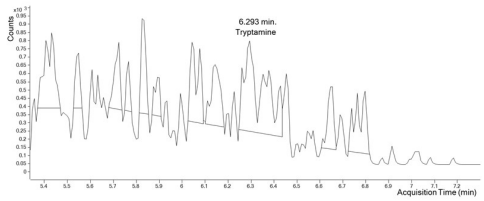
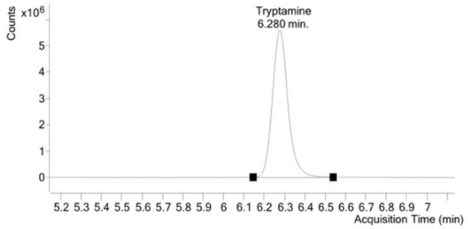
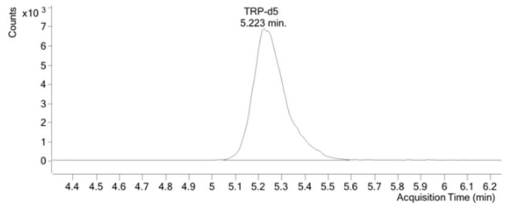
Blank chromatogram	QC sample chromatogram	Internal standard chromatogram
Neopterin (*m/z* 254 → *m/z* 206,1) 	Neopterin (*m/z* 254 → *m/z* 206,1) 	Neopterin-$_{13}$C5 (*m/z* 259,1 → *m/z* 211) 
Biopterin (*m/z* 238 → *m/z* 194) 	Biopterin (*m/z* 238 → *m/z* 194) 	
Tryptamine (*m/z* 161 → *m/z* 144,1) 	Tryptamine (*m/z* 161 → *m/z* 144,1) 	Tryptophan-d5 (*m/z* 208 → *m/z* 164) 

Figure 8 – Mass-chromatograms of the blank samples, QC samples, and internal standards (cont.)

3.2.2. Calibration curve and lowest limit of quantification

To investigate the dependence between the concentrations of the metabolites in the samples, calibration curves were made. The calibrators were made using blank samples that were previously spiked with the known concentrations of the analytes.

Calibration curves were made using six calibration levels, and each was analyzed in three replicates. Weighted linear regression models were used for the building of the calibration curves. The slope, intercept, and determination coefficient (R2) were calculated for each analyte. The calibration curve acceptance criterion was: R2 coefficient was >0.97.

Also, LLOQ was quantified in this assessment. LLOQ was defined as the concentration of the analyte that can be determined with high accuracy and precision; the analyte's signal in the assessment was more than five times higher than this in the blank sample. The calibration curve parameters are represented in Table 6.

Table 6 – Calibration curve characteristics for endogenous metabolites

Metabolite	Linear range	Slope	Intercept	Determination coefficient (r^2)
Tryptophan	10-1000 ug/ml	0,8531	-2,3589	0,9961
Kynurenine	10-50000 ng/ml	0,4001	-0,1024	0,9998
Choline	1-100 ug/ml	0,5206	0,1729	0,9936
Serotonin	1-25000 ng/ml	0,0990	-0,0052	0,9998
Quinolinic acid	1-1000 ng/ml	0,054	-0,0033	0,9999
Indole-3-propionic acid	1-1000 ng/ml	0,068	-0,0020	0,9996
Indole-3-lactic acid	10-10000 ng/ml	0,2631	-0,027	0,9954
Indole-3-acetic acid	10-1000 ng/ml	0,1058	0,0039	0,9991
Xanthurenic acid	0,2-2000 ng/ml	10,415	-0,1453	0,9995
Tryptamine	0,02-200 ng/ml	2,118	0,0105	0,9980
Biopterin	0,2-200 ng/ml	0,0073	-0,0002	0,9953
Kynurenic acid	0,2-200 ng/ml	1,632	-0,010	0,9968
Indole-3-carboxaldehyde	0,2-200 ng/ml	1,257	0,028	0,9981
Indole-3-butyric acid	0,2-200 ng/ml	1,216	0,011	0,9918
Indole-3-acrylic acid	0,2-200 ng/ml	4,097	0,040	0,9967
5-hydroxyindole acetic acid	0,2-200 ng/ml	1,077	0,045	0,9852
Dopamine	0,2-200 ng/ml	0,8632	-0,014	0,9987
Neopterin	0,2-200 ng/ml	0,0030	0,00002	0,9918
Anthranilic acid	0,2-200 ng/ml	2,118	0,671	0,9993
GABA	1-500 ng/ml	0,0543	-0,0002	0,9995
5-hydroxytryptophan	1-1000 ng/ml	1,936	-0,054	0,9970

Metabolite	Linear range	Slope	Intercept	Determination coefficient (r^2)
Acetylcholine	0,1-100 ng/ml	12,940	7,490	0,9981
Asparagine	1-1000 ng/ml	0,070	0,029	0,9868
Aspartic acid	1-1000 ng/ml	0,097	0,139	0,9733
Citrulline	50-5000 ng/ml	4,190	3,367	0,9970
Cortisol	0,1-1000 ng/ml	0,103	-0,0041	0,9936
Epinephrine	1-500 ng/ml	13,597	3,995	0,9820
Glutamic acid	10-5000 ng/ml	0,396	0,113	0,9970
Glutamine	10-5000 ng/ml	0,225	0,111	0,9923
L-DOPA	1-1000 ng/ml	0,417	0,042	0,9996
Metanephrine	10-5000 ng/ml	7,423	1,025	0,9762
Norepinephrine	1-1000 ng/ml	12,988	-0,427	0,9839
Normetanephrine	1-500 ng/ml	24,801	-0,098	0,9896
Phenylalanine	1-100 ug/ml	1,821	0,762	0,9993
Picolinic acid	1-1000 ng/ml	3,876	1,249	0,9993
Tyrosine	1-100 ug/ml	0,484	0,308	0,9992

3.2.3. Carryover

The carryover of the analytes was assessed through the injection of the blank sample right after the injection of the calibration sample with the highest concentration. The carryover was taken as acceptable if the analyte signal in the blank sample was less than 20% of the LLOQ.

3.2.4. Precision and accuracy

Precision and accuracy assessment of the developed method was performed using QC samples. The precision and accuracy were quantified for within-runs and between-runs. Within-run precision and accuracy were assessed by measuring QC samples at four concentration levels during one analytical cycle. Evert QC sample was analyzed six times. Between-run precision and accuracy were evaluated by measuring QC samples at four concentration levels during three analytical cycles. The measured concentrations were assessed using calibration curves. Precision and accuracy were considered acceptable if:

1. Mean concentrations of the analytes were in the limit of ± 20% of the nominal values;

2. Within- and between-runs coefficients of variation were in the limit of ± 20%.

The results are shown in Table 7.

Table 7 – Accuracy and precision of the method

Metabolite	**Percent from the nominal concentration of QC, % (RSD, %)**				
		LLOQ QC	**LQC**	**MQC**	**HQC**
Tryptophan	1 cycle	91,9 (7,9)	100,4 (5,8)	98,8 (7,9)	102,3 (5,0)
	2 cycle	101,8 (6,3)	102,7 (5,9)	100,3 (5,1)	103,0 (5,4)
	3 cycle	105,9 (8,0)	112,8 (5,3)	92,4 (6,6)	105,3 (6,6)
	Between cycles	**102,3 (9,8)**	**105,5 (8,7)**	**97,1 (9,6)**	**103,6 (10,9)**
Kynurenine	1 cycle	80,3 (6,5)	104,3 (5,2)	95,0 (5,3)	99,6 (5,7)
	2 cycle	119,1 (6,9)	87,2 (6,2)	93,9 (6,4)	100,9 (5,5)
	3 cycle	87,5 (7,7)	106,8 (6,4)	91,6 (6,9)	100,6 (8,0)
	Between cycles	**95,6 (11,5)**	**99,4 (8,7)**	**93,5 (9,7)**	**100,3 (9,2)**
Choline	1 cycle	91,9 (7,3)	84,0 (7,2)	103,9 (5,1)	87,4 (5,7)
	2 cycle	109,5 (7,3)	98,0 (7,0)	106,8 (5,1)	86,5 (7,0)
	3 cycle	108,0 (5,2)	114,4 (5,6)	101,1 (7,6)	93,4 (5,4)
	Between cycles	**103,1 (12,5)**	**98,8 (10,2)**	**104,0 (12,3)**	**89,1 (11,9)**
Serotonin	1 cycle	112,1 (7,8)	118,4 (6,2)	100,0 (6,5)	105,1 (7,7)
	2 cycle	115,9 (5,7)	113,4 (7,7)	103,5 (6,6)	107,8 (6,1)
	3 cycle	106,3 (6,4)	111,9 (7,8)	102,6 (5,3)	103,3 (6,2)
	Between cycles	**111,4 (10,9)**	**114,6 (11,5)**	**100,7 (10,5)**	**105,3 (8,7)**
Quinolinic acid	1 cycle	93,8 (6,8)	106,9 (6,5)	112,1 (7,6)	111,3 (5,1)
	2 cycle	113,7 (7,8)	99,7 (5,4)	108,0 (7,6)	110,1 (6,1)
	3 cycle	97,4 (5,1)	111,4 (5,1)	111,1 (7,4)	109,8 (7,4)
	Between cycles	**101,6 (11,1)**	**106,0 (9,8)**	**110,4 (12,4)**	**110,4 (8,9)**
Indole-3-propionic acid	1 cycle	96,0 (7,5)	111,6 (5,8)	99,4 (5,5)	96,7 (6,1)
	2 cycle	114,7 (5,3)	110,1 (5,6)	103,0 (5,4)	97,2 (6,0)
	3 cycle	93,1 (6,4)	109,0 (5,1)	100,6 (7,6)	96,5 (5,2)
	Between cycles	**101,2 (8,3)**	**110,2 (9,9)**	**101,0 (10,4)**	**96,8 (11,6)**
Indole-3-lactic acid	1 cycle	99,8 (7,2)	115,9 (5,4)	100,8 (5,3)	105,2 (6,1)
	2 cycle	90,6 (7,6)	103,0 (6,6)	103,5 (6,8)	107,8 (5,0)
	3 cycle	107,9 (7,6)	107,5 (6,3)	102,6 (6,6)	103,3 (6,4)
	Between cycles	**99,5 (8,3)**	**108,8 (9,9)**	**100,8 (10,4)**	**105,2 (11,6)**
Indole-3-acetic acid	1 cycle	108,4 (7,3)	117,5 (6,3)	106,6 (8,0)	96,8 (6,1)

Metabolite	Percent from the nominal concentration of QC, % (RSD, %)				
		LLOQ QC	LQC	MQC	HQC
	2 cycle	81,7 (7,4)	113,0 (7,3)	102,0 (6,7)	100,9 (8,0)
	3 cycle	119,2 (6,8)	84,0 (6,0)	106,4 (5,9)	100,4 (7,1)
	Between cycles	**103,1 (12,4)**	**104,9 (10,1)**	**105,0 (8,8)**	**99,3 (9,3)**
Xanthurenic acid	1 cycle	104,6 (5,7)	105,3 (7,9)	99,6 (5,0)	96,8 (5,9)
	2 cycle	113,5 (7,3)	92,5 (5,2)	100,8 (7,0)	98,9 (7,7)
	3 cycle	86,0 (5,1)	99,9 (6,0)	100,8 (5,9)	99,0 (5,0)
	Between cycles	**101,3 (8,4)**	**99,2 (9,8)**	**100,4 (10,3)**	**98,3 (9,8)**
Tryptamine	1 cycle	97,1 (6,5)	92,0 (5,6)	106,6 (5,4)	111,3 (6,1)
	2 cycle	119,1 (7,7)	116,1 (5,1)	102,0 (7,0)	110,1 (7,3)
	3 cycle	109,5 (5,5)	106,2 (6,4)	106,4 (6,3)	109,8 (6,2)
	Between cycles	**94,4 (11,2)**	**104,7 (8,0)**	**105,0 (9,1)**	**110,4 (9,4)**
Biopterin	1 cycle	101,6 (6,3)	91,8 (5,5)	108,9 (7,7)	96,8 (8,0)
	2 cycle	99,0 (7,2)	109,4 (7,3)	111,3 (7,6)	98,9 (7,7)
	3 cycle	116,1 (7,5)	92,0 (5,6)	109,0 (5,3)	99,0 (6,0)
	Between cycles	**117,4 (9,9)**	**97,7 (10,5)**	**109,8 (9,5)**	**98,3 (9,7)**
Kynurenic acid	1 cycle	116,7 (5,8)	106,0 (7,2)	100,8 (7,0)	105,2 (6,7)
	2 cycle	93,1 (6,9)	94,9 (6,6)	103,5 (7,4)	107,8 (7,6)
	3 cycle	97,1 (6,8)	100,0 (6,6)	102,6 (5,8)	103,3 (7,1)
	Between cycles	**93,5 (8,3)**	**100,3 (9,1)**	**100,8 (8,1)**	**105,2 (8,4)**
Indole-3-carboxaldehyde	1 cycle	87,7 (7,9)	101,7 (6,6)	112,1 (5,3)	110,2 (6,6)
	2 cycle	109,5 (7,4)	99,1 (6,9)	108,0 (7,2)	112,0 (6,8)
	3 cycle	87,6 (6,5)	80,7 (7,8)	111,1 (5,6)	105,6 (7,3)
	Between cycles	**82,3 (8,6)**	**93,8 (9,5)**	**110,4 (12,8)**	**109,3 (11,2)**
Indole-3-butyric acid	1 cycle	116,2 (5,8)	106,2 (6,8)	101,0 (5,7)	101,6 (7,8)
	2 cycle	118,9 (5,5)	111,2 (7,6)	102,8 (5,3)	104,6 (5,2)
	3 cycle	84,3 (5,7)	85,5 (6,4)	100,4 (5,6)	100,9 (5,5)
	Between cycles	**111,8 (11,6)**	**100,9 (11,5)**	**101,4 (9,9)**	**102,8 (9,0)**
Indole-3-acrylic acid	1 cycle	103,7 (6,8)	88,1 (6,4)	104,1 (6,4)	96,7 (6,1)
	2 cycle	110,7 (6,5)	100,4 (5,3)	104,8 (7,9)	97,2 (5,8)
	3 cycle	93,9 (5,2)	112,0 (6,4)	106,6 (6,7)	96,5 (6,8)

Metabolite	Percent from the nominal concentration of QC, % (RSD, %)				
		LLOQ QC	LQC	MQC	HQC
	Between cycles	**100,3 (11,0)**	**100,2 (9,5)**	**105,1 (10,4)**	**96,8 (8,5)**
5-hydroxyindole acetic acid	1 cycle	91,8 (7,2)	89,7 (7,4)	100,8 (7,5)	96,8 (7,4)
	2 cycle	118,4 (6,1)	80,7 (7,1)	103,5 (5,8)	98,9 (6,4)
	3 cycle	115,6 (7,5)	112,8 (5,9)	102,6 (5,7)	99,0 (7,6)
	Between cycles	**81,9 (10,0)**	**94,4 (10,5)**	**100,8 (11,6)**	**98,3 (12,1)**
Dopamine	1 cycle	84,7 (6,6)	106,4 (6,8)	100,6 (6,3)	106,8 (5,8)
	2 cycle	80,3 (7,6)	106,9 (6,2)	105,4 (5,4)	107,6 (6,7)
	3 cycle	99,3 (6,0)	103,9 (6,3)	103,6 (5,9)	102,1 (7,0)
	Between cycles	**111,4 (12,1)**	**105,7 (9,3)**	**103,3 (10,2)**	**105,4 (8,6)**
Neopterin	1 cycle	114,5 (6,1)	83,2 (6,0)	106,6 (5,2)	106,8 (5,4)
	2 cycle	86,2 (5,5)	112,3 (6,1)	102,0 (7,1)	107,6 (6,1)
	3 cycle	117,7 (8,0)	114,3 (6,6)	106,4 (6,7)	102,1 (7,4)
	Between cycles	**112,7 (9,4)**	**103,2 (10,5)**	**105,0 (9,3)**	**105,4 (10,4)**
Anthranilic acid	1 cycle	88,8 (6,3)	103,5 (5,2)	96,4 (5,4)	103,9 (5,1)
	2 cycle	88,4 (5,9)	86,6 (6,4)	98,4 (6,0)	105,8 (7,3)
	3 cycle	117,5 (6,7)	106,7 (8,0)	96,5 (5,9)	99,3 (7,2)
	Between cycles	**81,4 (11,6)**	**98,9 (12,4)**	**97,3 (10,0)**	**103,0 (9,4)**
GABA	1 cycle	111,0 (5,5)	97,8 (5,3)	105,8 (6,3)	99,6 (5,1)
	2 cycle	104,4 (5,5)	98,5 (7,8)	93,4 (7,8)	101,1 (5,9)
	3 cycle	84,8 (5,8)	110,3 (6,7)	94,1 (6,1)	99,0 (6,7)
	Between cycles	**100,1 (9,4)**	**102,2 (11,4)**	**97,8 (12,5)**	**100,0 (8,3)**
5-hydroxytryptophan	1 cycle	92,2 (5,3)	98,5 (7,3)	99,7 (5,7)	98,0 (5,7)
	2 cycle	86,1 (6,7)	95,6 (7,9)	96,0 (6,8)	96,2 (7,9)
	3 cycle	109,4 (7,1)	100,2 (6,2)	98,8 (6,9)	95,6 (6,7)
	Between cycles	**95,9 (9,2)**	**98,1 (10,1)**	**98,1 (8,9)**	**96,6 (11,5)**
Acetylcholine	1 cycle	81,8 (6,4)	109,3 (6,7)	96,8 (5,3)	104,9 (7,2)
	2 cycle	80,4 (5,7)	110,3 (7,1)	98,9 (6,5)	108,3 (5,5)
	3 cycle	113,0 (5,5)	100,8 (5,7)	96,9 (7,0)	108,4 (5,5)
	Between cycles	**85,6 (9,4)**	**106,8 (8,1)**	**97,6 (9,6)**	**107,2 (12,5)**
Asparagine	1 cycle	81,6 (6,0)	105,3 (6,5)	98,8 (6,7)	105,6 (7,5)

Metabolite	Percent from the nominal concentration of QC, % (RSD, %)				
		LLOQ QC	LQC	MQC	HQC
	2 cycle	101,7 (6,3)	84,8 (5,1)	100,1 (6,0)	105,8 (6,3)
	3 cycle	94,3 (7,5)	107,5 (5,8)	100,1 (6,3)	105,8 (6,3)
	Between cycles	**104,4 (9,1)**	**99,2 (8,2)**	**100,0 (8,6)**	**105,7 (12,2)**
Aspartic acid	1 cycle	115,8 (7,9)	115,3 (7,2)	112,1 (6,6)	108,0 (6,7)
	2 cycle	109,5 (5,3)	81,7 (6,7)	108,0 (8,0)	106,2 (6,3)
	3 cycle	90,9 (6,5)	118,0 (6,0)	111,1 (5,5)	105,6 (5,2)
	Between cycles	**105,4 (10,4)**	**105,0 (12,7)**	**110,4 (12,4)**	**106,6 (9,8)**
Citrulline	1 cycle	116,9 (5,3)	100,3 (6,4)	95,0 (5,7)	99,6 (5,5)
	2 cycle	92,1 (6,3)	91,9 (6,6)	93,9 (6,2)	100,9 (5,7)
	3 cycle	83,2 (7,0)	81,9 (6,7)	91,6 (7,8)	100,5 (7,0)
	Between cycles	**97,4 (12,4)**	**91,4 (9,7)**	**93,5 (10,1)**	**100,3 (9,0)**
Cortisol	1 cycle	92,9 (6,0)	97,8 (6,1)	104,2 (5,6)	105,5 (7,2)
	2 cycle	111,5 (5,4)	86,0 (5,3)	109,2 (7,3)	105,6 (6,4)
	3 cycle	85,2 (6,0)	111,4 (6,3)	103,0 (6,9)	105,4 (7,9)
	Between cycles	**116,2 (10,0)**	**98,4 (12,7)**	**101,8 (9,5)**	**105,5 (10,4)**
Epinephrine	1 cycle	92,0 (5,9)	112,8 (6,1)	100,7 (6,6)	91,3 (5,7)
	2 cycle	106,4 (7,4)	84,6 (5,1)	92,4 (7,4)	102,3 (5,3)
	3 cycle	118,8 (6,8)	100,8 (7,7)	100,2 (6,9)	101,2 (6,1)
	Between cycles	**105,8 (11,1)**	**99,4 (8,3)**	**97,8 (8,2)**	**98,0 (10,8)**
Glutamic acid	1 cycle	95,3 (5,9)	114,1 (7,2)	103,9 (5,6)	97,9 (6,7)
	2 cycle	113,4 (5,4)	87,2 (5,7)	106,9 (6,2)	95,2 (5,6)
	3 cycle	107,2 (6,1)	96,0 (6,2)	101,1 (6,0)	94,7 (5,3)
	Between cycles	**105,3 (8,4)**	**99,1 (8,3)**	**104,0 (12,0)**	**99,3 (9,3)**
Glutamine	1 cycle	83,2 (6,2)	86,6 (5,6)	95,0 (6,4)	99,7 (5,6)
	2 cycle	115,6 (5,4)	87,5 (5,1)	93,9 (7,2)	100,7 (7,2)
	3 cycle	87,6 (5,8)	111,6 (6,4)	91,6 (5,3)	100,4 (7,8)
	Between cycles	**95,5 (10,0)**	**95,2 (10,5)**	**93,5 (12,8)**	**100,2 (10,9)**
L-DOPA	1 cycle	106,6 (6,1)	91,6 (6,4)	100,8 (7,6)	108,3 (7,9)
	2 cycle	102,3 (6,2)	113,7 (5,8)	103,5 (7,7)	102,5 (6,4)
	3 cycle	105,5 (5,7)	101,7 (6,5)	102,6 (6,4)	105,3 (5,7)

Metabolite	Percent from the nominal concentration of QC, % (RSD, %)				
		LLOQ QC	LQC	MQC	HQC
	Between cycles	**104,8 (12,6)**	**102,3 (12,3)**	**100,8 (8,8)**	**108,3 (8,5)**
Metanephrine	1 cycle	86,6 (6,8)	112,3 (7,1)	95,0 (5,5)	119,7 (5,6)
	2 cycle	103,1 (6,0)	116,3 (6,9)	93,9 (6,7)	100,7 (7,9)
	3 cycle	89,9 (6,2)	95,7 (7,5)	91,6 (6,7)	100,4 (7,4)
	Between cycles	**93,2 (8,2)**	**108,1 (8,7)**	**93,5 (12,1)**	**100,2 (10,7)**
Norepinephrine	1 cycle	110,6 (7,8)	109,9 (7,6)	112,1 (5,3)	108,0 (5,2)
	2 cycle	105,7 (7,6)	115,0 (7,9)	108,0 (5,1)	106,2 (7,2)
	3 cycle	104,7 (7,2)	85,6 (7,9)	111,1 (5,8)	105,6 (5,4)
	Between cycles	**107,0 (9,2)**	**103,5 (12,3)**	**110,4 (12,7)**	**106,6 (9,8)**
Normetanephrine	1 cycle	108,0 (6,9)	89,6 (7,7)	106,6 (7,6)	109,6 (7,0)
	2 cycle	100,0 (7,0)	82,4 (5,8)	102,0 (5,0)	102,8 (6,4)
	3 cycle	106,0 (5,6)	93,2 (5,5)	106,4 (6,8)	101,4 (7,5)
	Between cycles	**104,7 (11,0)**	**88,4 (11,6)**	**105,0 (8,9)**	**117,6 (12,2)**
Phenylalanine	1 cycle	105,0 (6,1)	100,0 (5,4)	98,8 (6,6)	100,9 (5,9)
	2 cycle	105,1 (6,7)	100,3 (7,3)	100,3 (7,5)	101,2 (7,5)
	3 cycle	105,3 (6,7)	112,7 (6,3)	92,4 (7,7)	102,1 (5,9)
	Between cycles	**105,1 (9,0)**	**105,3 (10,1)**	**97,1 (12,8)**	**101,4 (12,2)**
Picolinic acid	1 cycle	99,1 (5,6)	111,4 (7,3)	100,8 (6,3)	108,3 (7,4)
	2 cycle	98,3 (6,5)	116,6 (6,2)	103,5 (6,7)	102,5 (7,9)
	3 cycle	113,3 (5,5)	93,7 (6,0)	102,6 (5,2)	105,3 (5,9)
	Between cycles	**103,5 (11,1)**	**107,3 (10,2)**	**100,8 (10,5)**	**108,3 (8,3)**
Tyrosine	1 cycle	100,5 (6,7)	93,3 (5,2)	103,9 (7,4)	100,9 (6,9)
	2 cycle	102,5 (7,7)	100,6 (6,3)	106,8 (7,9)	100,8 (6,5)
	3 cycle	104,5 (6,5)	101,6 (6,6)	101,1 (7,4)	100,7 (5,4)
	Between cycles	**103,8 (8,2)**	**102,3 (9,4)**	**104,0 (10,7)**	**101,0 (12,6)**

3.2.5. Matrix effect

Matrix effect assessment was made using six series of blank samples. Matrix effects were quantified for LQC and HQC levels. The level of matrix effect was applicable if the RSD was in the limits of ± 15%. The results are shown in Table 8.

Table 8 – Matrix effect for the measured metabolites

Metabolite	LQC		HQC	
	% from the nominal concentration	RSD, %	% from the nominal concentration	RSD, %
Tryptophan	96,1	7,9	101,1	6,8
Kynurenine	111,4	3,4	102,1	4,5
Choline	100,1	3,2	110,2	7,9
Serotonin	92,1	6,8	103,1	4,5
Quinolinic acid	95,7	2,3	96,1	6,8
Indole-3-propionic acid	97,1	3,4	93,3	3,4
Indole-3-lactic acid	103,1	1,1	100,1	4,5
Indole-3-acetic acid	98,1	3,4	95,9	1,1
Xanthurenic acid	96,1	2,3	106,2	6,8
Tryptamine	110,2	5,7	111,8	4,5
Biopterin	112,7	5,9	109,2	6,7
Kynurenic acid	99,1	7,9	100,1	6,0
Indole-3-carboxaldehyde	101,1	1,1	93,0	6,1
Indole-3-butyric acid	91,8	1,8	93,0	7,9
Indole-3-acrylic acid	99,1	4,5	109,2	4,5
5-hydroxyindole acetic acid	100,1	6,8	111,4	1,1
Dopamine	107,2	9,1	94,3	3,4
Neopterin	102,1	1,1	95,7	2,3
Anthranilic acid	91,4	6,8	92,7	7,9
GABA	111,2	5,7	104,1	7,9
5-hydroxytryptophan	110,2	3,4	99,1	3,4
Acetylcholine	106,2	9,1	104,1	4,5
Asparagine	95,5	7,9	91,0	4,5
Aspartic acid	98,1	5,7	105,2	7,9
Citrulline	103,1	4,5	111,2	2,3
Cortisol	96,1	7,9	92,0	2,3
Epinephrine	107,2	6,8	97,1	3,4
Glutamic acid	104,4	6,8	107,2	5,7
Glutamine	95,0	2,3	105,2	6,8
L-DOPA	96,1	7,9	104,1	5,7
Metanephrine	99,1	3,4	95,0	4,5
Norepinephrine	109,2	5,7	102,1	7,9
Normetanephrine	94,0	7,8	106,2	3,4
Phenylalanine	107,6	7,0	100,1	4,5
Picolinic acid	100,1	7,1	98,1	2,3
Tyrosine	93,0	6,8	106,2	1,1

3.2.6. Extraction coefficient

The extraction coefficient is calculated as a comparison of the peak areas of the analytes in the samples spiked with the calibration solutions before and after the extraction process. The assessment was made using LQC and HQC samples. The extraction coefficient was applicable if the RSD was in the limits of ± 15%. The results are shown in Table 9.

Table 9 – Extraction coefficient for the measured metabolites

Соединение	LQC		HQC	
	% from the nominal concentration	RSD, %	% from the nominal concentration	RSD, %
Tryptophan	91,0	2,3	97,1	3,4
Kynurenine	108,2	5,7	108,2	6,9
Choline	93,3	1,1	97,1	7,9
Serotonin	91,7	9,1	105,1	4,5
Quinolinic acid	95,0	5,7	98,1	7,1
Indole-3-propionic acid	99,1	9,1	104,2	5,7
Indole-3-lactic acid	91,9	6,8	95,0	9,1
Indole-3-acetic acid	92,5	2,3	98,1	3,4
Xanthurenic acid	104,1	1,1	105,2	5,7
Tryptamine	94,4	3,1	108,9	6,8
Biopterin	104,1	5,3	92,0	3,4
Kynurenic acid	93,7	1,1	97,1	6,8
Indole-3-carboxaldehyde	108,2	3,4	98,1	7,9
Indole-3-butyric acid	102,1	6,8	95,0	5,7
Indole-3-acrylic acid	93,1	2,3	106,2	9,1
5-hydroxyindole acetic acid	103,1	5,7	93,0	5,7
Dopamine	102,1	7,9	111,2	1,1
Neopterin	95,0	6,8	101,1	7,9
Anthranilic acid	109,2	2,3	106,2	7,8
GABA	101,1	7,9	92,0	7,7
5-hydroxytryptophan	95,0	1,1	100,1	3,4
Acetylcholine	103,1	3,4	91,0	1,1
Asparagine	97,1	7,9	107,2	2,3
Aspartic acid	107,2	3,4	100,1	3,4
Citrulline	104,1	5,7	98,1	9,1
Cortisol	100,1	4,5	97,1	5,7
Epinephrine	107,2	6,8	95,0	1,1
Glutamic acid	107,2	1,1	109,2	3,4
Glutamine	108,2	4,5	110,2	3,4
L-DOPA	111,2	9,1	91,0	2,3
Metanephrine	93,0	4,5	99,1	3,4
Norepinephrine	106,2	9,1	96,3	3,4
Normetanephrine	92,0	6,8	91,0	5,7

Phenylalanine	103,1	5,7	94,0	1,1
Picolinic acid	105,2	7,9	108,2	9,1
Tyrosine	109,2	1,1	103,1	9,1

3.2.7. Stability

The stability of the analytes was assessed using LQC, and HQC samples were analyzed right after the sample preparation and after designed storage conditions. The stability was assessed for:

1. The working solutions of the analytes after 6 h storage at room temperature (21 ± 3ºC);

2. Biological samples, stored for 24 h in the autosampler at the temperature of 10 ± 0,5ºC;

3. Biological samples that underwent three freeze/thaw cycles;

4. Biological samples stored in the freezer with a temperature of minus 35 ± 1ºC for 30 days.

The stability was acceptable if the RSD of the measured concentrations were in the limit of ± 15 % of their nominal values. The results of the assessment are shown in Table 10.

Table 10 – Stability results for the measured metabolites

Metabolite	LQC				HQC			
	6 h, room temp (standard)	24 h, autosampler	30 days in -35±1°C	After three freeze-thaw cycles	6 h, room temp (standard)	24 h, autosampler	30 days in -35±1°C	After three freeze-thaw cycles
Tryptophan	100,9	103.3	98.8	112,2	102.6	90,7	102.6	108,1
Kynurenine	111.7	105.0	95.9	110,2	99.3	95.9	99.3	101,1
Choline	99.9	97.5	101.6	100,1	98.1	100,9	102.9	109,2
Serotonin	97.5	103.3	97.2	111,8	100,9	99.2	104.4	103,1
Quinolinic acid	99.2	105.0	98.8	107,2	101.5	97.0	102.3	108,8
Indole-3-propionic acid	97.0	101.6	95.9	101,1	97.7	96.0	102.6	108,2
Indole-3-lactic acid	96.0	97.5	101.6	110,2	105.4	102.6	99.3	97,1
Indole-3-acetic acid	102.6	101.1	97.2	92,0	97.7	106.4	102.9	100,1
Xanthurenic acid	106.4	100,9	100,9	111,2	98.1	103.1	104.4	105,2
Tryptamine	103.1	111.7	99.2	98,1	100,9	98.0	102.3	94,0
Biopterin	99.2	99.9	97.0	93,0	101.5	95.3	102.6	99,1
Kynurenic acid	97.0	103.3	96.0	97,1	97.7	100,0	101.6	95,0
Indole-3-carboxaldehyde	99.5	105.0	102.6	110,2	102.9	90,7	98.7	109,2
Indole-3-butyric acid	96.4	97.5	106.4	110,2	104.4	95.9	95.8	100,8
Indole-3-acrylic acid	98.7	103.3	100,9	100,1	102.3	100,9	96.8	92,0
5-hydroxyindole acetic acid	99.4	105.0	99.2	102,1	103.2	99.2	99.7	97,1
Dopamine	104.3	101.6	97.0	92,7	102.9	97.0	94.6	92,7
Neopterin	102.0	97.5	96.0	96,1	104.4	96.0	98.7	109,2
Anthranilic acid	96.3	106.4	104.3	103,1	102.3	101.1	105.4	101,9
GABA	101.6	98.0	106.4	97,1	100,9	99.2	101.6	99,1
5-hydroxytryptophan	97.5	95.3	103.1	111,2	99.2	97.0	97.5	102,3
Acetylcholine	101.1	100,0	98.0	92,0	97.0	96.0	101.1	98,1

Metabolite	LQC				HQC			
	6 h, room temp (standard)	24 h, autosampler	30 days in -35±1ºC	After three freeze-thaw cycles	6 h, room temp (standard)	24 h, autosampler	30 days in -35±1ºC	After three freeze-thaw cycles
Asparagine	100,9	90,7	95.3	102,1	96.0	102.6	100,9	101,1
Aspartic acid	111.7	95.9	100,0	105,8	102.6	106.4	111.7	102,8
Citrulline	99.9	100,9	90,7	105,0	106.4	103.1	99.9	108,2
Cortisol	103.3	99.2	95.9	107,2	100,9	99.2	103.3	103,1
Epinephrine	105.0	97.0	100,9	94,4	99.2	97.0	105.0	107,4
Glutamic acid	97.5	96.0	99.2	96,1	97.0	99.5	97.5	91,0
Glutamine	103.3	101.1	111.7	105,2	96.0	96.4	103.3	94,5
L-DOPA	97.0	102.3	99.9	108,2	104.3	98.7	102.3	109,2
Metanephrine	96.0	103.2	103.3	99,1	102.6	99.2	103.2	99,1
Norepinephrine	104.3	102.9	105.0	106,2	97,1	97.0	102.9	111,2
Normetanephrine	102.6	104.4	97.5	99,1	110,2	96.0	104.4	104,1
Phenylalanine	94.2	102.3	103.3	108,2	110,2	101.1	102.3	92,3
Picolinic acid	96.3	97.2	93.5	100,1	100,1	97.5	97.2	109,2
Tyrosine	101.2	98.7	92.6	96,1	102,1	99.7	102.4	95,4

3.3. Neurotransmitters metabolomics after short- and long-term exposure of diazepam in zebrafish larvae

To investigate the possibility of the usage of the zebrafish as a model organism in the assessment of the pharmacological action of the neurotropic drugs using the pharmacometabolomic approach, the concentrations of the metabolites of GABAergic, serotonergic, dopaminergic/adrenergic, cholinergic neurotransmitter systems, aspartic acid system, kynurenine, and indole pathway metabolites, and other related metabolites after the exposure of diazepam at the doses of 0.8, 1.6, 16, 160 ug/L for short- (2.5 h) and long-term (96 h). Diazepam is a pharmacological compound with clearly described properties and mechanism of action on the central nervous system. Diazepam is used as a reference compound in investigating potential anxiolytic drugs [5, 29].

The list of the measured compounds is represented in Table 1. The method of quantification of these compounds was developed and validated. The validation results of the method are presented in chapter 3, section 2.

The main statistical differences in the concentrations of endogenous metabolites after short-term diazepam exposure are presented in Table 11. The main statistical differences in the concentrations of endogenous metabolites after long-term diazepam exposure are shown in Table 12.

Table 11 – Detailed information on the statistical differences across experimental groups after short-term diazepam exposure

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
GABA	-	-	-	-	-	-	-	<0,05 ↓	<0,05 ↓	-
Glutamic acid	-	-	-	-	-	-	<0,01 ↑	-	-	-
Glutamine	-	-	-	-	-	-	-	-	-	-
Tryptophan	-	-	-	<0,05 ↑	-	<0,01 ↑	<0,001 ↑	-	-	-
5-hydroxytryptophan	<0,05 ↓	-	-	-	-	-	<0,001 ↑	-	-	-
Serotonin	-	-	<0,001 ↑	<0,001 ↑	-	<0,05 ↑	<0,05 ↑	-	-	-
5-hydroxyindole acetic acid	-	<0,001 ↓	-	-	-	-	-	-	<0,001 ↑	-
Tryptamine	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	<0,01 ↑	-	<0,01 ↑	-
Tyrosine	<0,05 ↓	-	-	-	-	-	<0,001 ↑	-	<0,01 ↑	-
L-DOPA	-	-	-	-	-	<0,05 ↓	<0,01 ↓	-	-	-
Dopamine	-	-	-	<0,05 ↓	<0,05 ↓	-	<0,001 ↓	-	-	-
Norepinephrine	<0,05 ↑	-	-	-	<0,001 ↓	-	-	-	-	-
Normetanephrine	-	<0,05 ↓	-	-	<0,001 ↓	<0,01 ↓	-	-	-	-
Epinephrine	-	-	-	-	<0,001 ↓	<0,05 ↓	<0,01 ↓	-	-	-
Metanephrine	<0,05 ↓	<0,05 ↓	-	<0,05 ↓	-	-	-	-	-	-
Aspartic acid	-	-	-	<0,05 ↑	-	<0,05 ↑	<0,001 ↑	-	-	-
Asparagine	-	-	-	-	-	-	-	-	-	-
Acetylcholine	-	-	-	-	-	-	-	<0,05 ↓	-	-

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
Choline	-	-	-	-	-	-	<0,05 ↑	-	<0,05 ↑	<0,01 ↑
Kynurenine	<0,05 ↓	<0,05 ↓	<0,001 ↓	-	-	-	-	-	-	<0,01↑
Kynurenic acid	-	<0,01 ↑	-	-	-	-	-	<0,001 ↓	-	<0,01 ↑
Anthranilic acid	-	-	-	-	-	-	-	<0,05 ↓	<0,01 ↓	-
Xanthurenic acid	-	-	-	-	-	-	-	-	-	-
Quinolinic acid	-	-	-	-	-	-	-	-	-	-
Picolinic acid	<0,001 ↑	-	-	<0,05 ↑	-	-	-	-	-	-
Indole-3-carboxaldehyde	-	-	-	-	-	-	-	-	-	-
Indole-3-acetic acid	-	-	-	-	-	-	<0,05 ↑	-	<0,05 ↑	-
Indole-3-butyric acid	-	-	-	-	-	-	-	-	-	-
Indole-3-lactic acid	-	-	-	-	-	<0,01 ↑	<0,001 ↑	-	<0,05 ↑	-
Indole-3-acrylic acid	-	-	-	-	-	-	-	-	-	-
Indole-3-propionic acid	-	-	-	-	-	-	-	-	-	-
Cortisol	-	-	-	-	-	-	-	-	-	-
Citrulline	-	-	-	<0,01 ↑	-	-	<0,05 ↑	-	-	-
Biopterin	-	<0,001 ↓	<0,01 ↓	-	-	-	-	-	<0,05 ↑	-
Neopterin	-	-	-	-	-	-	-	-	-	-

Table 12 – Detailed information on the statistical differences across experimental groups after long-term diazepam exposure

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
GABA	-	-	-	-	-	-	-	-	-	-
Glutamic acid	-	-	-	-	-	-	-	-	-	-
Glutamine	-	-	-	-	-	-	-	-	-	-
Tryptophan	<0,001 ↓	-	<0,05 ↓	<0,05 ↓	<0,01 ↑	-	-	-	-	-
5-hydroxytryptophan	<0,001 ↓	-	<0,01 ↓	<0,05 ↓	-	-	-	-	-	
Serotonin	-	0,01 ↑	-	<0,05 ↑	<0,001 ↑	-	<0,01 ↑	-	-	-
5-hydroxyindole acetic acid	<0,01 ↓	-	-	<0,001 ↓	-	-	-	-	<0,01 ↓	-
Tryptamine	-	-	-	-	-	-	-	-	-	-
Phenylalanine	<0,05 ↑	-	<0,05 ↑	<0,001 ↑	-	-	-	-	-	-
Tyrosine	-	-	<0,01 ↓	<0,001 ↓	-	-	-	-	<0,01 ↓	-
L-DOPA	-	-	-	-	-	-	-	-	-	-
Dopamine	-	-	-	<0,05 ↓	-	-	-	-	<0,01 ↓	-
Norepinephrine	-	-	-	<0,05 ↑	-	-	<0,05 ↑	-	-	-
Normetanephrine	-	-	-	-	<0,05 ↑	-	-	-	-	-
Epinephrine	-	<0,001 ↑	-	-	-	-	-	-	<0,01 ↓	-
Metanephrine	-	-	<0,01 ↑	<0,05 ↑	-	-	-	-	-	-
Aspartic acid	-	-	<0,05 ↑	<0,001 ↑	-	-	-	-	-	-
Asparagine	-	-	-	-	-	-	-	-	-	-
Acetylcholine	<0,05 ↓	-	-	<0,001 ↓	-	-	-	-	<0,01 ↓	<0,05 ↓

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
Choline	-	<0,05 ↑	-	-	<0,01 ↑	-	-	-	-	-
Kynurenine	-	<0,01 ↓	-	<0,001 ↓	-	-	-	-	-	<0,05 ↓
Kynurenic acid	-	-	<0,05 ↑	-	-	-	-	<0,05 ↑	-	-
Anthranilic acid	-	-	-	-	-	<0,05 ↓	-	-	-	-
Xanthurenic acid	-	-	-	-	-	-	-	-	-	-
Quinolinic acid	<0,05 ↑	-	-	-	-	-	-	-	-	-
Picolinic acid	-	-	-	<0,01 ↑	-	-	-	-	<0,01 ↑	-
Indole-3-carboxaldehyde	<0,01 ↑	-	-	-	-	-	-	-	-	-
Indole-3-acetic acid	<0,001 ↓	-	<0,001 ↓	-	<0,05 ↑	-	-	-	-	-
Indole-3-butyric acid	<0,05 ↓	-	-	-	-	-	-	-	-	-
Indole-3-lactic acid	<0,001 ↓	-	<0,05 ↓	<0,05 ↓	<0,01 ↑	-	-	-	-	-
Indole-3-acrylic acid	-	-	-	-	-	-	-	-	-	-
Indole-3-propionic acid	-	-	-	-	<0,01 ↓	-	-	-	-	-
Cortisol	-	-	-	-	-	-	-	-	-	-
Citrulline	<0,01 ↑	-	-	<0,01 ↑	-	-	-	-	-	-
Biopterin	<0,05 ↓	-	-	-	<0,001 ↑	-	-	-	-	-
Neopterin	-	-	-	-	-	-	-	-	-	-

3.3.1. Morphological characterictics

Morphologically, the zebrafish larvae did not present any significant alterations for the short or medium-term exposures. In particular, there were no disturbances in the body formation, tail formation, hyperpigmentation found; the fins, somites, and notochords were formed correctly; there were no disturbances in the internal organs' formation found (Figure 9).

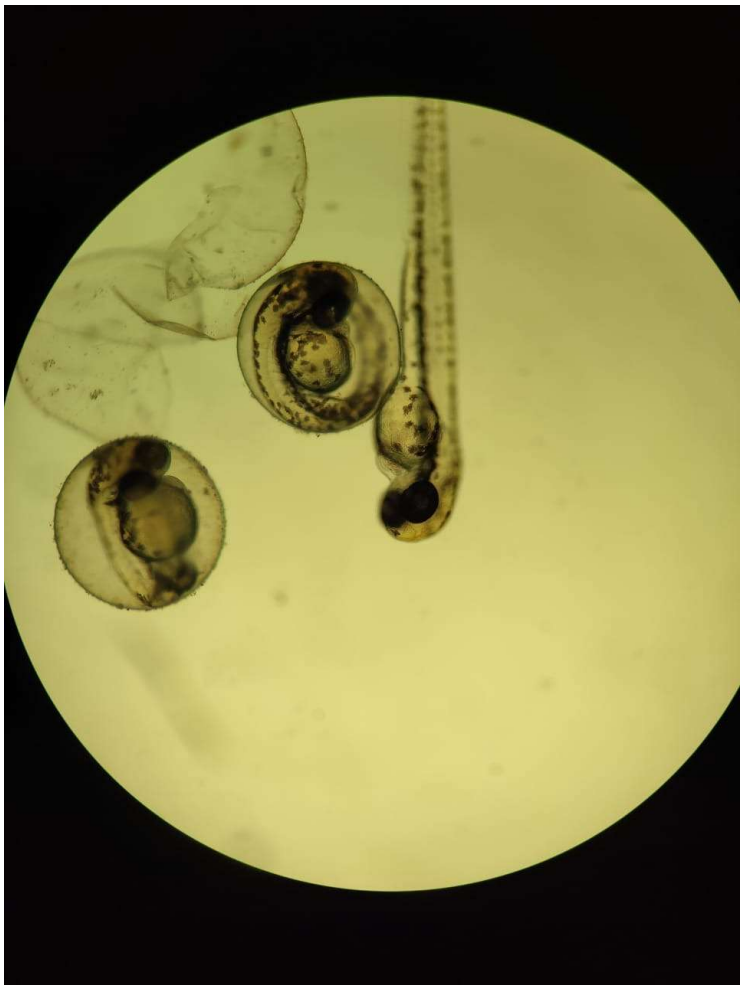


Figure 9 – Zebrafish larvae after the exposure to diazepam at a concentration of 160 ug/L for a long-term.

3.3.2. GABAergic system and aspartic acid system

This section presents the results of the short- and long-term exposure of diazepam on the levels of endogenous metabolites of the GABAergic and aspartic

acid systems. Figure 10 shows the scheme of aspartic and glutamic acid and GABA metabolism.

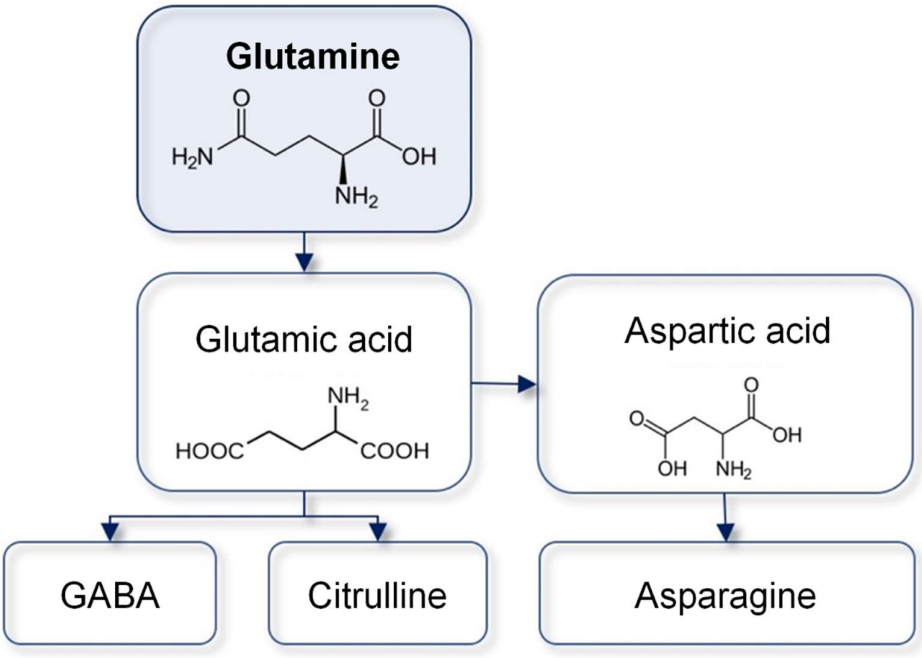


Figure 10 – GABAergic, aspartic acid, and glutamic acid system biochemical pathway.

GABAergic system

In short-term exposure, no dose-dependent trends in increasing or decreasing concentrations of GABA were found. The only group which presented higher ($p < 0.05$) concentrations of GABA was the group that was exposed to 1.6 µg/L versus the groups exposed to 16 and 160 µg/L (Figure 11A). Also, in 96 h exposure, none of the groups showed significant changes in GABA concentrations (Figure 11A). Concerning glutamic acid, the only group which showed a significant increase of concentrations ($p < 0.01$) was the group exposed to the highest dose (160 µg/L), versus the group receiving 0.8 µg/L of diazepam (Figure 11B). In long-term exposure, none of the groups were significantly different (Figure 11B). Glutamine concentrations did not change in any group in 2.5 h and 96 h exposures (Figure 11C).

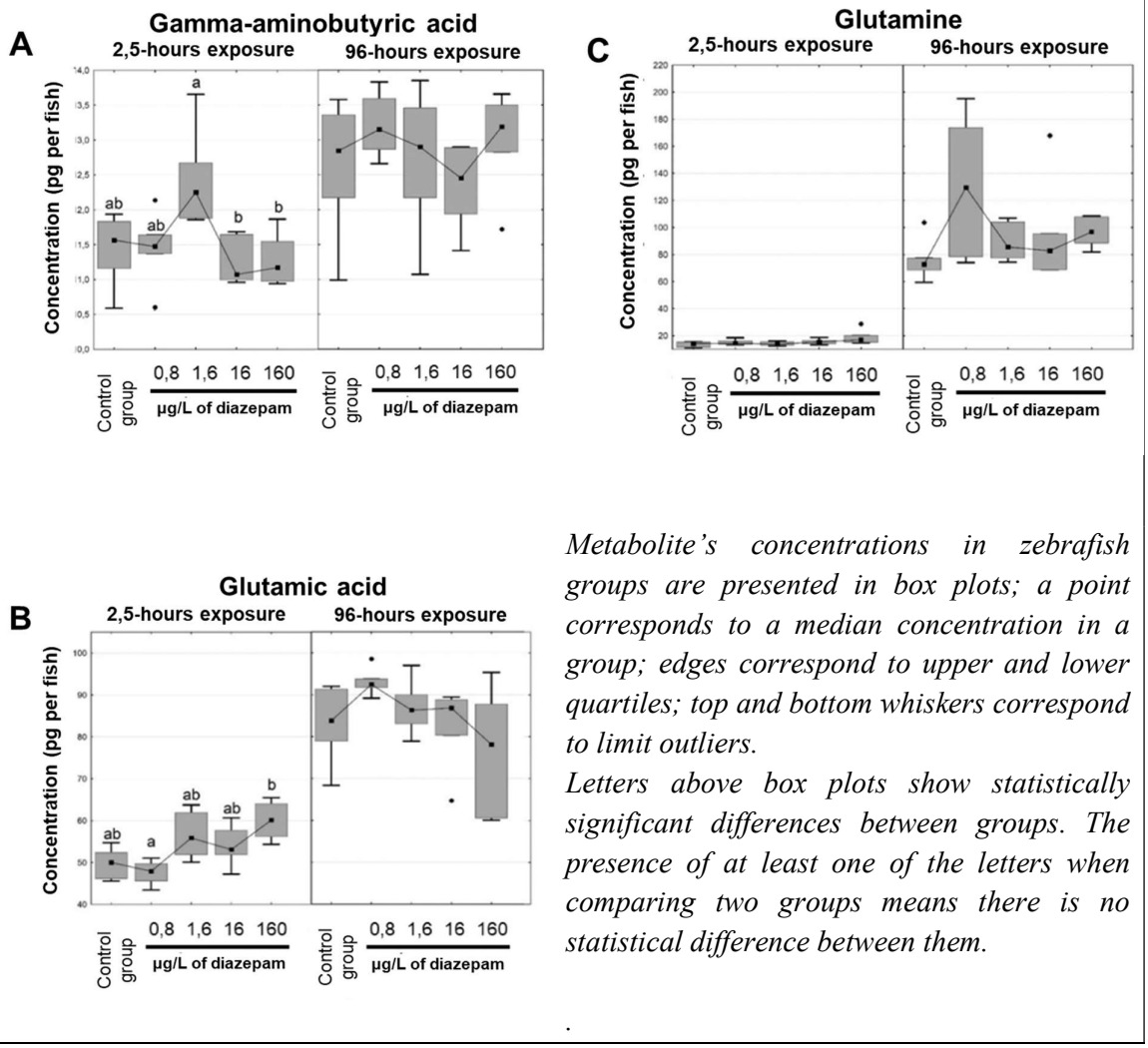
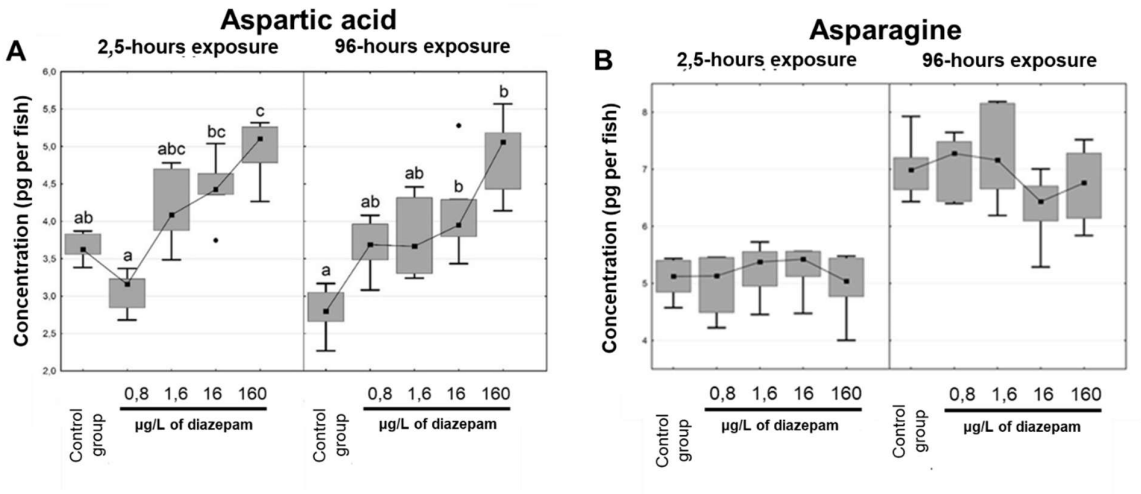


Figure 11 – Metabolites related to GABAergic and glutaminergic system.

Aspartic acid system

In 2.5 h exposure, aspartic acid concentrations presented higher concentrations at higher doses. The group that received 160 µg/L of diazepam showed higher concentrations than the vehicle control group and compared to the group that received 0.8 µg/L ($p < 0.05$, and $p < 0.001$, respectively). The group treated with 16 µg/L showed significantly higher aspartic acid concentrations than those exposed to 0.8 µg/L (Figure 12A). After 96 h exposure, aspartic acid concentrations were higher in the groups exposed to 16 and 160 µg/L of diazepam compared to the vehicle control ($p < 0.05$, and $p < 0.001$, respectively) (Figure 12A).

The metabolite asparagine, known to be a precursor of aspartic acid, did not show any significant difference or trend for short- or medium-term exposures (Figure 12B).



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
Letters above box plots show statistically significant differences between groups. The presence of at least one of the letters when comparing two groups means there is no statistical difference between them.

Figure 12 – Metabolites related to aspartic acid system.

3.3.3. Serotonergic system and kynurenine pathway

This section presents the results of the short- and long-term exposures of diazepam on the levels of endogenous metabolites of the serotonergic system and kynurenine pathway. Figure 13 shows the scheme of the tryptophan metabolism through serotonin, kynurenine, and indole pathways.

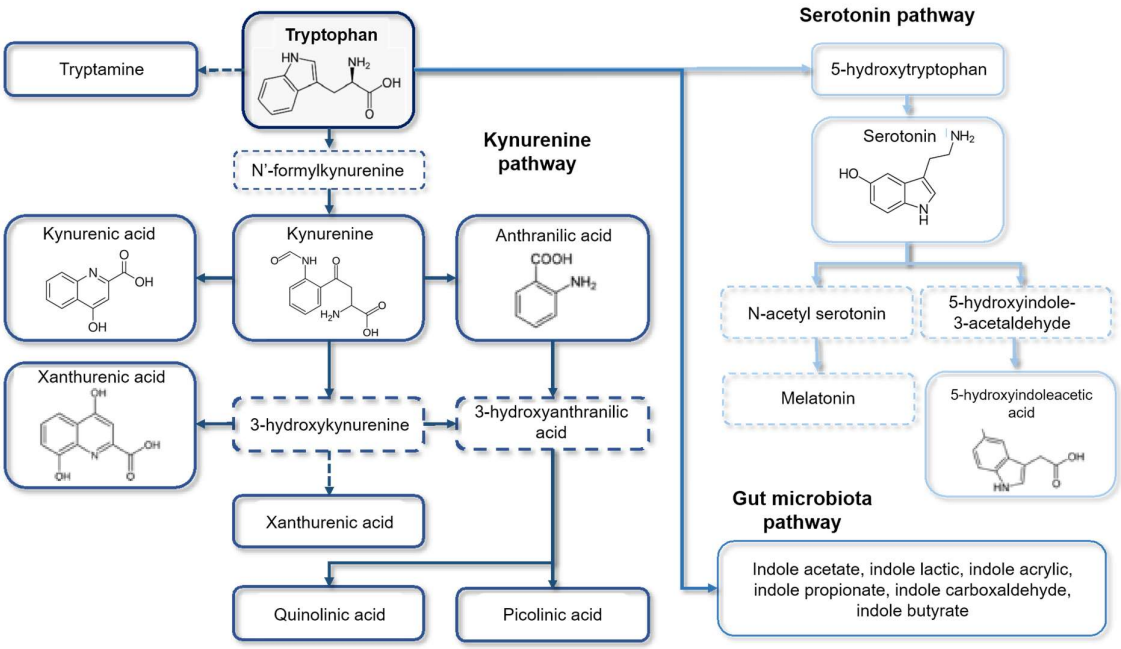


Figure 13 – The scheme of tryptophan metabolism.

Serotonergic system

In short-term exposure, tryptophan presented higher concentrations at higher doses. The group treated with 160 µg/L of diazepam presented higher ($p < 0.05$) concentrations than the vehicle control group. Also, the groups that received 16 µg/L and 160 µg/L presented higher ($p < 0.01$ and $p < 0.001$, respectively) concentrations of tryptophan than the group that received 0.8 µg/L of diazepam (Figure 14A). In contrast, tryptophan presented decreased concentration in the long-term treatments at higher doses of exposure. Indeed, the groups that received 0.8, 16 and 160 µg/L showed lower ($p < 0.001$, $p < 0.05$, and $p < 0.05$, respectively) concentrations than the vehicle control group (Figure 14A). In the case of serotonin, under 2.5 h treatment, this metabolite presented a trend of increasing concentrations at higher doses of exposure. The groups that received 16 and 160 µg/L presented higher ($p < 0.001$) concentrations than the control group and also higher ($p < 0.05$) than the group that received 0.8 µg/L (Figure 14B). Overall, in the 96 h treatments, there was also a trend of increasing serotonin concentrations at higher doses of exposure. The groups that received 1.6 and 160 µg/L diazepam showed higher ($p < 0.05$ and $p <$

0.01, respectively) concentrations than the vehicle control group, and also higher than the group that received 0.8 μg/L of diazepam ($p < 0.01$, and $p < 0.001$, respectively) (Figure 14B). Other metabolites such as 5-hydroxytryptophan, 5-hydroxyindole acetic acid, and tryptamine involved in the serotonergic system-serotonin pathway were also measured; however, these metabolites presented inconsistent responses (Figures 14C-E).

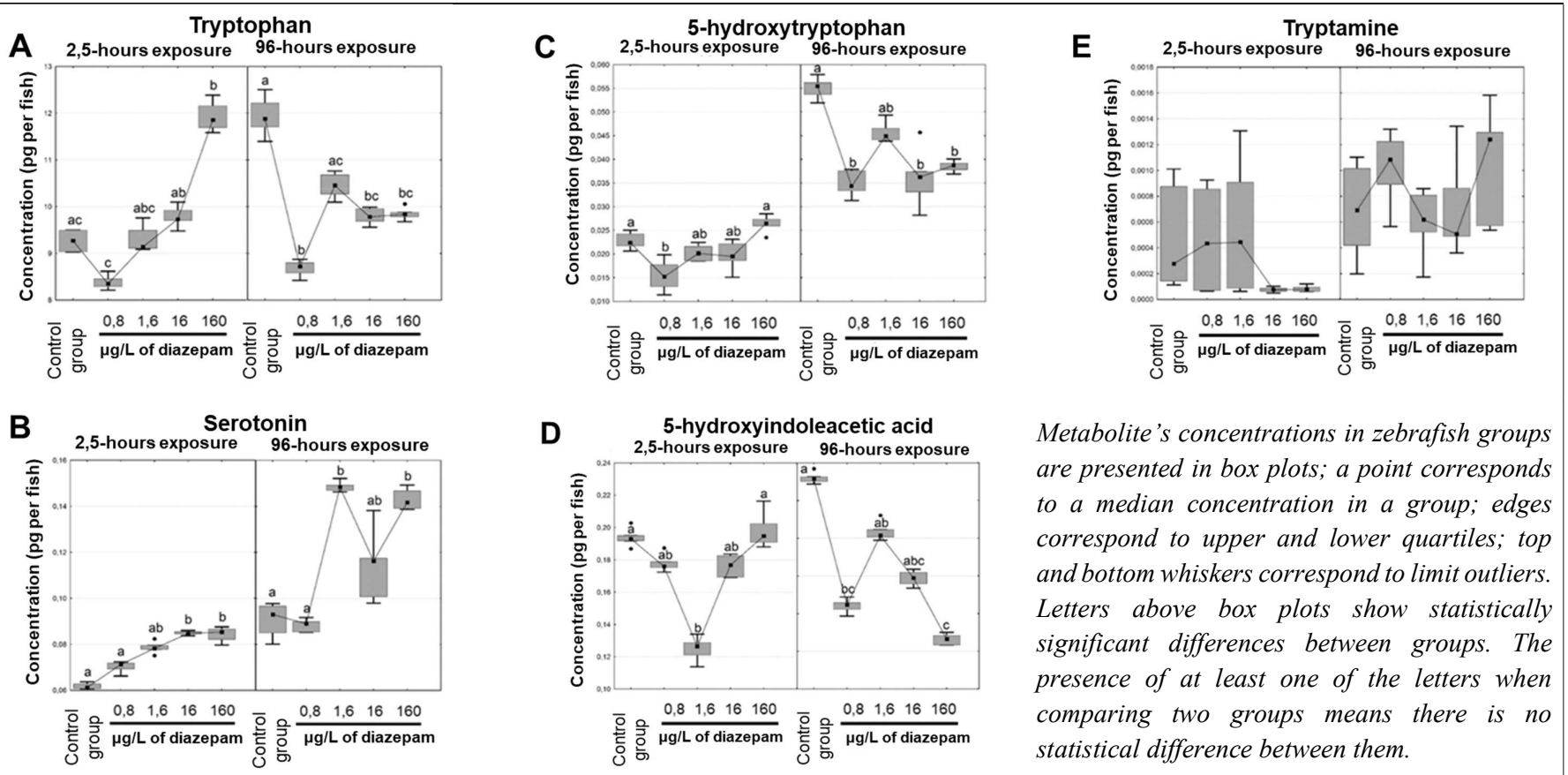
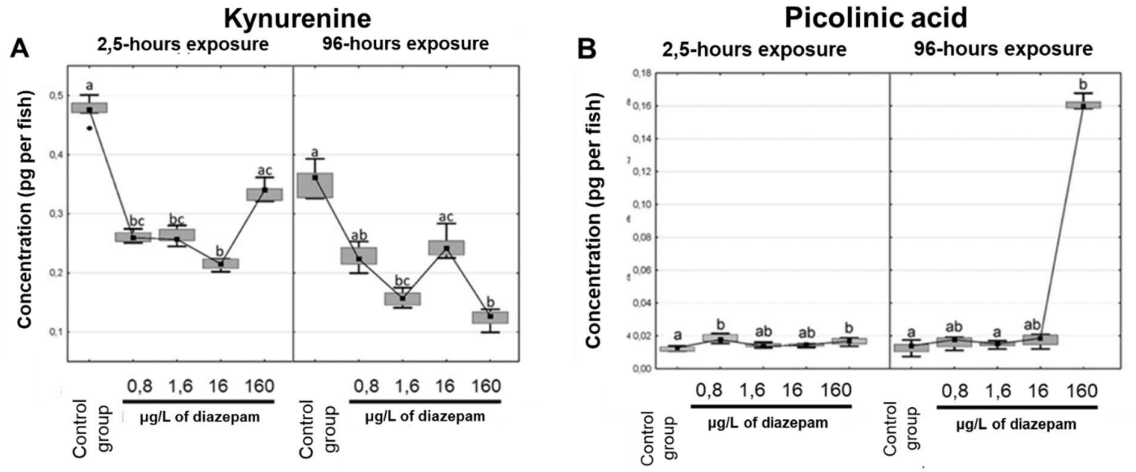


Figure 14 – Metabolites related to serotonergic system.

Kynurenine pathway

The kynurenine and picolinic acid concentrations changed after both short- and long-term exposures to diazepam. Short-term exposure led to the decrease in the levels of kynurenine in the group exposed to 16 ug/L compared to the 160 ug/L and vehicle control groups (Figure 15A). Long-term diazepam exposure led to the decrease of the kynurenine concentrations in the group exposed to 1.6 ug/L compared to the control group, but also in the group exposed to 160 ug/L of diazepam compared to the 16 ug/L and vehicle control groups (Figure 15A). Short-term diazepam exposure led to increased concentrations of the picolinic acid in the groups exposed to 0.8 and 160 ug/L compared to the vehicle control group. Long-term diazepam exposure led to the increase in the levels of picolinic acid in the group exposed to 160 ug/L compared to 1.6 ug/L and the vehicle control group (Figure 15B).



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
Letters above box plots show statistically significant differences between groups. The presence of at least one of the letters when comparing two groups means there is no statistical difference between them.

Figure 15 – Metabolites related to kynurenine pathway.

3.3.4. Dopaminergic system

This section presents the results of the short- and long-term exposures of diazepam on the levels of endogenous metabolites of the dopaminergic/adrenergic system. Figure 16 shows the scheme of the dopaminergic/adrenergic system metabolism.

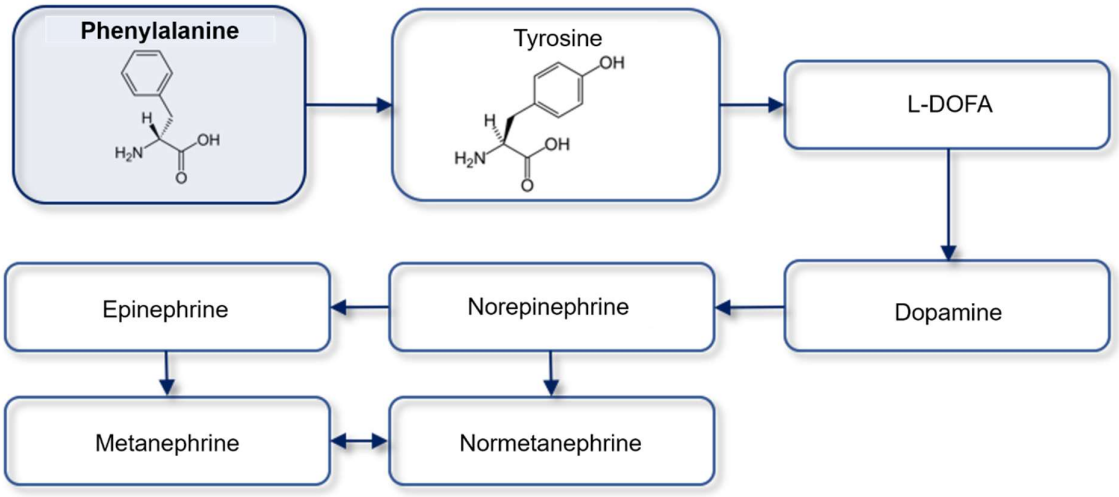


Figure 16 – The scheme of dopaminergic/adrenergic system metabolism.

In short-term exposure, phenylalanine presented significantly higher concentrations in the groups receiving higher doses of diazepam. The group that received 160 µg/L of diazepam showed higher ($p < 0.01$) concentrations than the groups that received 0.8 and 1.6 µg/L (Figure 17A). Under long-term exposure, phenylalanine concentrations in the group that received 160 µg/L of diazepam presented increased concentrations versus the vehicle control ($p < 0.001$). Also, the groups that received 0.8 and 16 µg/L of diazepam presented higher concentrations of phenylalanine compared to the vehicle control ($p < 0.05$) (Figure 17A). After 2.5 h exposure, tyrosine concentrations were significantly higher in the group that received 160 µg/L of diazepam compared to the groups that received 0.8 and 1.6 µg/L of diazepam ($p < 0.001$, and $p < 0.01$, respectively). In addition, the concentrations of tyrosine in the group exposed to 0.8 µg/L of diazepam were lower than those in the vehicle control group (Figure 17B). In contrast, tyrosine

concentrations after 96 h were lower in the group exposed to 16 and 160 μg/L of diazepam than the vehicle control ($p < 0.01$, and $p < 0.001$, respectively). Further, the group treated with 160 μg/L of diazepam presented lower concentrations of diazepam compared to the group exposed to 1.6 μg/L of diazepam ($p < 0.01$) (Figure 17B). The other metabolites of the dopaminergic/adrenergic system, including L-DOPA, dopamine, norepinephrine, epinephrine, normetanephrine, and metanephrine, presented occasional statistical differences in their concentrations in both short- and long-term exposures. However, the only systematic trend observed was an increased concentration at 0.8 μg/L doses in short-term exposure for most of these metabolites, but without a clear consistency with the diazepam concentrations (Figures 17C-E and 18A-C).

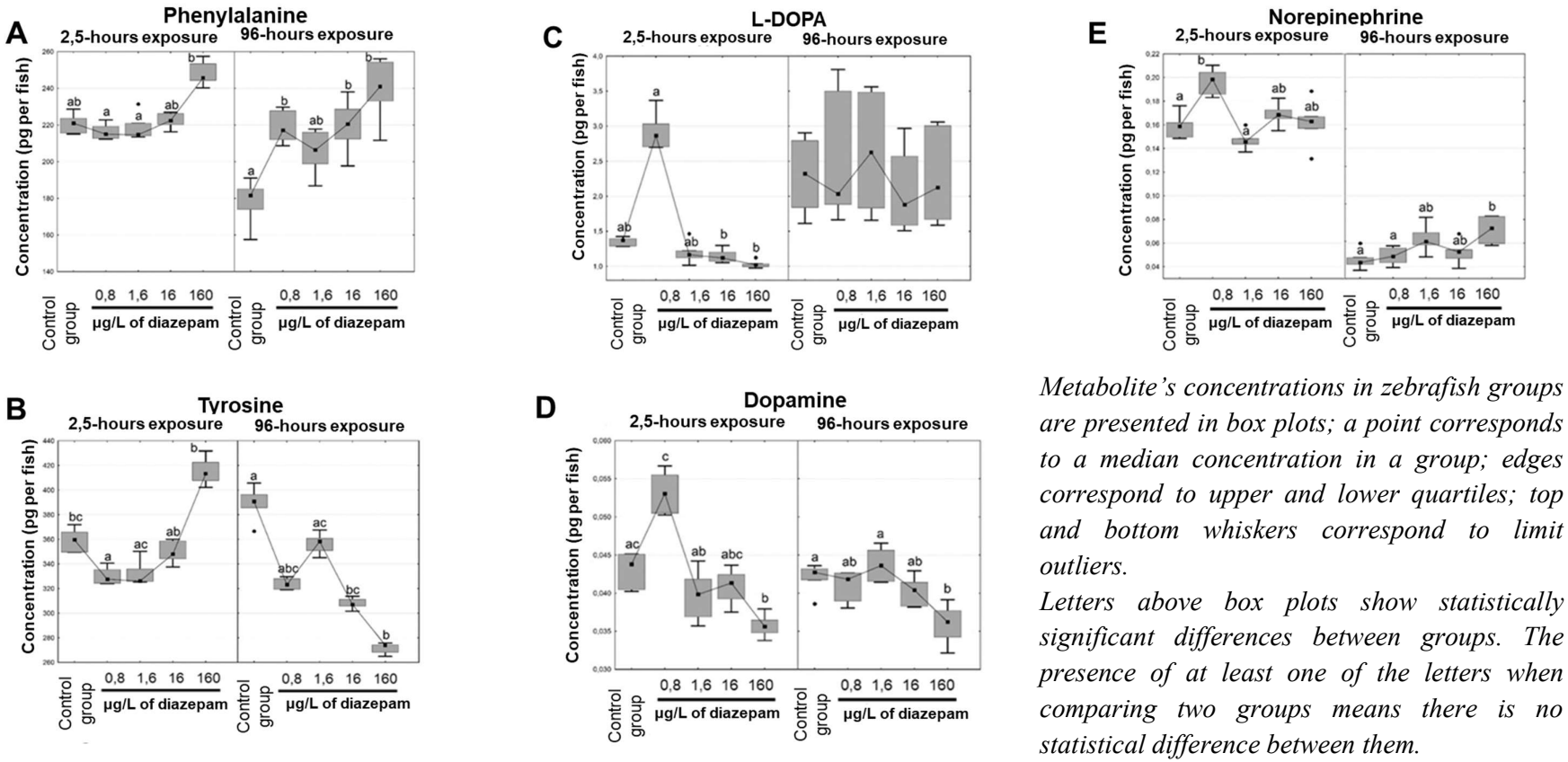
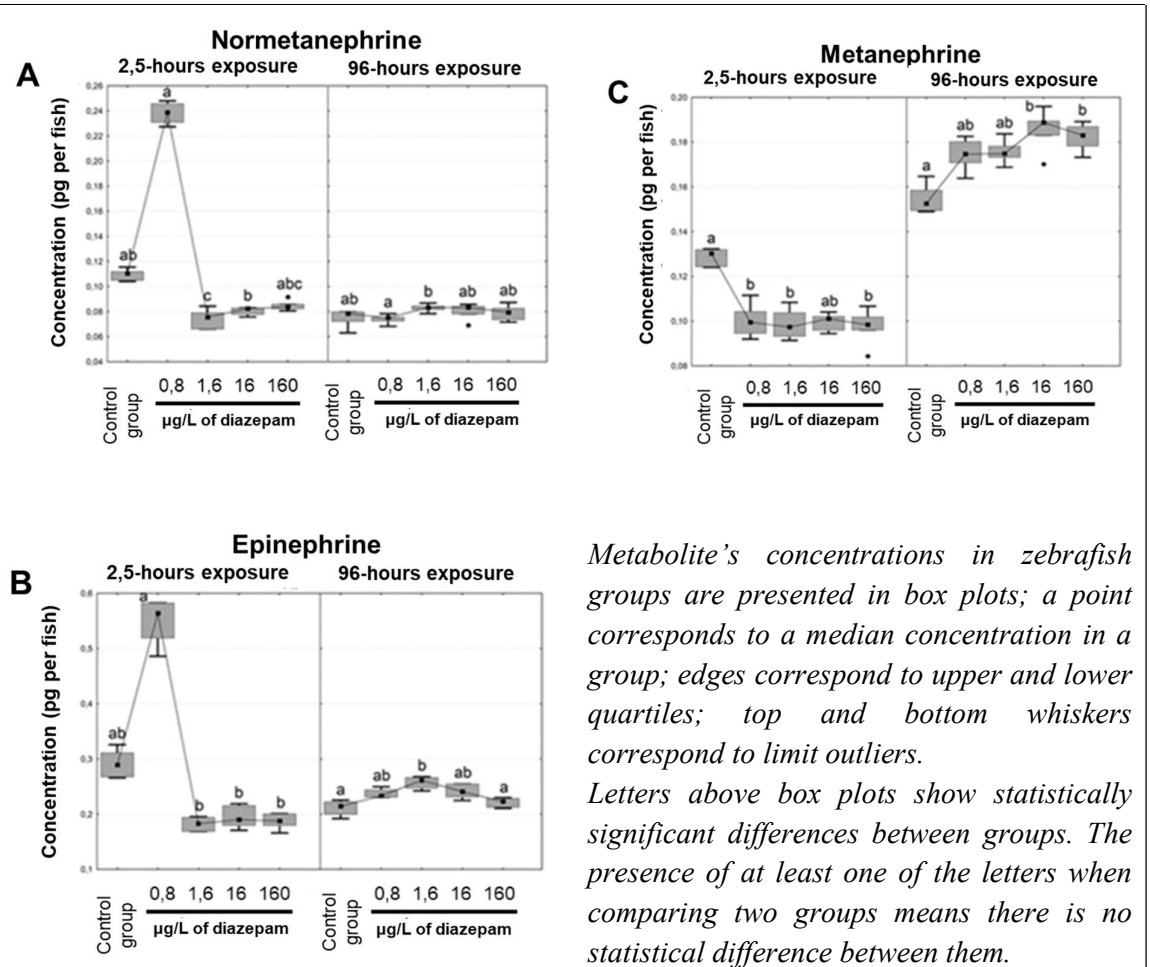


Figure 17 – Metabolites related to dopaminergic/adrenergic system (part 1).



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
Letters above box plots show statistically significant differences between groups. The presence of at least one of the letters when comparing two groups means there is no statistical difference between them.

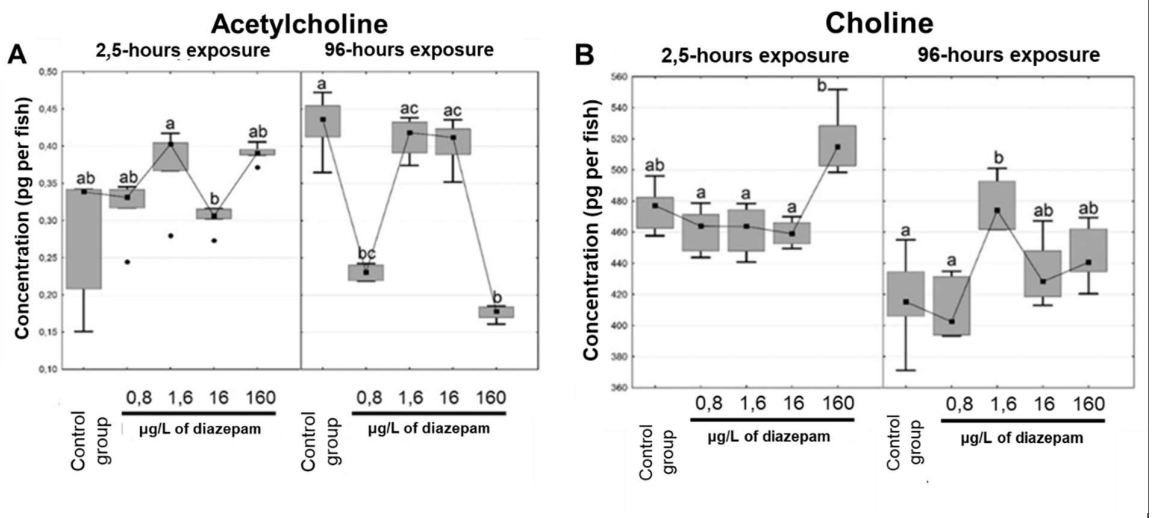
Figure 18 – Metabolites related to dopaminergic/adrenergic system (part 2).

3.3.5. Cholinergic system

This section presents the results of the short- and long-term exposures of diazepam on the levels of endogenous metabolites of the cholinergic system.

Choline and acetylcholine concentrations changed both after short- and long-term exposures. In particular, the concentrations of acetylcholine in the group exposed to 16 ug/L of diazepam for a short period were lower than those exposed to 1.6 ug/L. Long-term exposure led to the decrease in acetylcholine concentrations in the groups exposed to 0.8 and 160 ug/L of diazepam compared to vehicle control (Figure 19A). Choline concentrations were higher in the group exposed to 160 ug/L

of diazepam for a short period compared to other exposure groups. Long-term exposure led to the increase of the choline concentrations in the group exposed to 1.6 ug/L of diazepam compared to the group exposed to 0.8 ug/L and vehicle control group (Figure 19B).



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
Letters above box plots show statistically significant differences between groups. The presence of at least one of the letters when comparing two groups means there is no statistical difference between them.

Figure 19 – Metabolites related to cholinergic system.

3.3.6. Other metabolites related to neurotransmission

This section presents the results of the short- and long-term exposures of diazepam on the levels of endogenous metabolites of the neurotransmission-related pathways.

Microbial tryptophan catabolism metabolites (indole-3-carboxaldehyde, indole-3-acetic acid, indole-3-butyric acid, indole-3-lactic acid, indole3-acrylic acid, and indole-3-propionic acid) and other biological compounds (cortisol, citrulline, biopterin, and neopterin) involved in neurotransmission displayed inconsistent

changes and were hard to interpret across different diazepam concentrations for both the 2.5 h and 96 h experiments.

3.4. Determination of the half-lethal concentration of 5F-APINAC

To determine the concentrations of the working solutions of 5F-APINAC, half-lethal concentration (LC_{50}) of 5F-APINAC was determined. The results of the assessment are presented in Table 13 and Figure 20.

Table 13 – Survival rates of zebrafish larvae in the LC_{50} assessment

Experimental group	№ of embryos in the group	№ of dead embryos	Survival rate (%)
10 uM 5F-APINAC	20	0	100
20 uM 5F-APINAC	20	2	90
40 uM 5F-APINAC	20	5	75
80 uM 5F-APINAC	20	13	35
100 uM 5F-APINAC	20	18	10
Positive control (4 ug/l 3,4-dichloroaniline in 1% solution of DMSO in E3 medium)	20	15	25
Negative control (E3 medium)	20	1	95
Carrier solvent control (1% DMSO in E3 medium)	20	0	100

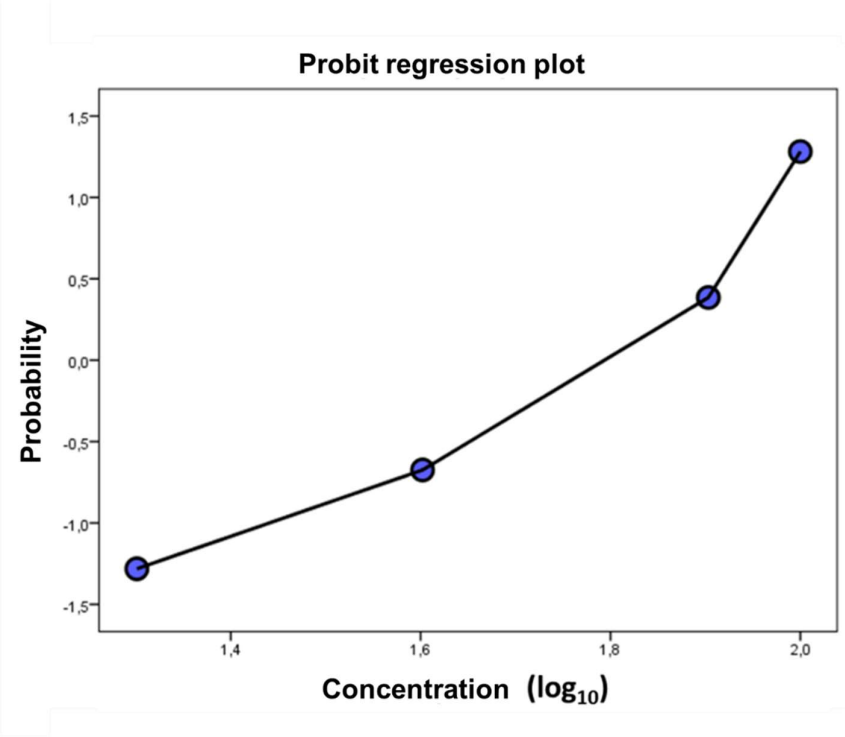


Figure 20 – Probit-regression analysis of half-lethal concentration assessment of 5F-APINAC.

Probit-regression analysis had shown that LC_{50} for 5F-APINAC was 55.0 uM (95% CI: 44.3-68.7 uM). The survival rate of the embryos in the negative control – 95%, in the vehicle control – 100%; lethality in the positive control was 75%. This data is within the acceptance criteria for the test (survival rate in the negative control and vehicle control > 90%, lethality in the positive control > 30%).

5F-APINAC has demonstrated that it has levels of toxicity compared to other cannabinoids. Thus, for zebrafish larvae, the lethality of Δ^9-THC and synthetic cannabinoids CP 55,940 and WIN 55,212-2 are 10, 50, and 3 uM, respectively [45].

Based on the obtained results and the results about the toxicity of the defluorinated analog of 5F-APINAC – APINAC [210] – 10 uM concentration of 5F-APINAC was used as the highest.

3.5. Neurotransmitters metabolomics after short- and long-term exposure of 5F-APINAC in zebrafish larvae

In order to investigate the possibility of the usage of the zebrafish as a model organism in the assessment of the pharmacological action of the neurotropic drugs of cannabimimetic action using the pharmacometabolomic approach, the concentrations of the metabolites of GABAergic, serotonergic, dopaminergic/adrenergic, cholinergic neurotransmitter systems, aspartic acid system, kynurenine, and indole pathway metabolites, and other related metabolites after the exposure of 5F-APINAC at the doses of 0.001, 0.01, 0.1, 1.0, and 10 uM for short- (4 h) and long-term (96 h). 5F-APINAC is a pharmacological compound with cannabimimetic properties. There were no complex investigations made in the field of the action of cannabimimetics action on the CNS.

. The list of the measured compounds is represented in Table 1. The method of quantification of these compounds was developed and validated. The validation results of the method are presented in chapter 3, section 2.

The main statistical differences in the concentrations of endogenous metabolites after short-term 5F-APINAC exposure are presented in Table 14. The main statistical differences in the concentrations of endogenous metabolites after long-term 5F-APINAC exposure are shown in Table 15.

Table 14 – Detailed information on the statistical differences across experimental groups after short-term 5F-APINAC exposure

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	Contr. vs 5	1 vs 2	1 vs 3	1 vs 4	1 vs 5	2 vs 3	2 vs 4	2 vs 5	3 vs 4	3 vs 5	4 vs 5
GABA	-	-	-	-	<0,01 ↓	-	<0,01 ↓	-	<0,001 ↓	-	-	<0,01 ↓	-	-	<0,01 ↓
Glutamic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glutamine	-	-	-	-	<0,001 ↓	-	-	-	<0,001 ↓	-	-	<0,01 ↓	-	<0,05 ↓	<0,05 ↓
Tryptophan	-	-	-	-	-	-	-	-	<0,05 ↓	-	-	-	-	-	-
5-hydroxytryptophan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serotonin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-hydroxyindole acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptamine	-	-	-	<0,05 ↓	<0,001 ↓	-	-	-	<0,001 ↓	-	-	<0,001↓	-	<0,001 ↓	<0,01 ↓
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	-	-	-	-	<0,05 ↓	-	-	-	<0,01 ↓	-	-	-	-	-	<0,05 ↓
L-DOPA	-	-	<0,05 ↑	-	-	-	-	-	-	-	-	-	-	-	-
Dopamine	<0,01 ↑	-	<0,001 ↑	<0,05 ↑	<0,05 ↑	-	-	-	-	<0,01 ↑	-	-	-	-	-
Norepinephrine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Normetanephrine	-	-	-	-	<0,01 ↓	-	-	-	<0,001 ↓	-	-	<0,05 ↓	-	-	<0,001 ↓
Epinephrine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metanephrine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic acid	-	-	<0,01 ↓	-	<0,01 ↓	-	<0,001 ↓	-	<0,001 ↓	<0,01 ↓	-	<0,05 ↓	-	-	-
Asparagine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetylcholine	<0,01 ↑	<0,001 ↑	<0,001 ↑	<0,001 ↑	<0,001 ↑	-	<0,001 ↑	-	-	<0,001 ↑	-	-	<0,001 ↓	<0,001 ↓	-

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	Contr. vs 5	1 vs 2	1 vs 3	1 vs 4	1 vs 5	2 vs 3	2 vs 4	2 vs 5	3 vs 4	3 vs 5	4 vs 5
Choline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kynurenine	<0,01 ↓	<0,01 ↑	-	<0,05 ↓	-	<0,001 ↑	<0,001 ↑	-	-	-	<0,001 ↓	<0,001 ↓	<0,01 ↓	<0,01 ↓	-
Kynurenic acid	<0,001 ↓	<0,001 ↑	-	<0,001 ↓	-	<0,001 ↑	<0,001 ↑	-	<0,001 ↑	<0,001 ↓	<0,001 ↓	<0,001 ↓	<0,001 ↓	<0,05 ↓	<0,001 ↑
Anthranilic acid	-	-	-	-	-	-	-	-	-	-	-	<0,05 ↓	<0,01 ↓	<0,001 ↓	-
Xanthurenic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Quinolinic acid	-	-	-	-	-	-	<0,05 ↑	-	-	-	-	-	<0,05 ↓	<0,01 ↓	-
Picolinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-carboxaldehyde	-	-	-	-	<0,05 ↓	-	-	-	-	-	-	-	-	-	-
Indole-3-acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-butyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-lactic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-acrylic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-propionic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cortisol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrulline	<0,05 ↓	<0,01 ↓	<0,001 ↓	<0,001 ↓	<0,001 ↓	-	<0,001 ↓	-	<0,001 ↓	<0,01 ↓	-	<0,001 ↓	<0,05 ↑	-	<0,01 ↓
Biopterin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neopterin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 15 – Detailed information on the statistical differences across experimental groups after short-term 5F-APINAC exposure

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	Contr. vs 5	1 vs 2	1 vs 3	1 vs 4	1 vs 5	2 vs 3	2 vs 4	2 vs 5	3 vs 4	3 vs 5	4 vs 5
GABA	-	-	-	-	<0,001 ↓	-	<0,05 ↑	<0,001 ↑	<0,001 ↓	-	<0,05 ↑	<0,001 ↓	-	<0,001 ↓	<0,001 ↓
Glutamic acid	-	-	-	<0,05 ↑	-	-	-	<0,001 ↑	-	-	<0,01 ↑	-	<0,01 ↑	-	<0,05 ↑
Glutamine	-	-	-	-	<0,05 ↑	-	-	<0,05 ↑	<0,01 ↑	-	-	-	-	<0,05 ↑	-
Tryptophan	-	-	<0,05 ↓	<0,05 ↓	<0,001 ↑	-	-	-	<0,001 ↑	-	-	<0,001 ↑	-	<0,001 ↑	<0,001 ↑
5-hydroxytryptophan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serotonin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-hydroxyindole acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptamine	-	-	<0,01 ↓	-	-	-	-	-	<0,05 ↑	-	-	-	-	<0,001 ↑	<0,05 ↑
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	-	-	<0,01 ↓	-	-	<0,05 ↓	-	-	-	<0,001 ↓	-	-	-	<0,05 ↑	-
L-DOPA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dopamine	<0,05 ↑	<0,05 ↑	<0,001 ↑	-	-	-	-	<0,001 ↓	<0,001 ↓	-	<0,001 ↓	<0,001 ↓	<0,001 ↓	<0,001 ↓	-
Norepinephrine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Normetanephrine	-	-	-	-	-	-	<0,05 ↑	-	-	-	-	-	-	-	-
Epinephrine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metanephrine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic acid	-	-	-	-	-	-	-	-	<0,01 ↑	-	-	-	-	<0,05 ↑	-
Asparagine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetylcholine	-	-	<0,001 ↑	<0,001 ↓	<0,001 ↓	-	<0,001 ↑	<0,001 ↓	<0,001 ↓	<0,05 ↑	<0,001 ↓	<0,001 ↓	<0,001 ↓	<0,001 ↓	-

Metabolite	Contr. vs 1	Contr. vs 2	Contr. vs 3	Contr. vs 4	Contr. vs 5	1 vs 2	1 vs 3	1 vs 4	1 vs 5	2 vs 3	2 vs 4	2 vs 5	3 vs 4	3 vs 5	4 vs 5
Choline	-	-	-	-	-	-	-	<0,01 ↓	<0,01 ↓	-	-	-	<0,001 ↓	<0,01 ↓	-
Kynurenine	<0,001 ↓	-	<0,001 ↓	-	<0,001 ↓	-	-	<0,01 ↑	-	<0,001 ↓	-	-	<0,001 ↑	-	<0,01 ↓
Kynurenic acid	-	<0,001 ↑	-	-	-	<0,001 ↑	<0,01 ↑	<0,05 ↑	<0,01 ↑	<0,01 ↓	<0,001 ↓	<0,01 ↓	-	-	-
Anthranilic acid	<0,01 ↓	<0,05 ↓	<0,001 ↓	<0,05 ↑	-	-	-	<0,001 ↑	-	-	<0,001 ↑	-	<0,001 ↑	-	<0,001 ↓
Xanthurenic acid	-	-	-	<0,05 ↓	<0,05 ↑	-	-	-	<0,001 ↑	-	-	<0,001 ↑	-	<0,001 ↑	<0,001 ↑
Quinolinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picolinic acid	-	<0,001 ↑	<0,001 ↓	<0,001 ↑	<0,001 ↑	<0,001 ↑	<0,001 ↓	<0,001 ↑	<0,001 ↑	<0,001 ↓	<0,001 ↑	<0,001 ↑	<0,001 ↑	<0,001 ↑	<0,001 ↑
Indole-3-carboxaldehyde	-	-	<0,01 ↓	<0,01 ↑	<0,001 ↓	-	-	<0,001 ↑	-	-	<0,001 ↑	<0,05 ↓	<0,001 ↑	-	<0,001 ↓
Indole-3-acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-butyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-lactic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-acrylic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole-3-propionic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cortisol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrulline	-	-	-	-	<0,001 ↑	-	-	<0,001 ↑	<0,001 ↑	-	-	<0,001 ↑	<0,001 ↑	<0,001 ↑	-
Biopterin	<0,01 ↑	-	<0,001 ↑	<0,001 ↑	<0,001 ↑	-	-	<0,01 ↑	-	<0,001 ↑	<0,001 ↑	<0,05 ↑	-	-	-
Neopterin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.5.1. Morphological characteristics

In acute exposure, there were no visible malformations in any group. In chronic exposure, the groups of larvae exposed to 5F-APINAC at the highest concentration (10 μM) presented multiple morphological and developmental alterations. Approximately 60% of the larvae were unhatched, 20% had a curved tail related to spinal cord malformations, and all of them presented hyperpigmentation (Figure 21). The groups of larvae exposed to all other concentrations of 5F-APINAC and the vehicle control groups in chronic exposure did not present alterations in morphology or development.

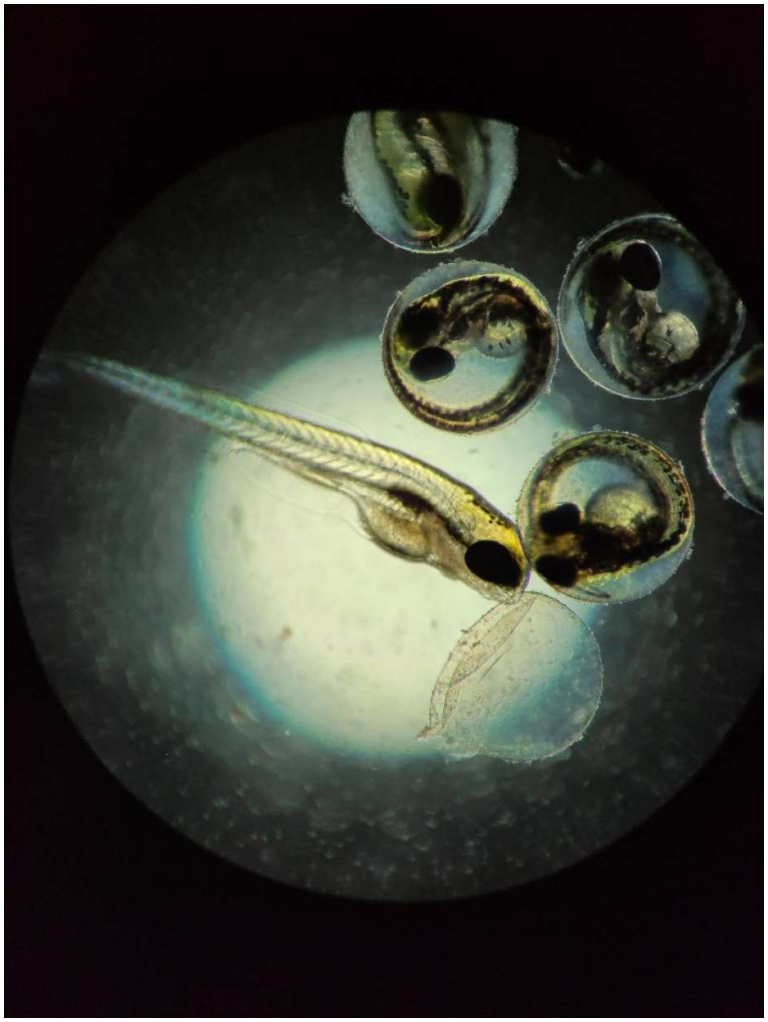
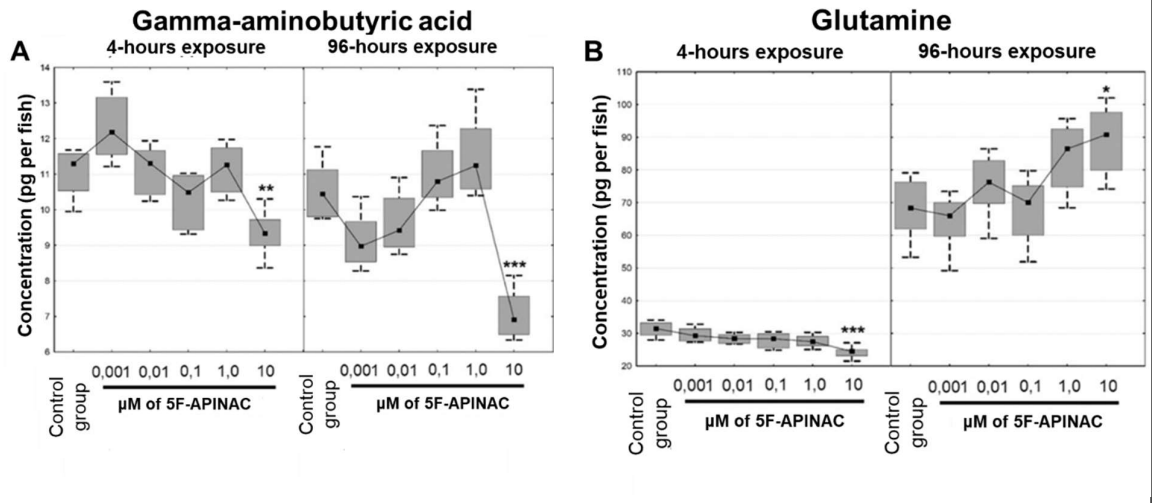


Figure 21 – Zebrafish larvae after the exposure to 5F-APINAC at a concentration of 10 uM for a long-term.

3.5.2. GABAergic system

This section presents the results of the short- and long-term exposure of 5F-APINAC on the levels of endogenous metabolites of the GABAergic and aspartic acid systems.

GABA presented the lowest concentration at the highest dose of exposure (10 µM 5F-APINAC) in the acute ($p < 0.01$) and chronic ($p < 0.001$) experiments compared to the vehicle control group (Figure 22A). In acute exposure, there was a descending trend of glutamine concentrations, being the group that received 10 µM 5F-APINAC the one that had the lowest value ($p < 0.001$) compared to the vehicle control (Figure 22B). In contrast, in chronic exposure, glutamine concentrations presented an overall ascending trend being higher at the highest doses of exposure ($p < 0.05$) (Figure 22B). Glutamic acid concentrations demonstrated several individual significant differences in acute and chronic exposures but without clear dose-dependent trends.



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
*Asterisks above box plots show statistically significant differences between the treatment group and the control group. * - p<0,05; ** - p<0,01; *** - p<0,001.*

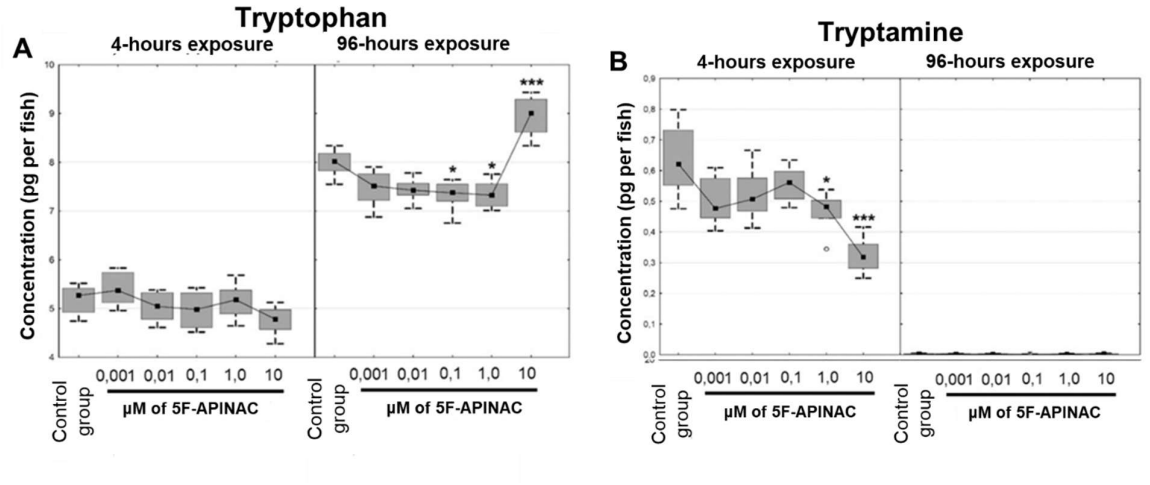
Figure 22 – Metabolites related to GABAergic and glutaminergic system.

3.5.3. Serotonergic system and kynurenine pathway

This section presents the results of the short- and long-term exposures of 5F-APINAC on the levels of endogenous metabolites of the serotonergic system and kynurenine pathway.

Serotonergic system

Tryptophan concentrations in acute exposure did not have significant differences among groups. In chronic exposure, tryptophan concentrations presented an overall descending trend with a statistically neat increase of concentration at 10 μM 5F-APINAC ($p < 0.001$) as compared to the vehicle control group (Figure 23A). Tryptamine concentrations in acute exposure presented lower ($p < 0.05$) concentrations in the intervention groups at higher doses versus the vehicle control. This was especially clear in the group receiving 10 μM 5F-APINAC ($p < 0.001$) (Figure 23B). The concentrations in the chronic exposure experiment were characterized by very low values in all groups (below 0.005 pg/fish) (Figure 23B). 5-Hydroxytryptophan, serotonin, and 5-hydroxyindole acetic acids did not present any statistical differences among groups.

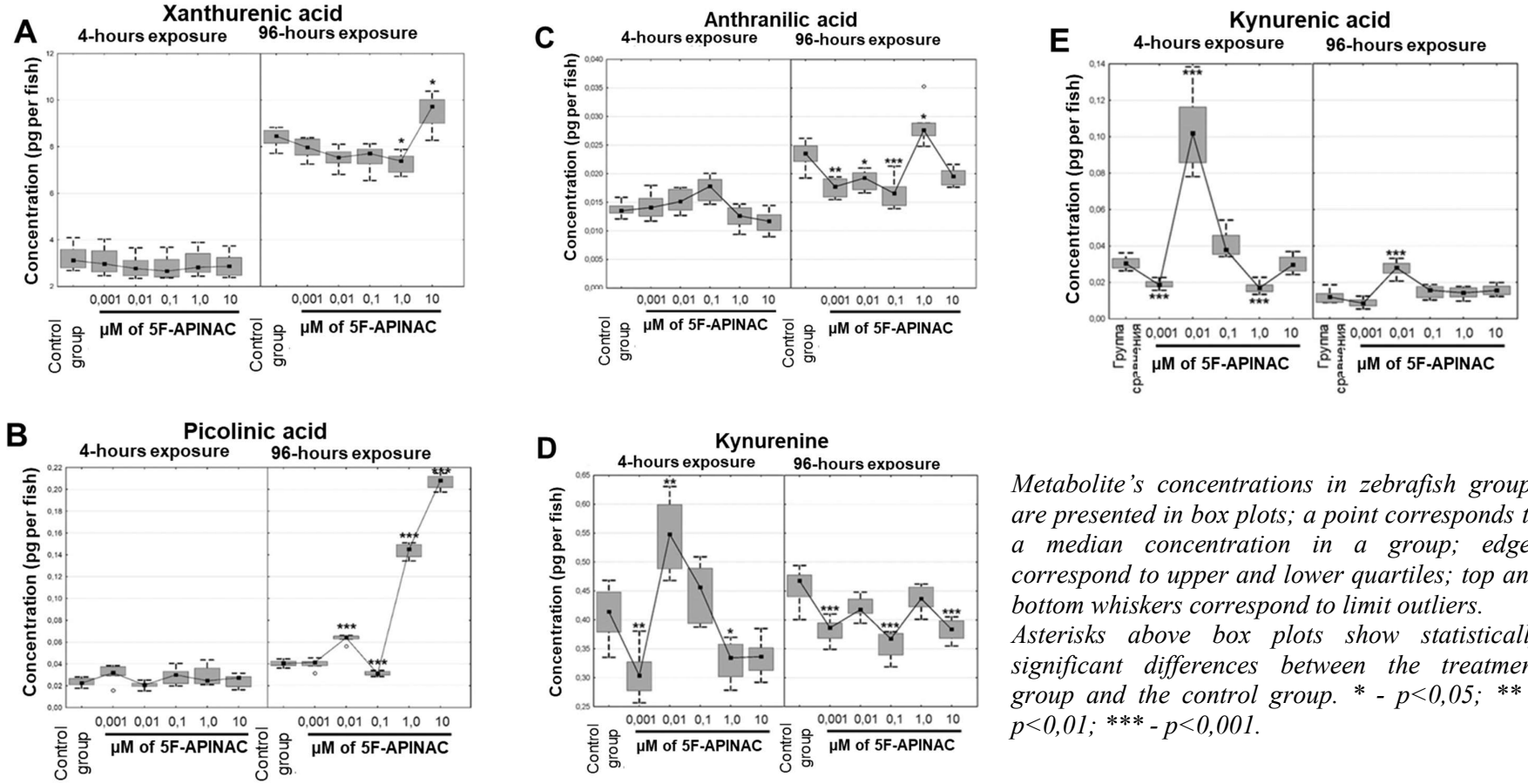


Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
*Asterisks above box plots show statistically significant differences between the treatment group and the control group. * - p<0,05; ** - p<0,01; *** - p<0,001.*

Figure 23 – Metabolites related to serotonergic system.

Kynurenine pathway

Xanthurenic acid and picolinic acid were the only metabolites that presented clear trajectories in this pathway. Xanthurenic acid concentrations were not different among groups in acute exposure (Figure 24A), but in chronic exposure, there was a descending trend with an apparent increase in the group that received 10 µM 5F-APINAC ($p < 0.05$ compared to control) (Figure 24A). Picolinic acid concentrations were not affected during acute exposure (Figure 24B), but in chronic exposure, the groups receiving the highest doses (1.0 and 10 µM 5F-APINAC) had drastic increases ($p < 0.001$) (Figure 24B). Other metabolites involved in the kynurenine pathway (kynurenine, kynurenic acid, anthranilic acid, and quinolinic acid) did not have clear trends (Figure 24 C-E).



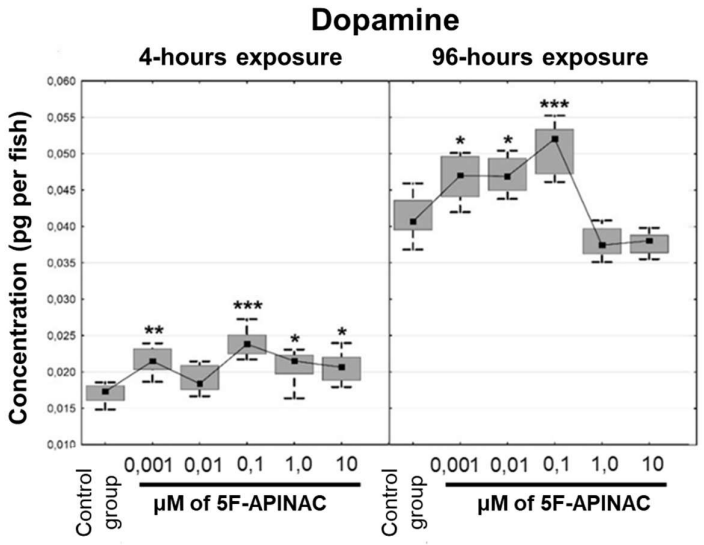
*Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers. Asterisks above box plots show statistically significant differences between the treatment group and the control group. * - p<0,05; ** - p<0,01; *** - p<0,001.*

Figure 24 – Metabolites related to kynurenine pathway.

3.5.4. Dopaminergic system

This section presents the results of the short- and long-term exposures of 5F-APINAC on the levels of endogenous metabolites of the dopaminergic/adrenergic system.

The only metabolite in this neurotransmitter system that presented consistent trajectories was dopamine. In acute exposure, most of the intervention groups had higher ($p < 0.05$) concentrations than the vehicle control (Figure 25). In chronic exposure, there was also a similar trend of increases in the concentrations at higher doses; however, the groups receiving the highest doses (1.0 μM and 10 μM) presented a concentration drop (Figure 25). Phenylalanine, tyrosine, L-DOPA, norepinephrine, normetanephrine, epinephrine, and metanephrine did not have clear trends among groups.



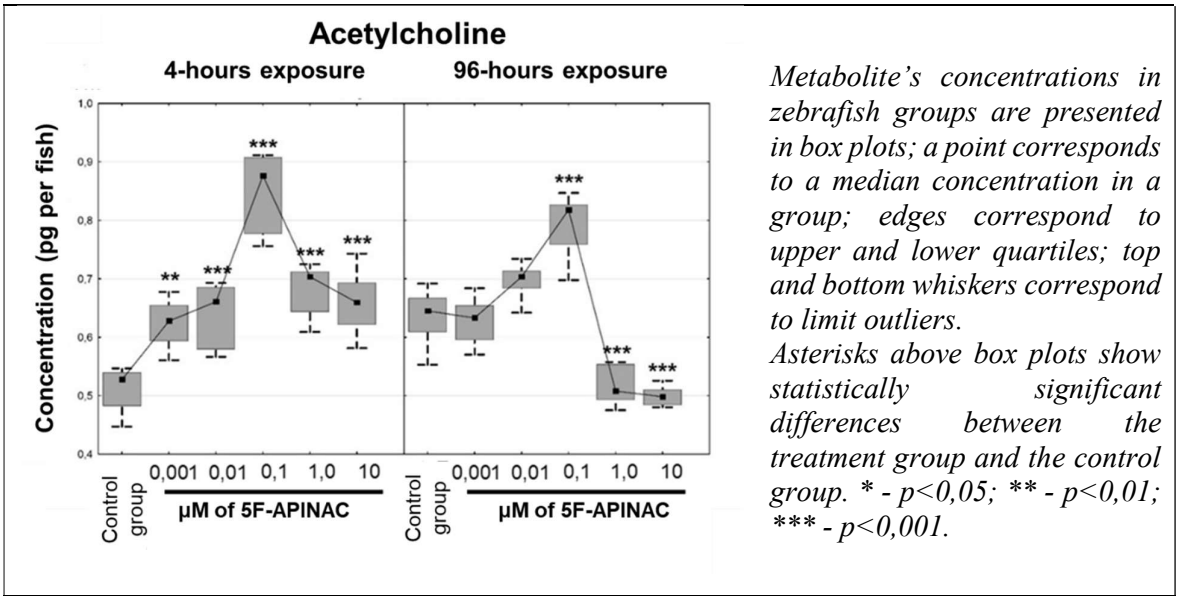
Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
*Asterisks above box plots show statistically significant differences between the treatment group and the control group. * - p<0,05; ** - p<0,01; *** - p<0,001.*

Figure 25 – Metabolites related to dopaminergic system.

3.5.5. Cholinergic system

This section presents the results of the short- and long-term exposures of 5F-APINAC on the levels of endogenous metabolites of the cholinergic system.

In acute exposure, the neurotransmitter acetylcholine had significantly higher concentrations in all intervention groups compared to the vehicle control ($p < 0.01$) (Figure 26). There was a similar ascending trend in chronic exposure, but there was a significant drop in the groups exposed to 1.0 and 10 µM 5F-APINAC compared to the vehicle control ($p < 0.001$). (Figure 26). Choline, a precursor of acetylcholine, presented no trend among groups.



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
*Asterisks above box plots show statistically significant differences between the treatment group and the control group. * - p<0,05; ** - p<0,01; *** - p<0,001.*

Figure 26 – Metabolites related to cholinergic system.

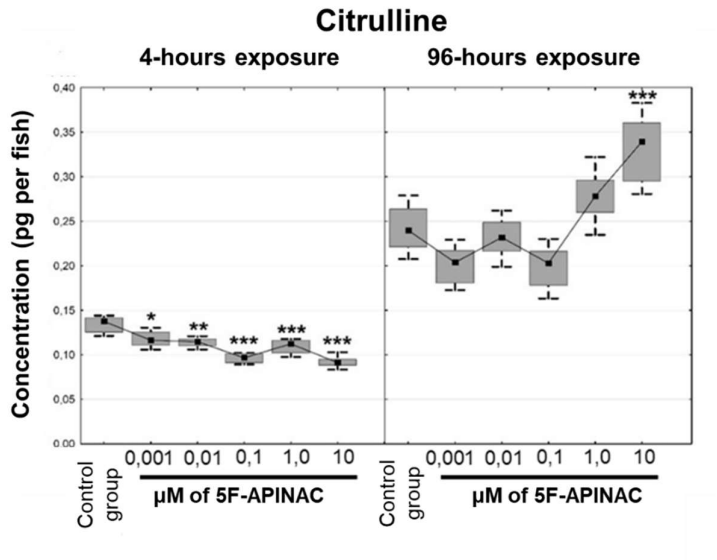
3.5.6. Other metabolites related to neurotransmission

This section presents the results of the short- and long-term exposures of 5F-APINAC on the levels of endogenous metabolites of the neurotransmission-related pathways.

Concentrations of aspartic acid were lower in the groups exposed to 0.1 and 10 uM of 5F-APINAC for the short-term compared to the vehicle control group.

None of the microbial tryptophan catabolism metabolites (indole-3-carboxaldehyde, indole-3-acetic acid, indole-3-butyric acid, indole-3-lactic acid, indole-3-acrylic acid, and indole-3-propionic acid) were significantly altered in acute or chronic experiments.

Citrulline was the only metabolite that presented interpretable results from the extra available metabolites related to neurotransmission, which, however, were not classified in any pathway or system. During acute exposure, citrulline concentrations presented a descending trend within all the intervention groups versus the vehicle control ($p < 0.05$) (Figure 27). In chronic exposure, the group that was exposed to the highest dose (10 μM 5F-APINAC) had a dramatically higher concentration compared to the control group ($p < 0.001$) (Figure 27). Cortisol, biopterin, and neopterin were not informative.



Metabolite's concentrations in zebrafish groups are presented in box plots; a point corresponds to a median concentration in a group; edges correspond to upper and lower quartiles; top and bottom whiskers correspond to limit outliers.
*Asterisks above box plots show statistically significant differences between the treatment group and the control group. * - p<0,05; ** - p<0,01; *** - p<0,001.*

Figure 27 – The concentration graph of citrulline.

3.6. Behavioral study of 5F-APINAC exposure in zebrafish larvae

To confirm the effects of 5F-APINAC of the CNS and to investigate the correlations between behavioral effects and metabolomics changes induced by 5F-APINAC, behavioral studies were conducted.

Short-term 5F-APINAC exposure resulted in the dose-dependent decrease of the total distance traveled by zebrafish larvae (Table 16 and Figures 28 and 30) [23, 25, 40]. Long-term 5F-APINAC exposure was also connected to lowering the total distance traveled by zebrafish (Table 16 and Figures 29 and 30) [23, 199]. The

exposure of the highest dose of 5F-APINAC resulted in a dramatic decrease of locomotor activity, which lasted until the end of the exposure on the second day of the experiment (Figures 28 and 29) [45, 224].

Table 16 – The values of total distance traveled by zebrafish larvae exposed to different concentrations of 5F-APINAC for short and long periods

Total distance travelled		
Groups	**Mean ± SD (mm)**	***p*-value compared to vehicle control**
4 h exposure		
Vehicle control	9957,3 ± 885,5	—
Experimental group №1 (0,001 uM)	8140,2±882,7	NS*
Experimental group №2 (0,01 uM)	7969,8±530,4	NS*
Experimental group №3 (0,1 uM)	7518,0±533,5	>0,01
Experimental group №4 (1,0 uM)	5496,1±461,3	>0,001
Experimental group №5 (10 uM)	3608,4±285,8	>0,001
48 h exposure		
Vehicle control	51718,4±3018,6	—
Experimental group №1 (0,001 uM)	47797,4±2271,9	NS*
Experimental group №2 (0,01 uM)	45747,2±2515,5	>0,01
Experimental group №3 (0,1 uM)	46283,6±2156,1	>0,05
Experimental group №4 (1,0 uM)	43150,3±2098,0	>0,001
Experimental group №5 (10 uM)	29406,8±1783,8	>0,001

* - not significant (p>0,05).

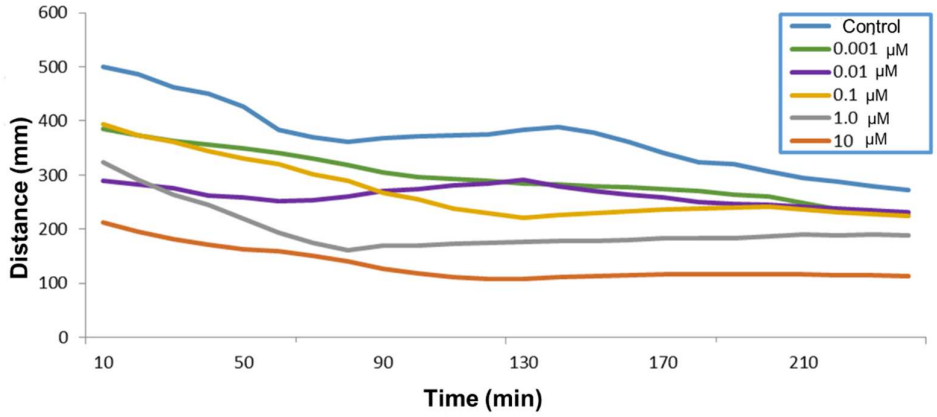


Figure 28 – Total distance traveled by zebrafish larvae in 4 h exposure to 5F-APINAC.

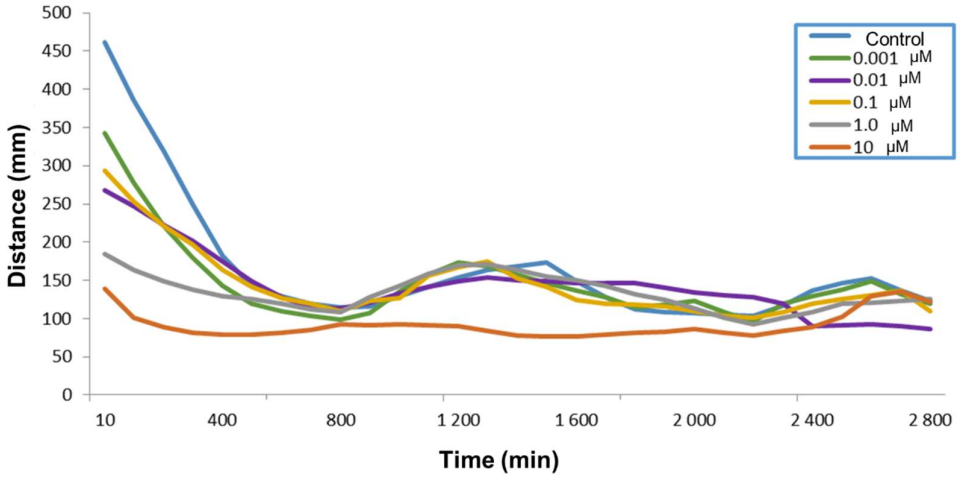


Figure 29 – Total distance traveled by zebrafish larvae in 48 h exposure to 5F-APINAC.

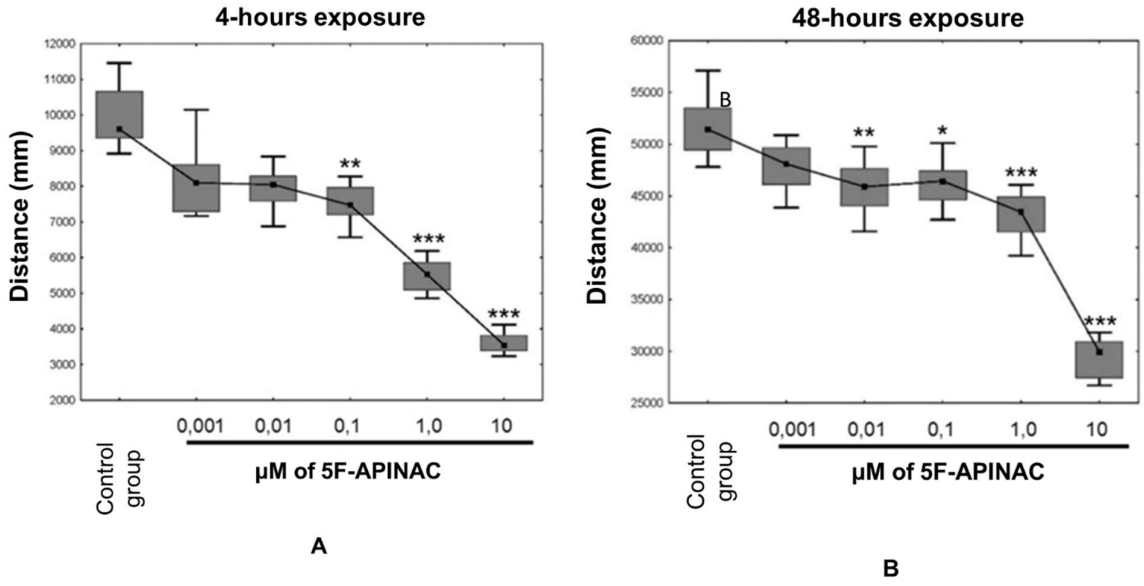


Figure 30 – Total distance traveled by zebrafish larvae in 4 and 48 h exposures to 5F-APINAC.

Several neurotransmitters have been shown to have statistically significant correlations with the total distance traveled. The results of the correlation analysis are presented in Table 17 and Figures 31-37.

Table 17 – Correlations between the concentrations of endogenous metabolites and total distance traveled after 4 h exposure to 5F-APINAC

Metabolite	**Spearman's rank correlation coefficient r**	***p*-value**
GABA	0,58	<0,001
Glutamic acid	0,22	—
Glutamine	0,81	<0,001
Tryptophan	0,47	<0,01
5-hydroxytryptophan	0,06	—
Serotonin	0,11	—
5-hydroxyindole acetic acid	0,13	—
Tryptamine	0,82	<0,001
Phenylalanine	0,52	<0,01
Tyrosine	0,57	<0,001
L-DOPA	-0,08	—
Dopamine	-0,18	—
Norepinephrine	0,21	—

Metabolite	Spearman's rank correlation coefficient r	*p*-value
Normetanephrine	0,50	<0,01
Epinephrine	0,05	—
Metanephrine	0,16	—
Aspartic acid	0,62	<0,001
Asparagine	0,33	<0,05
Acetylcholine	-0,25	—
Choline	0,53	<0,001
Kynurenine	0,42	<0,05
Kynurenic acid	0,23	—
Anthranilic acid	0,51	<0,01
Xanthurenic acid	0,27	—
Quinolinic acid	0,45	<0,01
Picolinic acid	0,04	—
Indole-3-carboxaldehyde	0,55	<0,001
Indole-3-acetic acid	0,06	—
Indole-3-butyric acid	0,22	—
Indole-3-lactic acid	0,29	—
Indole-3-acrylic acid	0,35	<0,05
Indole-3-propionic acid	0,39	<0,05
Cortisol	0,25	—
Citrulline	0,77	<0,001
Biopterin	0,06	—
Neopterin	0,25	—

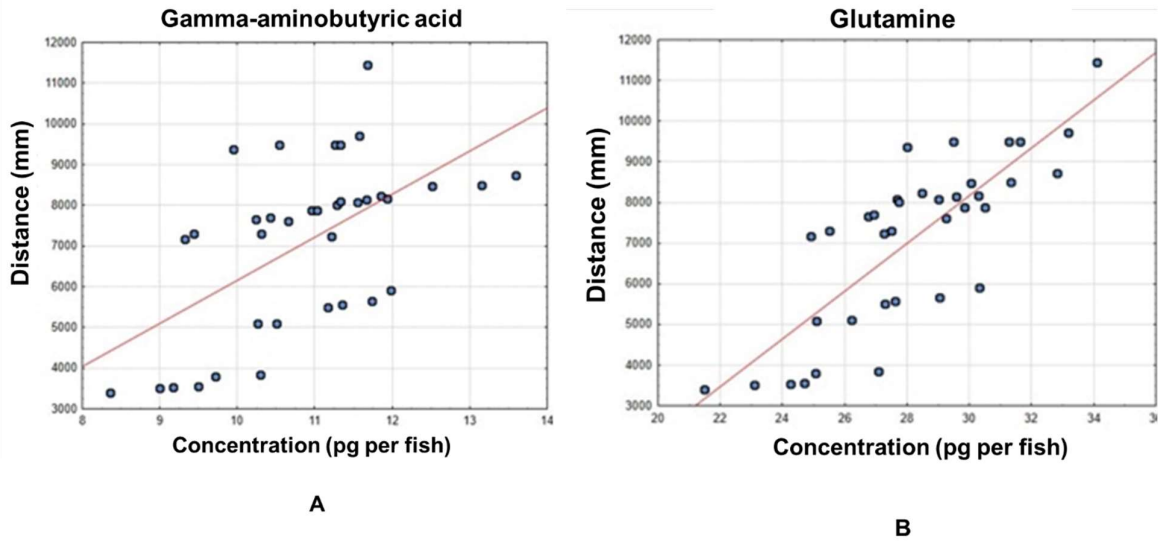


Figure 31 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 1).

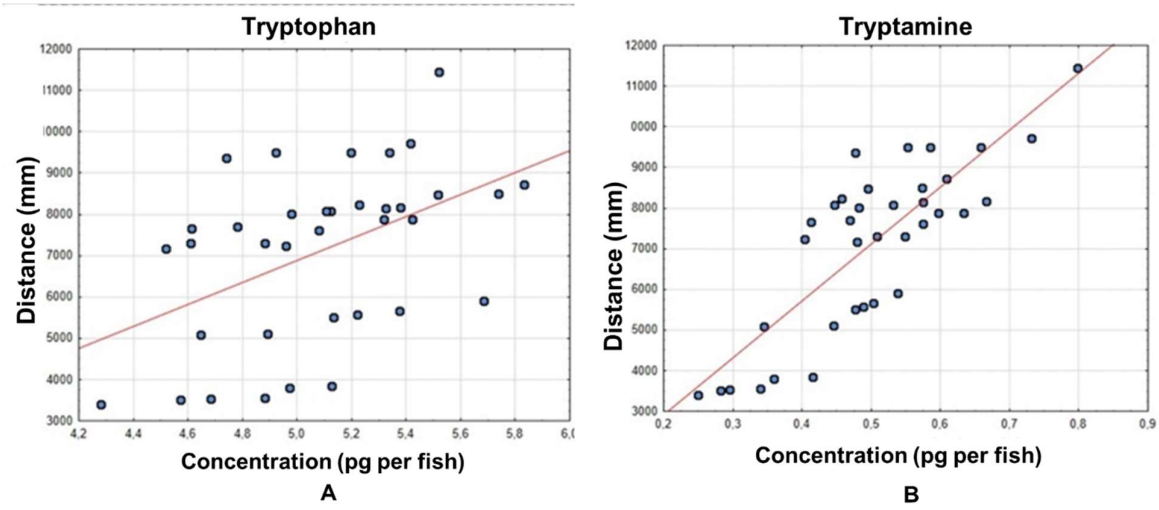


Figure 32 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 2).

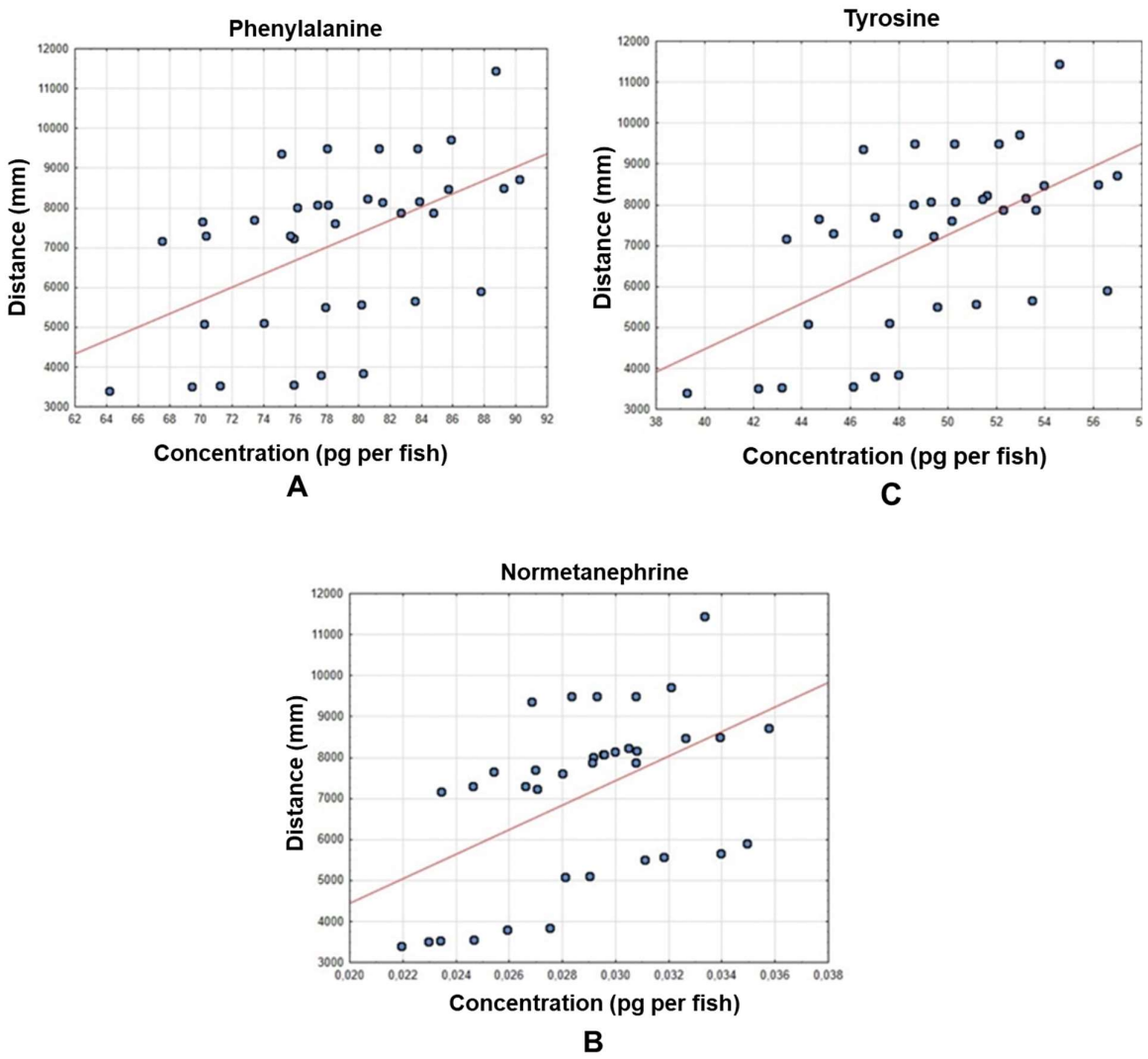


Figure 33 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 3).

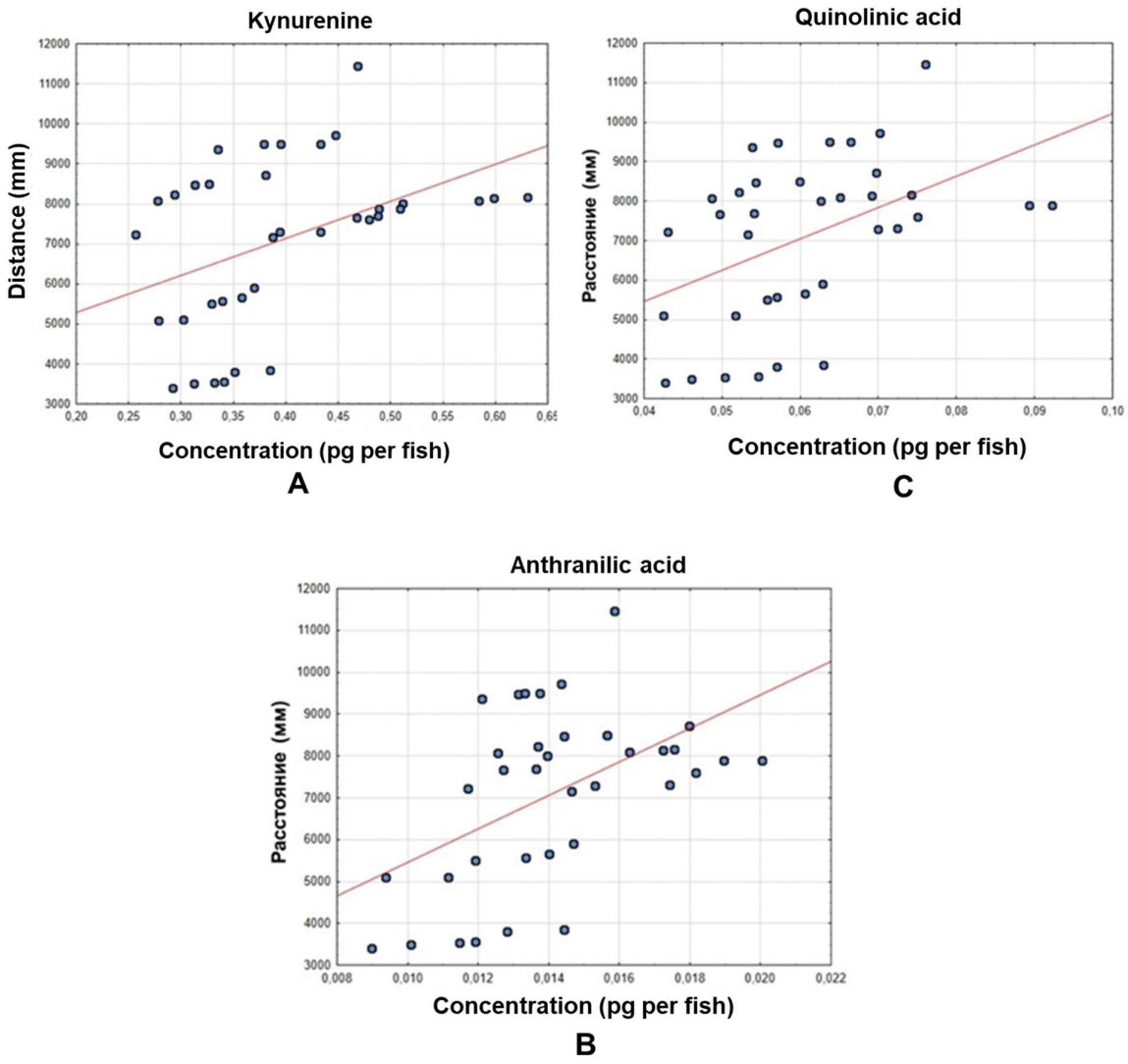


Figure 34 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 4).

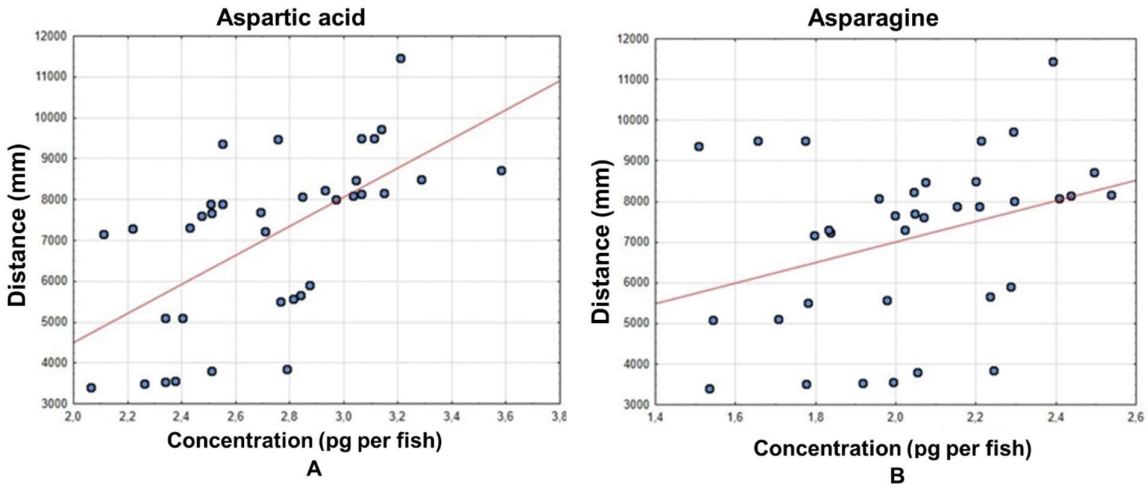


Figure 35 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 5).

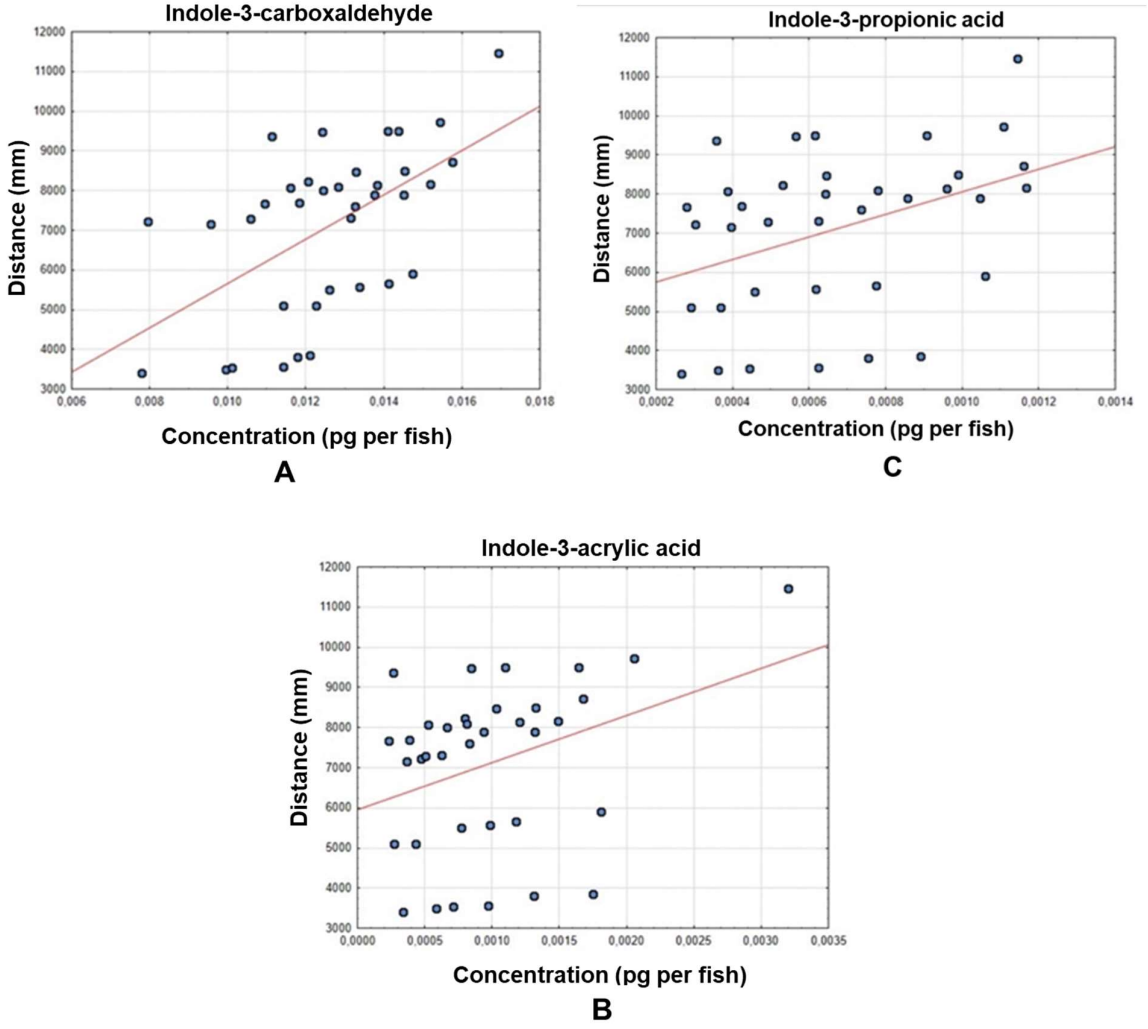


Figure 36 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 6).

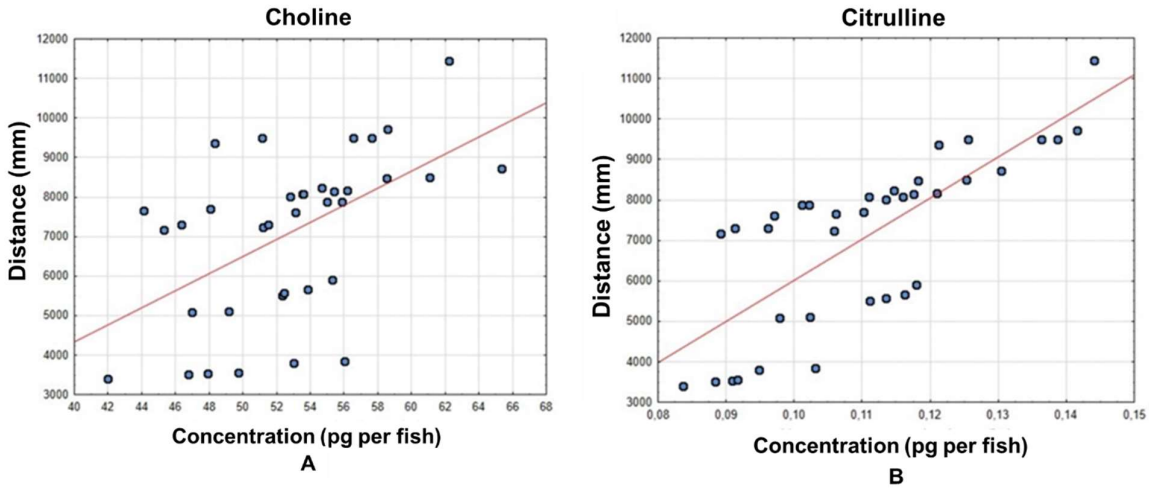


Figure 37 – Correlation between metabolites related to neurotransmission and total distance traveled by zebrafish larvae after 4 h exposure to 5F-APINAC (part 7).

4. Discussion

The use of novel approaches in search of new pharmacological substances and potential drugs of abuse and the extensive search of new animal models is an integral part of the modernization of experimental pharmacology. The studies that were carried out made it possible to develop the main methodological approaches for studying pharmacological effects of physiologically active substances using *Danio rerio* as a model organism in the pharmacometabolomics investigations.

At the first step, it is crucial to establish the most significant metabolic pathways that may provide information about the mode of action of the studied substances. When studying the effects of potentially neurotropic drugs using an untargeted metabolomics approach, it is necessary to pay special attention to the endogenous compounds potentially associated with neuronal transmission. Examples of such compounds would be amino acids – precursors for neurotransmitters synthesis (e.g., phenylalanine, tyrosine, tryptophan), organic acids, steroids, etc. The untargeted pharmacometabolomics approach had shown that diazepam caused alterations in the concentrations of endogenous metabolites related to neurotransmission, which made it possible to hypothesize that diazepam affects multiple metabolomics pathways related to neurotransmitters synthesis; also, this assessment has proven the ability to use zebrafish larvae as a model organism for studying the pharmacological effects of neurotropic drugs using pharmacometabolomics approach.

After establishing the metabolic pathways of interest through untargeted metabolomics, it is necessary to develop and validate the methods of targeted metabolomics approach to determine the concentrations of endogenous metabolites involved in the metabolic pathways. In the case of studying the pharmacological effects of neurotropic drugs, it is necessary to develop a method for the quantitative determination of endogenous compounds involved in various neurotransmitter systems. When the targeted metabolomics approach is conducted on a large number of metabolites, it is possible to assess a wide range of potential effects of the drug on the CNS.

To assess the effects of the pharmacological substances on targeted metabolic pathways, it is necessary to select a reference pharmacologically active substance with a well-described impact on the organism; besides, it has to affect the metabolic pathways studied. Thus, diazepam was chosen to study the pharmacometabolomics effects of the neurotropic substances using *Danio rerio* as a model animal. Diazepam is a pharmacological substance with well-described effects and the mechanism of action on the central nervous system. It is frequently used as a reference drug in the assessments of searching for new anxiolytic drugs [5, 6, 15].

There were no dose-dependent changes in the levels of neurotransmitters of the GABAergic system found in the experiments of diazepam exposure. The obtained results may be explained by the fact that diazepam's primary mode of action is binding to the $GABA_A$-receptors, thus allosterically enhancing their responsiveness to GABA [47]. It is known that diazepam can modulate $GABA_A$-receptors when combined with low GABA concentrations [236]. Therefore, these results suggest that the drug's effectiveness relies far more on its impact on GABA receptors rather than the amount of the synthesis of GABA.

Glutamic acid in its' ionized form (glutamate) is one of the most abundant amino acids found in the CNS and is also one of the primary excitatory neurotransmitters [333]. Also, glutamic acid is a precursor of GABA. The reaction of glutamate conversion into GABA is regulated with the glutamate decarboxylase enzyme [110]. Several of the authors found that glutamate plays a pivotal role in forming benzodiazepine dependence; moreover, the injection of glutamate antagonists led to a much rapid formation of dependence [280]. There was a tendency to increase glutamic acid concentrations in the zebrafish groups exposed to diazepam for a short period. The increase in glutamic acid levels after short-term exposure to diazepam may indicate the lowering of GABA synthesis due to an increase in GABAergic innervation.

The concentrations of aspartic acid were increased after both short-term and long-term exposure to diazepam. Aspartic acid is an alpha-amino acid that plays an important role in the biosynthesis of proteins, urea cycle, and gluconeogenesis, and

also is an agonist of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. The obtained results are consistent with the previous studies, which report increasing aspartate levels in the rodent's brain [45]. The activation of NMDA- and AMPA-receptors leads to the formation of excitotoxic effects; in fact, aspartic acid effects are opposite to those from GABA. It was found that the elevated levels of aspartic acid are connected to the excitotoxic effects [251]. It was also reported that the increased NMDA receptors stimulation takes place in diazepam dependence [250]. Therefore, it is conceivable that diazepam-induced elevated aspartic acid levels may contribute to its debilitating withdrawal signs such as anxiety, muscle rigidity, and seizures.

Diazepam had an influence on serotonergic system metabolites concentrations. Interestingly, tryptophan presented increased concentrations at higher doses of diazepam in short-term exposure but lower concentrations in long-term exposure. Consistent with our findings observed in the short-term exposure, studies performed in mice have shown that the acute administration of diazepam may cause an elevation of tryptophan concentrations in the brain [192]. Furthermore, long-term studies have observed reduced tryptophan concentrations in the brains of rats exposed to diazepam for a long period [62, 243]. Tryptophan is an alpha-amino acid that is a precursor of the neurotransmitter serotonin and several other derivatives. Tryptophan is an essential amino acid, so its concentrations in zebrafish larvae are related only to the nutrients in the yolk sac. Increased concentrations of tryptophan in the groups exposed to the highest dose of diazepam for a short term may be explained by the lower conversion rate of this amino acid into its' metabolites, which may be connected with the lowering of tryptophan hydroxylase activity. Decreasing tryptophan concentrations after long-term exposure may be interpreted as an enhancement of tryptophan catabolism into serotonin as a consequence of increasing tolerance to diazepam. This may be supported by the increased serotonin levels after 96 h exposure and decreased levels of its precursor, 5-hydroxytryptophan.

It is worth noting that serotonin is involved in an important neurotransmitter system regulating multiple physiological processes, including mood, reducing depression and anxiety [281]. Several researchers reported that diazepam's anxiolytic action involves the serotonergic system, specifically serotonin and its precursor [99, 234]. Other data indicate that diazepam exposure reduces the release of serotonin in neuronal cells [236, 241]. The lowering of serotonin release and the increase of its synthesis may be connected to the alterations in the levels of this metabolite.

Long-term diazepam exposure decreased the levels of 5-hydroxyindole acetic acid. The studies in this field present controversial data. Pratt et al. and Rastogi et al. postulate that the exposure of several benzodiazepines (clonazepam, diazepam, chlordiazepoxide, clobazam) led to the increase in the levels of 5-hydroxyindole acetic acid in rats' brains [236, 241]. In contrast, Boix et al. reported that intraperitoneal injection of diazepam did not cause the effect on the levels of 5-hydroxyindole acetic acid [62]. In the experiment on zebrafish larvae, 5-hydroxyindole acetic acid concentrations were lower in the groups exposed to diazepam for 96 h, indicating the lowering of the serotonin biotransformation rate. However, because the data about this metabolite is controversial, the results should be proven by using different animal models.

The concentrations of the metabolites of the kynurenine pathway were affected by diazepam exposure. The kynurenine concentrations were lower in the groups of zebrafish exposed to diazepam, while picolinic acid concentrations were higher in the groups of zebrafish exposed to the highest dose of diazepam for a long period.

Both kynurenine and picolinic acid are potential antagonists of NMDA-receptors. The lowering of the kynurenine concentrations may indicate that the increase of serotonergic system metabolites anabolism and kynurenine synthesis took place. These metabolites had shown to exhibit neuroprotective action [92]. In the meantime, the increase of the picolinic acid concentrations may postulate

compensatory reactions forwarded to decrease the excitotoxic effects of aspartic acid.

Phenylalanine and tyrosine concentrations similarly increased after 2.5 h exposure but had differential changes after 96 h exposure. Phenylalanine and tyrosine are amino acids that are precursors of the synthesis of the monoamines of the dopamine family. The information on the behavior of these metabolites during diazepam exposure is scarce, but some investigations have suggested that the expression of tyrosine hydroxylase (the enzyme that catalyzes the conversion of tyrosine into L-DOPA) is increased after diazepam administration [273]. Also, long-term exposure of diazepam to pregnant Long-Evans rats led to the decrease of the tyrosine levels in the brains of offspring [124]. The conversion of tyrosine into L-DOPA and subsequent metabolites and the lowering of tyrosine hydroxylase expression may explain the decrease in the tyrosine levels after long-term diazepam exposure. The increase in phenylalanine levels may be explained by the drop in the conversion rate of this metabolite into tyrosine through the inhibition of phenylalanine hydroxylase. The increase of phenylalanine and tyrosine levels after short-term exposure may be connected to the decrease in the conversion rate of these metabolites. This theory is proven by the general lowering trend of the dopamine family metabolites.

It is worth noting that the concentrations of L-DOPA, dopamine, normetanephrine and epinephrine were increased in the experimental group №1, followed by a subsequent decreasing trend relative to the increasing concentrations of diazepam after short-term exposure. Some authors report that diazepam elevates locomotor activity in mice, showing a “biphasic” response with increased activity at low doses and reduced activity at higher doses [101, 217, 330]. In addition, there is evidence that administration of a catecholamine synthesis inhibitor and dopamine receptor antagonists inhibit the diazepam-related increase in locomotor activity [279]. In this context, the results further support the hypothesis of diazepam's influence on dopamine levels linked to locomotor activity through the dopaminergic system. There is an acute biphasic response for most dopaminergic/adrenergic

metabolites at low diazepam concentration. Also, a similar trend of the concentrations of the dopaminergic/adrenergic metabolites may indicate that the biphasic behavioral response may be connected to dopamine and other metabolites of this system. The long-term diazepam exposure led to a decrease in dopamine levels in zebrafish larvae. The lowering of the concentrations of this metabolite may be connected to the depletion of dopaminergic synapses due to the formation of diazepam dependence.

The information about the influence of benzodiazepines on the levels of epinephrine and norepinephrine is scarce at this moment. Thus, Stein et al. found that alprazolam exposure led to the reduction of epinephrine turnover in the rats CNS [277]. At the same time, Rastogi et al. postulate that the chronic exposure of diazepam and bromazepam caused an increase in the levels of norepinephrine in several brain regions of rats [241]. So, the assumption can be made that the increase in the levels of norepinephrine after a long-term exposure may be connected to the lowering of its' catabolism; also, the increase of the epinephrine and norepinephrine concentrations may be interpreted as a lowering of dopamine catabolism. The abovementioned conclusions are in agreement with the trending of metanephrine, which was the decrease after short-term and the increase after long-term exposures to diazepam.

Acetylcholine is one of the main neurotransmitters in the CNS. There is a lack of information about the influence of diazepam on the cholinergic system. Lundgren et al. had shown that diazepam exposure led to the increase in the levels of acetylcholine, wherein did not cause the disruption in the levels of choline and did not alter the activity of choline transferase and acetylcholine transferase in the experiments on mice [93]. The elevated levels of acetylcholine may be connected to a slight anticholinergic activity of diazepam that develops due to the lowering of the release of acetylcholine from the preganglionic nerve cells, which leads to the decrease of its cleavage [93, 231]. To further investigate the true causes of the obtained results, more targeted investigations are needed.

There is no information about the effect of diazepam on the levels of microbiota tryptophan conversion pathway metabolites. However, there are data about the possibility of these metabolites binding to the benzodiazepines' site with the subsequent allosteric modification of GABA receptors [289]. Thus, changes in the levels of these metabolites may bring information about yet unknown action on the CNS.

When investigating the pharmacometabolomics effects of the poorly studied drugs, one should consider the information about the effects of the drugs of the same pharmacological group. Thus, when interpreting the results obtained after 5F-APINAC exposure, the data were compared with those received after exposure to other synthetic cannabinoids.

Besides the metabolomics changes, the exposure group №5 that received 10 uM solution of 5F-APINAC for a long period presented multiple morphological and developmental alterations. Approximately 60% of the larvae were unhatched, 20% had a curved tail related to spinal cord malformations, and all of them presented hyperpigmentation. Similar alterations were found in the experiments with different cannabimimetics. For example, the exposure of Δ^9-THC and synthetic cannabinoids CP 55940 and WIN 55,212-2 caused spinal curvatures, yolk sac edema, and pericardial edema [125]. Also, adverse effects of the cannabimimetics on embryo development were found using different animal models. For example, it was found that the fetus death rate was higher in the pregnant rabbits that were exposed to the Δ^9-THC in concentrations of 0.7 and 1.4 mg/kg [267]. Further investigations had shown that exposure to Δ^9-THC and marijuana increased levels of miscarriage, the rates of skeletal and soft tissues development (e.g., hydronephrosis, hydrocephalus, orofacial cleft, etc.) in rodents [214]. The exposure of the chick embryos to synthetic cannabinoid HU 210 led not only to the increase in death rates but also to the decrease in the mass and length of the body and the mass of the brain of embryos [89]. In humans, consumption of Δ^9-THC is reported to be connected with the number of miscarriages, fetal alcohol syndrome, and lower memory and studying skills in children [210]. According to WHO reports, case-control studies indicate

that using cannabinoids during pregnancy is connected to negative consequences for an embryo, e.g., lower weight and length of the body, sleep quality, verbal activity, and the memory of a child [150]. In the experiments of 5F-APINAC exposure, it was found that this cannabimimetic presents teratogenic activity; nevertheless, there was no increase in the death rates found. These data are in agreement with ones about a non-fluorinated analog of 5F-APINAC – APINAC [60, 270]. Though it should be noted that the assessment of the stability of this drug in the E3 medium was not studied, additional investigations are needed to address the question about the consistency of 5F-APINAC concentrations in the solutions.

GABA presented the lowest concentrations at the highest dose of exposure (10 μM 5F-APINAC) in the acute and chronic experiments compared to the vehicle control group. Cannabinoids have been found to inhibit the release of several neurotransmitters, including GABA, and inhibit the GABAergic innervation system [98]. Synthetic cannabinoids have been postulated to depress locomotor activity by inhibiting GABA uptake. Also, it is known that the endocannabinoid system modulates GABA receptors. It has been postulated that endocannabinoids can potentiate the stimulation of $GABA_A$ receptors at lower doses of GABA [79]. Also, evidence shows that prolonged treatment with cannabimimetics causes hyperexcitability and an increase in GABA release [54, 137]. Decreased levels of GABA in the groups treated with the highest dose of 5F-APINAC may indicate that cannabimimetics can increase GABAergic activity, which potentially leads to the lowering of GABA synthesis. Also, lower levels of GABA may be connected to the increased levels of its cleavage in the synaptic cleft due to its higher release.

Glutamine concentrations were also found to be altered, being lower at higher doses in acute exposure. In the chronic exposure experiment, glutamine concentrations were higher at higher exposure doses. Glutamine is a precursor of the neurotransmitters glutamate and GABA. Previous research did show that Δ^9-THC administration increased the total levels of glutamate + glutamine in humans [107, 152]. Also, it has been suggested that cannabinoid receptors agonists inhibit glutamine release [209]. Despite the lacking information about glutamine levels in

the CNS, it may be postulated that 5F-APINAC short-term exposure caused the decrease of glutamine release from neuronal cells, lowering its synthesis, while long-term exposure led to the increase of the synthesis, possibly through the rise of the GABA catabolism.

Several significant changes in the tryptophan and tryptamine levels attributable to 5F-APINAC exposure were observed in the study. The serotonergic system is key to regulating multiple neurophysiological processes, particularly mood, perception, anger, aggression, attention, and anxiety [226]. There is research suggesting that the endocannabinoids system is involved in the regulation of mood and depression via the serotonergic system [206]. It was found that Δ^9-THC and a synthetic cannabinoid WIN 55,212-2 inhibited the synthesis of 5-hydroxytryptophan and serotonin through the effect on tryptophan hydroxylase activity in different brain regions of rats [285]. In our study, elevated concentrations of tryptophan after long-term exposure may be explained by the inhibition of tryptophan hydroxylase, leading to the lowering of tryptophan conversion.

Nowadays, tryptamine is referred to as a group of "trace amines", compounds that are structurally and metabolically related to classical monoamines and that have agonist action to the trace amine-associated receptor 1 (TAAR1) [60]. Trace amines play a role in the regulation of the number of monoamines in the synaptic cleft, particularly stimulating TAAR1 receptors, which cause the release of neurotransmitters and prevent their reuptake [209]. There is a dearth of reliable information about tryptamine levels or their synthesis after exposure to cannabimimetics. Although, works report that tryptamine is readily metabolized by the brain monoamine oxidases, so it has a very short half-life period [79]. Overuse of this trace amine due to the increase of the effects of monoamines in the brain may explain its lowering levels in acute exposure, and very low levels after chronic exposure to 5F-APINAC may have resulted in nearly undetectable concentrations.

Several statistical differences on concentration levels of the kynurenine pathway were also found. These metabolites are connected to the kynurenine pathway, the main metabolic pathway of tryptophan conversion. Most of the

metabolites of the kynurenine pathway are connected to NMDA receptors transmission. Excessive levels of NMDA receptors agonists have been found to be connected with excitotoxicity in the brain [122]. Cannabinoids have been shown to reduce NMDA activity through NMDA receptors antagonism, thus decreasing excitotoxicity. Opposite to these findings, other studies have shown that exposure to synthetic cannabinoid WIN 55,212-2 and endocannabinoid anandamide enhance the effects of NMDA [49, 196, 197, 210, 264]. Kynurenic acid presents an antagonism to the NMDA receptors, thus performing neuroprotective and neuroinhibitory actions [92]. Xanthurenic acid is believed to have neuromodulator action by increasing neurotransmission rate [326]. It is also known that anthranilic and picolinic acids decrease excitotoxic effects by increasing the polarization of neuronal cells membranes [284]. Despite these facts, in our study, we found that the metabolites of the kynurenine pathway that are reported to be connected with neuroprotective effect were significantly increased after 5F-APINAC exposure. It may be interpreted and a realization of neuroprotective action as a response to the exposure to toxic agent – 5F-APINAC.

In the present study, higher dopamine levels after acute exposure and lower levels after chronic exposure at the highest doses of 5F-APINAC were observed. Dopamine, one of the main neurotransmitters, plays a significant role in reward-motivated behavior, and many addictive abuse substances increase dopamine release or block its reuptake into neurons. It is known that dopamine receptors are modulated by the endocannabinoid system and that acute Δ^9-THC exposure is associated with increased dopaminergic cell firing, increased dopamine synthesis, and increased dopamine release [298]. Also, acute Δ^9-THC and WIN 55,212-2 treatments have been shown to increase dopamine and noradrenaline synthesis in different regions of the brains of rats [302]. Repeated administration of synthetic cannabinoids increased the expression of tyrosine hydroxylase, the enzyme that catalyzes the reaction of tyrosine conversion into the metabolites of the dopamine family, in the brain of animals [118]. The increase of dopamine levels after a short-term 5F-APINAC exposure may be connected to the increase of its' synthesis. In the long-

term exposure, the increase of dopamine levels in the experimental groups №1-3 may also be linked to its' synthesis increase. In contrast, the decrease of dopamine in the experimental groups №4-5 may be potentially explained by the exhaustion of its' reserves in the cells during the formation of dependence.

Acetylcholine presented higher concentrations after acute exposure and lower concentrations after chronic exposure. It has been observed that the administration of Δ^9-THC can cause significant elevations in acetylcholine and choline levels in multiple brain regions in mice [100]. Administration of WIN 55,212–2 at a low dose induced stimulation of hippocampal acetylcholine efflux, while a higher dose exposure resulted in prolonged inhibition of efflux [101, 217]. Acetylcholine release in hippocampal synaptosomes was inhibited by the synthetic cannabinoid WIN 55,212-2 [108, 258]. Also, classic studies in this field have shown that acetylcholine levels were strongly reduced, especially in the hippocampus [205]. Despite the contradictory results obtained in the earlier studies, it may be postulated that short-term exposure of 5F-APINAC caused an increase of acetylcholine synthesis, while long-term exposure decreased its' efflux into the synaptic cleft.

Citrulline concentrations were lower in the groups of zebrafish exposed to 5F-APINAC for a short-term, while long-term exposure increased concentrations in the experimental group №5 compared to control. Citrulline is a substrate for the synthesis of the metabolites of the GABAergic innervation system [327]. Currently, there are no data describing the changes of citrulline concentrations after cannabimimetics exposure. The disturbance of citrulline concentrations may be connected to both the alterations of the GABAergic pathway metabolites catabolism and the difference in the balance of other biochemical pathways (e.g., urea cycle).

To confirm the action of 5F-APINAC on the CNS and investigate the connections between metabolomics data and the effect of 5F-APINAC, the behavioral assessment using zebrafish larvae was done.

A dose-dependent decrease of total locomotor activity was observed in the behavioral study. The activity was decreasing as the concentrations of 5F-APINAC were increasing. According to the available literature data, the locomotor activity of

zebrafish is changed in the form of a biphasic response, i.e., the increase of the activity at the lower doses of cannabimimetics, followed by the decrease at higher doses [102]. This type of response was found when exposing *Danio rerio* to such agonists of cannabinoid receptors as anandamide [223, 224], Δ^9-THC [73], WIN 55,212-2 [74], JWH-018 [247], AKB48, 5F-AKB48 [247], 5F-ADBINACA, AB-FUBINACA and STS-135 [261], and presupposes that the biphasic activity response is typical not just for a specific cannabinoid, but for all of the potential agonists of cannabinoid receptors [23, 211, 224]. Movement alterations up to akinesia are the most common side effects observed in cannabinoids intake [23, 24, 211]. The alterations in locomotor activity caused by cannabimimetics may be connected to their ability to increase the dopamine release in the nucleus accumbens [153, 280]. 5F-APINAC exposure, however, did not cause a biphasic response. This discrepancy may be connected to the concentrations tested in this study.

The exposure to 5F-APINAC caused a dose-dependent decrease of a total distance traveled both at 4 h exposure and 48 h exposure. These results are in agreement with ones obtained for Δ^9-THC in zebrafish [152, 288, 306]. The inhibiting effect of cannabinoid receptor agonists on locomotor activity is traditionally considered as a development of anxiety-like behavior. In contrast, recent studies have shown that cannabimimetics induce synaptic activity in the neuromuscular junctions, decreased heart rate, and the strength of the reaction on acute stress [113, 275]. These results show that the lowering of the zebrafish activity during cannabimimetics exposure may be interpreted not only as a building of anxiety.

Several investigations show that the lowering of the levels of GABA [90, 193], glutamine [132], tryptophan [187], choline [59], among others, is connected to anxiety behavior. Since the lowering of traveled distance is postulated as an indicator of stress [151, 152], the obtained results are in agreement with the literature data

Conclusions

The main stages of studying the pharmacological effects of biologically active substances through the pharmacometabolomics approach using *Danio rerio* as an animal model were developed. The applicability of the developed methodology was proven by studying the effects caused by the compounds with neurotropic action.

The analytical method for determining the metabolomic panel of endogenous metabolites related to neurotransmission was developed and validated. The developed method allows one to determine many of the essential endogenous compounds and makes it possible to assess the type and the strength of action of biologically active substances on the CNS.

The assessment of the diazepam action on the concentrations of neurotransmitters was made to investigate the possibility of *Danio rerio* usage in the investigations of pharmacodynamics of neurotropic drugs through the pharmacometabolomics approach. Diazepam exposure caused alterations in the levels of metabolites of different neurotransmitter systems. It is interesting to note that GABA concentrations did not change (or did not show a dose-dependent response) across all of the experimental groups, which suggests that the mechanism of action of diazepam is only realized through the modulation of $GABA_A$ receptors, wherein not changing the rate of synthesis of GABA.

The exposure of a novel synthetic cannabinoid 5F-APINAC caused alterations in the levels of neurotransmitters and related metabolites. The most notable changes were found in GABA, dopamine, tryptamine, and acetylcholine levels. The obtained results are in agreement with the effects of other cannabinoid receptor agonists, proving the effects of cannabimimetics on the turnover of neurotransmitters. The exposure of 5F-APINAC for 96 h led to the formation of morphological alterations in zebrafish embryos, proving the embryotoxic effect of this drug.

5F-APINAC exposure caused a dose-dependent decrease of locomotor activity of zebrafish larvae; besides that, 10 uM concentration of 5F-APINAC led to the dramatic decrease of activity, which indicates that this dose is toxic. The

correlations between traveled distance and the concentrations of neurotransmitters were found. This data is in agreement with the anxiogenic effect of cannabinoids.

The perspective of the further investigations on this topic may be connected to the evaluation of pharmacometabolomics effects of different neurotropic drugs to accumulate the data to predict the effects of unknown chemicals with a potential neurotropic mode of action. It is also needed to conduct additional behavioral studies to establish the type of neurotropic action. In addition, it is possible to develop other analytical methods for studying the effects of different drugs on the other organ systems (cardiovascular, digestive, etc.). Also, further studies in the field of "omics" approaches may be conducted to make complex investigations of the drugs of interest on the organism.

List of abbreviations

ED_{50} – half-effective dose

LD_{50} – half-lethal dose

LC_{50} – half-lethal concentration

RNA – ribonucleic acid

mRNA – matrix ribonucleic acid

DNA – desoxyribonucleic acid

GC – gas chromatography

GC-MS – gas chromatography coupled to mass-spectrometry

NMR – nuclear magnetic resonance

MS – mass-spectrometry

HPLC – high-performance liquid chromatography

HPLC-MS – high-performance liquid chromatography coupled with mass-spectrometry

HPLC-MS/MS – high-performance liquid chromatography coupled with tandem mass-spectrometry

PET – positron emission tomography

MALDI – matrix-assisted laser desorption/ionization

DM – diabetes mellitus

IEMs – inborn errors of metabolism

5F-APINAC – adamantan-1-yl-1-(5-fluoropentyl)-1H-indazole-3-carboxylate

E3 – zebrafish embryo medium

dpf – days post fertilization

MRM – multiple reaction monitoring

CNS – central nervous system

GABA – gamma-aminobutyric acid

L-DOPA – L-dioxyphenylalanine

NMDA-receptor – receptor of N-methyl-D-aspartate

AMPA-receptor – receptor of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

$GABA_A$-receptor – type A GABA receptor

Δ^9-THC – tetrahydrocannabinol

WHO – World Health Organization

TAAR1 – trace amine associated receptors, type 1

References

1. Abdrashitov R. “The review of the methods of quantifying the activity of CYP2D6 using exogenous and endogenous markers”. *Pharmakokinetika i pharmakodinamika*. 1 (2015). [In Russ.].

2. Babchenko V. “Traumatic brain injury models in Zebrafish (Danio rerio)’. *Rossiyskiy fisiologicheskiy jurnal im. IM Sechenova*. 107.9 (2021) [In Russ.].

3. Barulin N. “Impact of different incubation media on embryonic development of Denio rerio in in vivo experiments”. *Zhivotnovodstvo i veterinarnaya medicina*. 4 (2020). [In Russ.].

4. Belyaeva N. “Zebrafish as a model in biomedical studies”. *Biomedicinskaya himiya*. 56.1 (2010). [In Russ.].

5. Voronina T. “Outlook for new anxiolytics discovery”. *Experimentalnaya i klinicheskaya farmakologiya*. 65.5 (2002). [In Russ.].

6. Devyashin A. “Dose-dependent effect assessment of benzodiapine-type anxiolytics in Danio rerio”. *Obzori po klinicheskoy farmakologii i lekarstvennoy terapii*. 18.1 (2020). [In Russ.].

7. Dedov I. “Personalized medicine: current state and outlook”. *Vestnik Rossiyskoy akademii medicinskih nauk*. 67.12 (2012). [In Russ.].

8. Dryagina N. “Brain metabolome”. *Rossiyskiy nevrologicheskiy jurnal*. 25.1 (2020). [In Russ.].

9. Zastrogin M. “Carbamazepine impact on the activity of cytochrome P450 3A4 isoenzyme in patients with alcoholism”. *Experimentalnaya i klinicheskaya farmakologiya*. 79.10 (2016). [In Russ.].

10. Zastrogin M. “Association between performance profile and safety of bromodihydrochlorophenylbenzodiazepine and CYP2C19* polymorphisms in patients with anxiety disorders and alcoholism comorbidity”. *Narkologiya*. 17.4 (2018). [In Russ.].

11. Zub A. “The possibility of using biological model of freshwater fish Danio rerio in preclinical studies”. *Vestnik Volgogradskogo gosudarstvennogo medicinskogo universiteta*. 1 (73) (2020). [In Russ.].

12. Karcova L. “Chromatography and electrophoresis application in metabolomic studies”. *Jurnal analiticheskoy himii*. 74.4 (2019). [In Russ.].

13. Kachanov D. “Danio rerio (Zebrafish) as a versatile model in preclinical studies”. *Forcipe*. 1 (2018). [In Russ.].

14. Kachanov D. “Comparative review of possibilities of using Danio rerio (Zebrafish) as preclinical studies model”. *Mezhunarodniy nauchno-issledovatelskiy jurnal*. 6-2 (96) (2020). [In Russ.].

15. Korolev A. “Investigation of GABA A receptors role in the mechanism of psychotropic effects of pyrrolo [1,2-a][1,4] benzodiazepine derivatives in operant drug differentiation model in Wistar rats”. *Experimentalnaya i klinicheskaya farmakologiya*. 77.6 (2014). [In Russ.].

16. Krotova N. “Zebradanio (zebrafish) as a new promising model in translational neurobiology”. *Rossiyskiy fisiologicheskiy jurnal im. IM Sechenova*. 105.11 (2019). [In Russ.].

17. Kukes V. “Personalized medicine in clinical pharmacology”. *Biomedicina*. 3 (2010). [In Russ.].

18. Kukes V. “Personalized medicine in internal medicine”. *Klinicheskaya medicina*. 95.3 (2017). [In Russ.].

19. Lebedev V. “Possibility of using behavioral responses of Danio rerio in dose-dependent effects assessment of phenazepam”. *Laboratornie zhivotnie dlya nauchnih issledovaniy*. 1 (2018). [In Russ.].

20. Levikin K. “Comparative effects of antidepressants impact of different pharmacological groups on adult Danio rerio behavior”. *Obzory po klinicheskoy farmakologii i lekarstvennoy terapii*. 18.1 (2020). [In Russ.].

21. Livanova A. “Danio rerio as an experimental model in radiobiology”. *Radiacionnaya biologia. Radioekologiya*. 60.2 (2020) [In Russ.].

22. Markin P. “New psychoactive substance 5F-APINAC impact on endobiotics concentrations of different neurotransmitter systems analysis in Zebrafish (Danio rerio) and rabbit models”. *International Journal of Medicine and Psychology*. 3.5 (2020). [In Russ.].

23. Markin P. “Comparative analysis of behavioral models of Zebrafish and mice in response to new psychoactive substance APINAC exposure”. www. issledo. ru *Redakcionnaya kollegiya*. (2019): 52. [In Russ.].

24. Markin P. “Analysis of behavioral model of Zebrafish in response to new psychoactive substance 5F-APINAC exposure”. apni. ru. *Redakcionnaya kollegiya*. (2020): 15. [In Russ.].

25. Markin P. “Metabolomic profiling as an evaluation method of safety and/or efficiency of physiologically active substances”. *IV Vserossiyskaya konferenciya “Analiticheskaya hromatografiya I kapillyarniy elektroforez” s mezhdunarodnim uchastiem.* (2020). [In Russ.].

26. Maslov D. “Pharmacometabonimics is a new approach to personalization of drug therapy”. *Biomedicinskaya himiya*. 63.2 (2017). [In Russ.].

27. Mironov A. “Guideline for conducting preclinical studies of drugs”. (2012). [In Russ.].

28. Morozov S. “Challenges of complex chemical profiling of medicinal plants”. *Himiya rastitelnogo sirya*. 4 (2018). [In Russ.].

29. Neznamov G. “New anxiolytic drug Afobazol: results of comparative clinical study with diazepam in generalized anxiety disorder”. *Psihiatriya i psihofarmakologiya*. 8.4 (2006). [In Russ.].

30. Orlova A. “Using metabolomics approaches in medicinal plants and phytomedicines analysis (a review)”. *Razrabotka i registraciya lekarstvennih sredstv*. 10.1 (2021). [In Russ.].

31. Smirnov V. “Impact of CYD2D6 isoenzyme on drug metabolism and methods for determination its activity”. *Vedomosti Nauchnogo centra expertizi sredstv medicinskogo primeneniya*. 3 (2015). [In Russ.].

32. Smirnov V. “Determination of enzyme activity in drug metabolism – outlook for application in clinical practice”. *Vedomosti Nauchnogo centra expertizi sredstv medicinskogo primeneniya*. 4 (2016). [In Russ.].

33. Sichev D. “Clinical pharmacogenetics: Training manual”. *GEOTAR-Media*. 2007. [In Russ.].

34. Sichev D. “Stages of development and technological implementations of personalized medicine in clinical practice”. *World Journal of Personalized Medicine*. 1.1 (2017). [In Russ.].

35. Sichev D. “Personalized medicine: a clinical pharmacologist’s perspective”. *Consilium medicum*. 19.1 (2017). [In Russ.].

36. Sichev D. “Clinical and pharmacological technologies of drug therapy personalization in cardiovascular diseases: a focus on direct oral anticoagulants”. *Annals of the Russian Academy of Medical Sciences*. 74.5 (2019). [In Russ.].

37. Furina R. “Metabolomic studies in medicine”. *Kazanskiy medicinskiy jurnal*. 95.1 (2014). [In Russ.].

38. Shabanov P. “Novelty stress effect on behavioral responses of Danio rerio and dose-dependent effects evaluation of benzodiazepine-type anxiolytics using phenazepam as an example”. *Obzory po klinicheskoy farmakologii i lekarstvennoy terapii*. 15.3 (2017). [In Russ.].

39. Shestakova K. “Studying pharmacokinetics of new synthetic cannamimetic 5F-APINAC and its impact on tryptophan metabolism”. *IV Vserossiyskaya konferenciya “Analiticheskaya hromatografiya I kapillyarniy elektroforez” s mezhdunarodnim uchastiem*. (2020). [In Russ.].

40. Shestakova K. “Studying metabolism of new synthetic cannamimetic 5F-APINAC”. *Cennost estestvenno-nauchnogo i tehnicheskogo znaniya v usloviyah sovremennoy tehnogennoy civilizacii: sbornik nauchnih trudov po materialam Mezhdunarodnoy nauchno-prakricheskoy konferencii*. (2020). [In Russ.].

41. Shimshirt A. “Investigation of diazepam impact on anxiety responses, caused by indomethacin in mice”. *Rossiyskiy bioterapevticheskiy jurnal*. 11.1 (2012). [In Russ.].

42. Shimshirt A. “Studying the effects of indomethacin and its combination with diazepam on anxiety reactions impact”. *Visokie tehnologii, fundamentalnie i prikladnie issledovaniya v fisiologii i medicine*. (2012). [In Russ.].

43. Achenbach J.C. "Analysis of the uptake, metabolism, and behavioral effects of cannabinoids on zebrafish larvae." *Zebrafish*. 15.4 (2018).

44. Ahn E. "Temporal fluxomics reveals oscillations in TCA cycle flux throughout the mammalian cell cycle." *Molecular systems biology*. 13.11 (2017).

45. Akhtar M.T. "Developmental effects of cannabinoids on zebrafish larvae." *Zebrafish.* 10.3 (2013).

46. Akhtar M.T. "Metabolic effects of cannabinoids in zebrafish (Danio rerio) embryos determined by 1 H NMR metabolomics." *Metabolomics.* 12.3 (2016)

47. Akhtar M.T. "Zebrafish as a model for systems medicine R&D: rethinking the metabolic effects of carrier solvents and culture buffers determined by 1H NMR metabolomics." *Omics: a journal of integrative biology*. 20.1 (2016).

48. Anichtchik O. "Distinct structure and activity of monoamine oxidase in the brain of zebrafish (Danio rerio)." *Journal of Comparative Neurology.* 498.5 (2006).

49. Appolonova S.A. "In vivo and in vitro metabolism of the novel synthetic cannabinoid 5F-APINAC." *Forensic Toxicology.* 38.1 (2020).

50. Assem M. "The impact of uremic toxins on cerebrovascular and cognitive disorders." *Toxins.* 10.7 (2018).

51. Badyal D.K. and Chetna D. "Animal use in pharmacology education and research: The changing scenario." *Indian journal of pharmacology*. 46.3 (2014).

52. Barth A.L., Justice N.J., Ngai J. "Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system." *Neuron*. 16.1 (1996).

53. Bayés À. "Evolution of complexity in the zebrafish synapse proteome." *Nature communications.* 8.1 (2017).

54. Berger M., Gray J.A., Roth B.L. "The expanded biology of serotonin." *Annual review of medicine*. 60 (2009).

55. Berman J., Payne E., Hall C. "The zebrafish as a tool to study hematopoiesis, human blood diseases, and immune function." (2012).

56. Bhattacharya S. "The Role of Computational Systems Biology Models in Toxicity Testing in the 21st Century: an Example with Predictive Multi-Scale Models of the Liver." *New Horizons in Predictive Toxicology*. (2011).

57. Bhattacharyya S. "Metabolomic signature of exposure and response to citalopram/escitalopram in depressed outpatients." *Translational psychiatry* 9.1 (2019).

58. Bilici R. "Synthetic cannabinoids." *Northern clinics of Istanbul*. 1.2 (2014).

59. Bjelland I. "Choline in anxiety and depression: the Hordaland Health Study." *The American journal of clinical nutrition*. 90.4 (2009).

60. Bloomfield M. AP "The effects of Δ 9-tetrahydrocannabinol on the dopamine system." *Nature*. 539.7629 (2016).

61. Boekelheide K., Andersen M. "A mechanistic redefinition of adverse effects–a key step in the toxicity testing paradigm shift." *ALTEX-Alternatives to animal experimentation*. 27.4 (2010).

62. Boix, F. "Handling-habituation prevents the effects of diazepam and alprazolam on brain serotonin levels in rats." *Behavioural brain research*. 36.3 (1990).

63. Bouatra S. "The human urine metabolome." *PloS one*. 8.9 (2013).

64. Braford Jr, Mark R. "Comparative aspects of forebrain organization in the ray-finned fishes: touchstones or not?." *Brain, Behavior and Evolution.* 46.4-5 (1995).

65. Brand M., Granato M., Nüsslein-Volhard C. "Keeping and raising zebrafish." *Zebrafish: a practical approach*. (2002).

66. Brindle J.T. "Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1 H-NMR-based metabonomics." *Nature medicine*. 8.12 (2002).

67. van den Brink, W. J. "Multivariate pharmacokinetic/pharmacodynamic (PKPD) analysis with metabolomics shows multiple effects of remoxipride in rats." *European Journal of Pharmaceutical Sciences*. 109 (2017).

68. Brown J.M., Hazen S.L. "The gut microbial endocrine organ: bacterially derived signals driving cardiometabolic diseases." *Annual review of medicine*. 66 (2015).

69. Burgess H.A., Granato M. "Sensorimotor gating in larval zebrafish." Journal of *Neuroscience*. 27.18 (2007).

70. Burgess H.A., Granato M. "Modulation of locomotor activity in larval zebrafish during light adaptation." *Journal of Experimental Biology*. 210.14 (2007).

71. Burt T., Nandal S. "Pharmacometabolomics in early-phase clinical development." *Clinical and translational science*. 9.3 (2016).

72. Calcaterra N.E., Barrow J.C. "Classics in chemical neuroscience: diazepam (valium)." *ACS chemical neuroscience*. 5.4 (2014).

73. Canazza I. "Effect of the novel synthetic cannabinoids AKB48 and 5F-AKB48 on “tetrad”, sensorimotor, neurological and neurochemical responses in mice. In vitro and in vivo pharmacological studies." *Psychopharmacology*. 233.21 (2016).

74. Canazza I. "Pharmaco-toxicological effects of the novel third-generation fluorinate synthetic cannabinoids, 5F-ADBINACA, AB-FUBINACA, and STS-135 in mice. In vitro and in vivo studies." *Human Psychopharmacology: Clinical and Experimental.* 32.3 (2017).

75. Candy J., Collet C. "Two tyrosine hydroxylase genes in teleosts." *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*. 1727.1 (2005).

76. Carpio Y., Estrada M. P. "Zebrafish as a genetic model organism." *Biotecnología Aplicada*. 23.4 (2006).

77. Cassani E. "Increased urinary indoxyl sulfate (indican): new insights into gut dysbiosis in Parkinson's disease." *Parkinsonism & related disorders*. 21.4 (2015).

78. Cassar S. "Use of zebrafish in drug discovery toxicology." *Chemical research in toxicology*. 33.1 (2019).

79. Castaneto M.S. "Synthetic cannabinoids: epidemiology, pharmacodynamics, and clinical implications." *Drug and alcohol dependence*. 144 (2014).

80. Chakravarty S. "Chronic unpredictable stress (CUS)-induced anxiety and related mood disorders in a zebrafish model: altered brain proteome profile implicates mitochondrial dysfunction." *PloS one.* 8.5 (2013).

81. Chávez M. N. "Zebrafish as an emerging model organism to study angiogenesis in development and regeneration." *Frontiers in physiology*. 7 (2016).

82. Chen C., Gonzalez F.J., Idle J.R. "LC-MS-based metabolomics in drug metabolism." *Drug metabolism reviews*. 39.2-3 (2007).

83. Chen R. "Personal omics profiling reveals dynamic molecular and medical phenotypes." *Cell*. 148.6 (2012).

84. Chih-Kang C. "Indoxyl sulfate, a representative uremic toxin, suppresses erythropoietin production in a HIF-dependent manner." *Laboratory investigation*. 91.11 (2011).

85. Chouchani E.T. "Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS." *Nature*. 515.7527 (2014).

86. Cicatiello A. G., Di Girolamo D., Dentice M. "Metabolic effects of the intracellular regulation of thyroid hormone: old players, new concepts." *Frontiers in endocrinology.* 9 (2018).

87. Clayton T. A. "Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism." *Proceedings of the National Academy of Sciences.* 106.34 (2009).

88. Clish C.B. "Metabolomics: an emerging but powerful tool for precision medicine." *Molecular Case Studies*. 1.1 (2015).

89. Cohen K. "Chronic use of synthetic cannabinoids is associated with impairment in working memory and mental flexibility." *Frontiers in psychiatry*. 11 (2020).

90. Colizzi M. "Delta-9-tetrahydrocannabinol increases striatal glutamate levels in healthy individuals: implications for psychosis." *Molecular psychiatry*. 25.12 (2020).

91. Collier S.R., Casey D.P., Kanaley J.A. "Growth hormone responses to varying doses of oral arginine." *Growth hormone & IGF research*. 15.2 (2005).

92. Connick, J. H., and T. W. Stone. "Quinolinic acid effects on amino acid release from the rat cerebral cortex in vitro and in vivo." *British journal of pharmacology*. 93.4 (1988).

93. Consolo S. "Effect of diazepam on mouse whole brain and brain area acetylcholine and choline levels." *European journal of pharmacology*. 27.2 (1974).

94. Costello L.C. "The important role of osteoblasts and citrate production in bone formation:“osteoblast citration” as a new concept for an old relationship." *The Open bone journal.* 4 (2012).

95. Couchman L. "A novel approach to quantitative LC-MS/MS: therapeutic drug monitoring of clozapine and norclozapine using isotopic internal calibration." *Analytical and bioanalytical chemistry*. 405.29 (2013).

96. Dang L. "Cancer-associated IDH1 mutations produce 2-hydroxyglutarate." *Nature*. 462.7274 (2009).

97. David A. "Concentrating mixtures of neuroactive pharmaceuticals and altered neurotransmitter levels in the brain of fish exposed to a wastewater effluent." *Science of the Total Environment*. 621 (2018).

98. Deshpande, L.S., R.E. Blair, R.J. DeLorenzo. "Prolonged cannabinoid exposure alters GABAA receptor mediated synaptic function in cultured hippocampal neurons." *Experimental neurology*. 229.2 (2011).

99. Djeridane Y., B. Lemmer, Y. Touitou. "Diazepam affects both level and amplitude of rat locomotor activity rhythm but has no effect on core body temperature." *Chronobiology international*. 22.6 (2005).

100. Domino E.F. "Cannabinoids and the cholinergic system." *The Journal of Clinical Pharmacology*. 21.S1 (1981).

101. Dong X., Y. Wang, Z. Qin. "Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases." *Acta Pharmacologica Sinica.* 30.4 (2009).

102. Drews E., M. Schneider, M. Koch. "Effects of the cannabinoid receptor agonist WIN 55,212-2 on operant behavior and locomotor activity in rats." *Pharmacology Biochemistry and Behavior*. 80.1 (2005).

103. Dunn, W. B., N. JC Bailey, H. E. Johnson. "Measuring the metabolome: current analytical technologies." *Analyst*. 130.5 (2005).

104. Elahi D. A. R. I. U. S. H. "Interaction of arginine and gastric inhibitory polypeptide on insulin release in man." *American Journal of Physiology-Endocrinology and Metabolism*. 242.5 (1982).

105. Ellero-Simatos S. "Pharmacometabolomics reveals that serotonin is implicated in aspirin response variability." *CPT: pharmacometrics & systems pharmacology*. 3.7 (2014).

106. Ellertsdóttir E. "Vascular morphogenesis in the zebrafish embryo." *Developmental biology*. 341.1 (2010).

107. Fantegrossi W.E., C.D. Wilson, M.D. Berquist. "Pro-psychotic effects of synthetic cannabinoids: interactions with central dopamine, serotonin, and glutamate systems." *Drug metabolism reviews*. 50.1 (2018).

108. Fattore L. "Synthetic cannabinoids—further evidence supporting the relationship between cannabinoids and psychosis." *Biological psychiatry*. 79.7 (2016).

109. FDA, US. "Bioanalytical method validation guidance for industry, US Department of Health and Human Services." *Food and Drug Administration, Center*

for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Biopharmaceutics. (2018).

110. Fenalti G. "GABA production by glutamic acid decarboxylase is regulated by a dynamic catalytic loop." *Nature structural & molecular biology.* 14.4 (2007).

111. Flores M. "P4 medicine: how systems medicine will transform the healthcare sector and society." *Personalized medicine*. 10.6 (2013).

112. Fu Jing Z.G., B.C. Kelly. "Metabolomic profiling of zebrafish (Danio rerio) embryos exposed to the antibacterial agent triclosan." *Environmental toxicology and chemistry.* 38.1 (2019).

113. Fujii H., S. Goto, M. Fukagawa. "Role of uremic toxins for kidney, cardiovascular, and bone dysfunction." *Toxins.* 10.5 (2018).

114. Fukui S. "Blood–brain barrier transport of kynurenines: implications for brain synthesis and metabolism." *Journal of neurochemistry.* 56.6 (1991).

115. Gabbs M. "Advances in our understanding of oxylipins derived from dietary PUFAs." *Advances in nutrition.* 6.5 (2015).

116. Gamazon E.R., A.D. Skol, M.A. Perera "The limits of genome-wide methods for pharmacogenomic testing." *Pharmacogenetics and genomics.* 22.4 (2012).

117. Gessel M.M., J.L. Norris, R.M. Caprioli "MALDI imaging mass spectrometry: spatial molecular analysis to enable a new age of discovery." *Journal of proteomics.* 107 (2014).

118. Gevi F. "Urinary metabolomics of young Italian autistic children supports abnormal tryptophan and purine metabolism." *Molecular autism.* 7.1 (2016).

119. Gifford A.N. "Cannabinoid receptor-mediated inhibition of acetylcholine release from hippocampal and cortical synaptosomes." *British journal of pharmacology.* 131.3 (2000).

120. Gill S. R."Metagenomic analysis of the human distal gut microbiome." *Science*. 312.5778 (2006).

121. Gore A.V. "Vascular development in the zebrafish." *Cold Spring Harbor perspectives in medicine.* 2.5 (2012).

122. Gowda GA N. "Metabolomics-based methods for early disease diagnostics." *Expert review of molecular diagnostics.* 8.5 (2008).

123. Grant R. S., S. E. Coggan, G. A. Smythe. "The physiological action of picolinic acid in the human brain." *International journal of tryptophan research.* 2 (2009).

124. Grebe S.KG, R.J. Singh. "LC-MS/MS in the clinical laboratory–where to from here?." *The Clinical biochemist reviews.* 32.1 (2011).

125. Greizerstein H.B., L.K. Aldrich. "Ethanol and diazepam effects on intrauterine growth of the rat." *Developmental pharmacology and therapeutics.* 6 (1983).

126. Gustafsson S.B., S. OP Jacobsson "Effects of cannabinoids on the development of chick embryos in ovo." *Scientific reports.* 9.1 (2019).

127. Guyon J.R. "Modeling human muscle disease in zebrafish." *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease.* 1772.2 (2007).

128. Han J.H. "Metabolomic profiling distinction of human nonalcoholic fatty liver disease progression from a common rat model." *Obesity.* 25.6 (2017).

129. Han Y. "Cardiotoxicity evaluation of anthracyclines in zebrafish (Danio rerio)." *Journal of Applied Toxicology.* 35.3 (2015).

130. Hansen R.L., Y. Jin Lee "High-Spatial Resolution Mass Spectrometry Imaging: Toward Single Cell Metabolomics in Plant Tissues." *The Chemical Record.* 18.1 (2018).

131. Hartung T. "Systems toxicology: real world applications and opportunities." *Chemical research in toxicology.* 30.4 (2017).

132. Hartung T., M. McBride "Food for thought… on mapping the human toxome." *ALTEX-Alternatives to animal experimentation.* 28.2 (2011).

133. Hasler G. "Association between prefrontal glutamine levels and neuroticism determined using proton magnetic resonance spectroscopy." *Translational psychiatry*. 9.1 (2019).

134. He J.H. "A zebrafish phenotypic assay for assessing drug-induced hepatotoxicity." *Journal of pharmacological and toxicological methods.* 67.1 (2013).

135. He X. "Gut microbiota and nonalcoholic fatty liver disease: insights on mechanism and application of metabolomics." *International journal of molecular sciences.* 17.3 (2016).

136. Hefti F.F. "Requirements for a lead compound to become a clinical candidate." *BMC neuroscience.* 9.3 (2008).

137. Hellmuth C. "Tyrosine is associated with insulin resistance in longitudinal metabolomic profiling of obese children." *Journal of diabetes research.* (2016).

138. Höglund E., Ø. Øverli, S. Winberg "Tryptophan metabolic pathways and brain serotonergic activity: a comparative review." *Frontiers in endocrinology.* 10 (2019).

139. Hol J.W. "The tryptophan kynurenine pathway, neopterin and IL-6 during vulvectomy and abdominal hysterectomy." *Journal of biomedical science.* 21.1 (2014).

140. Holmes E., I.D. Wilson, J.K. Nicholson. "Metabolic phenotyping in health and disease." *Cell*. 134.5 (2008).

141. Horzmann K.A., J.L. Freeman. "Zebrafish get connected: investigating neurotransmission targets and alterations in chemical toxicity." *Toxics.* 4.3 (2016).

142. Tuzankina I. "PATHOPHYSIOLOGIC GENE DEPENDENT MECHANISMS OF SEPARATE TYPES OF IMMUNE MEDIATED PATHOLOGY (English version)". *Diss. Russian Academy of Sciences*. (2019).

143. Howe K. "The zebrafish reference genome sequence and its relationship to the human genome." *Nature.* 496.7446 (2013).

144. Hu Y. "Changes of embryonic development, locomotor activity, and metabolomics in zebrafish co-exposed to chlorpyrifos and deltamethrin." *Journal of Applied Toxicology.* 41.9 (2021).

145. Hubbard T.D., I.A. Murray, G.H. Perdew "Indole and tryptophan metabolism: endogenous and dietary routes to Ah receptor activation." *Drug Metabolism and Disposition.* 43.10 (2015).

146. Hutton J. C., A. Sener, W.J. Malaisse "Interaction of branched chain amino acids and keto acids upon pancreatic islet metabolism and insulin secretion." *Journal of biological chemistry*. 255.15 (1980).

147. International Council for Harmonization. "ICH Guideline M10 on Bioanalytical Method Validation." (2019).

148. Irving B.A. "Effect of insulin sensitizer therapy on amino acids and their metabolites." *Metabolism.* 64.6 (2015).

149. Isogai S., M. Horiguchi, B., M. Weinstein "The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development." *Developmental biology*. 230.2 (2001).

150. Ito S., M. Yoshida "Protein-bound uremic toxins: new culprits of cardiovascular events in chronic kidney disease patients." *Toxins.* 6.2 (2014).

151. Iversen L. "Cannabis and the brain." *Brain.* 126.6 (2003).

152. Jenny M. "Δ9-Tetrahydrocannabinol and cannabidiol modulate mitogen-induced tryptophan degradation and neopterin formation in peripheral blood mononuclear cells in vitro." *Journal of neuroimmunology.* 207.1-2 (2009).

153. Jenny M. "The potential role of cannabinoids in modulating serotonergic signaling by their influence on tryptophan metabolism." *Pharmaceuticals.* 3.8 (2010).

154. Jensen H.M. "Cannabidiol effects on behaviour and immune gene expression in zebrafish (Danio rerio)." *PloS one*. 13.7 (2018).

155. Jia M. "Toxicity and metabolomics study of isocarbophos in adult zebrafish (Danio rerio)." *Ecotoxicology and environmental safety.* 163 (2018).

156. Jin Y. "The toxicity of chlorpyrifos on the early life stage of zebrafish: a survey on the endpoints at development, locomotor behavior, oxidative stress and immunotoxicity." *Fish & shellfish immunology.* 43.2 (2015).

157. Kaddurah-Daouk R. "Metabolomic mapping of atypical antipsychotic effects in schizophrenia." *Molecular psychiatry.* 12.10 (2007).

158. Kaddurah-Daouk R. "Lipidomic analysis of variation in response to simvastatin in the Cholesterol and Pharmacogenetics Study." *Metabolomics.* 6.2 (2010).

159. Kaddurah-Daouk R. "Enteric microbiome metabolites correlate with response to simvastatin treatment." *PLoS One*. 6.10 (2011).

160. Kaddurah-Daouk R., B.S. Kristal, R.M. Weinshilboum. "Metabolomics: a global biochemical approach to drug response and disease." *Annu. Rev. Pharmacol. Toxicol.* 48 (2008).

161. Kaddurah-Daouk R., R. Weinshilboum, Pharmacometabolomics Research Network. "Metabolomic signatures for drug response phenotypes: pharmacometabolomics enables precision medicine." *Clinical Pharmacology & Therapeutics.* 98.1 (2015).

162. Kaddurah-Daouk R., R.M. Weinshilboum, Pharmacometabolomics Research Network. "Pharmacometabolomics: implications for clinical pharmacology and systems pharmacology." *Clinical Pharmacology & Therapeutics.* 95.2 (2014).

163. Kalueff A.V. "Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond." *Zebrafish.* 10.1 (2013).

164. Kalueff A.V., A.M. Stewart, R. Gerlai. "Zebrafish as an emerging model for studying complex brain disorders." *Trends in pharmacological sciences.* 35.2 (2014).

165. Kanungo J. "Zebrafish model in drug safety assessment." *Current Pharmaceutical Design.* 20.34 (2014).

166. Karu Naama "A review on human fecal metabolomics: Methods, applications and the human fecal metabolome database." *Analytica chimica acta.* 1030 (2018).

167. Kim K. "Mealtime, temporal, and daily variability of the human urinary and plasma metabolomes in a tightly controlled environment." *PloS one.* 9.1 (2014).

168. Kim S. "Food metabolomics: from farm to human." *Current Opinion in Biotechnology*. 37 (2016).

169. Kimmel C.B. "Stages of embryonic development of the zebrafish." *Developmental dynamics.* 203.3 (1995).

170. Klein S., E. Heinzle "Isotope labeling experiments in metabolomics and fluxomics." *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* 4.3 (2012).

171. Koeth R.A. "Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis." *Nature medicine* 19.5 (2013).

172. Kokel D. "Rapid behavior-based identification of neuroactive small molecules in the zebrafish." *Nature chemical biology* 6.3 (2010).

173. Koppe L. "Insulin resistance in chronic kidney disease: new lessons from experimental models." *Nephrology Dialysis Transplantation* 29.9 (2014).

174. Krause D. "High kynurenine (a tryptophan metabolite) predicts remission in patients with major depression to add-on treatment with celecoxib." *Frontiers in psychiatry* 8 (2017).

175. Krauss R.M., H. Zhu, R. Kaddurah-Daouk "Pharmacometabolomics of statin response." *Clinical Pharmacology & Therapeutics* 94.5 (2013).

176. Küchler A.M. "Development of the zebrafish lymphatic system requires VEGFC signaling." *Current Biology* 16.12 (2006).

177. Kujala U.M. "Branched-chain amino acid levels are related with surrogates of disturbed lipid metabolism among older men." *Frontiers in medicine* 3 (2016).

178. Lee C. C. "Branched-chain amino acids and insulin metabolism: the Insulin Resistance Atherosclerosis Study (IRAS)." *Diabetes care* 39.4 (2016).

179. Lee J., J.L. Freeman. "Zebrafish as a model for investigating developmental lead (Pb) neurotoxicity as a risk factor in adult neurodegenerative disease: a mini-review." *Neurotoxicology* 43 (2014).

180. Lehotay D. C. "LC–MS/MS progress in newborn screening." *Clinical biochemistry* 44.1 (2011).

181. Lehto M., P. Groop. "The gut-kidney axis: putative interconnections between gastrointestinal and renal disorders." *Frontiers in endocrinology* 9 (2018).

182. Lei X.D. "Role of tumor necrosis factor-alpha in zebrafish retinal neurogenesis and myelination." *International journal of ophthalmology* 9.6 (2016).

183. Leibfritz D., W. Dreher, W. Willker. "In vivo NMR applications of metabonomics." *The Handbook of Metabonomics and Metabolomics* (2007).

184. Lele Z., P. H. Krone. "The zebrafish as a model system in developmental, toxicological and transgenic research." *Biotechnology advances* 14.1 (1996).

185. Lenzen, S., H. Formanek, U. Panten. "Signal function of metabolism of neutral amino acids and 2-keto acids for initiation of insulin secretion." *Journal of Biological Chemistry* 257.12 (1982).

186. L. Caixia "Comprehensive and quantitative proteomic analyses of zebrafish plasma reveals conserved protein profiles between genders and between zebrafish and human." *Scientific reports* 6.1 (2016).

187. Liabeuf S. "Clinical studies and chronic kidney disease: what did we learn recently?" *Seminars in nephrology*. Vol. 34. No. 2. WB Saunders. (2014).

188. Lindseth G., B. Helland, J. Caspers "The effects of dietary tryptophan on affective disorders." *Archives of psychiatric nursing* 29.2 (2015).

189. Linscheid N. "Quantitative proteome comparison of human hearts with those of model organisms." *PLoS biology* 19.4 (2021).

190. L. Shu, S.D. Leach "Zebrafish models for cancer." *Annual Review of Pathology: Mechanisms of Disease* 6 (2011).

191. L. XingYu "Developmental toxicity and neurotoxicity of synthetic organic insecticides in zebrafish (Danio rerio): A comparative study of deltamethrin, acephate, and thiamethoxam." *Chemosphere* 199 (2018).

192. Lopez-Castejon G., D. Brough "Understanding the mechanism of IL-1β secretion." *Cytokine & growth factor reviews* 22.4 (2011).

193. López-Rubalcava C., A. Saldivar, A. Fernandez-Guasti "Interaction of GABA and serotonin in the anxiolytic action of diazepam and serotonergic anxiolytics." *Pharmacology Biochemistry and Behavior* 43.2 (1992).

194. Lydiard R. B. "The role of GABA in anxiety disorders." *Journal of Clinical Psychiatry* 64 (2003).

195. MacPhail R. C. "Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol." *Neurotoxicology* 30.1 (2009).

196. Mahidol C. "Biodiversity and natural product drug discovery." *Pure and Applied Chemistry* 70.11 (1998).

197. Markin P. "Short-and long-term exposures of the synthetic cannabinoid 5F-APINAC induce metabolomic alterations associated with neurotransmitter systems and embryotoxicity confirmed by teratogenicity in zebrafish." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 243 (2021).

198. Markin P. "Short-and medium-term exposures of diazepam induce metabolomic alterations associated with the serotonergic, dopaminergic, adrenergic and aspartic acid neurotransmitter systems in zebrafish (Danio rerio) embryos/larvae." *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 38 (2021).

199. Markin P., Moskaleva N., Appolonova S. "Methodology of the new pharmacometabolic approach in the investigation of the pharmacological effects of physiologically active drugs using Danio rerio model: examples om neurotropic drugs". *Scientific research of the SCO countries: Synergy and Integration.* (2020).

200. Mathew A. K., Padmanaban V. C. "Metabolomics: The apogee of the omics trilogy". *International Journal of Pharmacy and Pharmaceutical Sciences*. (2013).

201. Mattes W.B. "Public consortium efforts in toxicogenomics." *Essential Concepts in Toxicogenomics*. Humana Press (2008).

202. Mazzali M. "Uric acid and hypertension: cause or effect?." *Current rheumatology reports* 12.2 (2010).

203. McClay J.L. "Large-scale neurochemical metabolomics analysis identifies multiple compounds associated with methamphetamine exposure." *Metabolomics* 9.2 (2013).

204. Medina, S. "Metabolomics and the diagnosis of human diseases-A guide to the markers and pathophysiological pathways affected." *Current medicinal chemistry* 21.7 (2014).

205. Gabbs M. "Advances in our understanding of oxylipins derived from dietary PUFAs." *Advances in nutrition* 6.5 (2015).

206. Mendiguren A., J. Pineda. "Cannabinoids enhance N-methyl-D-aspartate-induced excitation of locus coeruleus neurons by CB1 receptors in rat brain slices." *Neuroscience letters* 363.1 (2004).

207. Miller G.M. "The emerging role of trace amine-associated receptor 1 in the functional regulation of monoamine transporters and dopaminergic activity." *Journal of neurochemistry* 116.2 (2011).

208. Mills E., L. AJ O'Neill "Succinate: a metabolic signal in inflammation." *Trends in cell biology* 24.5 (2014).

209. Mokdad A.H. "Actual causes of death in the United States, 2000." *Jama* 291.10 (2004).

210. Moranta D., S. Esteban, J. García-Sevilla "Differential effects of acute cannabinoid drug treatment, mediated by CB 1 receptors, on the in vivo activity of tyrosine and tryptophan hydroxylase in the rat brain." *Naunyn-Schmiedeberg's archives of pharmacology* 369.5 (2004).

211. Morbiato E. "Potential of the zebrafish model for the forensic toxicology screening of NPS: A comparative study of the effects of APINAC and methiopropamine on the behavior of zebrafish larvae and mice." *Neurotoxicology* 78 (2020).

212. Mu X. "Developmental effects and estrogenicity of bisphenol A alternatives in a zebrafish embryo model." *Environmental science & technology* 52.5 (2018).

213. Mueller T. "The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei)." *Brain research* 1381 (2011).

214. Mueller T., P. Vernier, M.F. Wullimann "The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish Danio rerio." *Brain research* 1011.2 (2004).

215. Murray R.M. "Traditional marijuana, high-potency cannabis and synthetic cannabinoids: increasing risk for psychosis." *World Psychiatry* 15.3 (2016).

216. Muthulakshmi S. "Zearalenone induced embryo and neurotoxicity in zebrafish model (Danio rerio): role of oxidative stress revealed by a multi biomarker study." *Chemosphere* 198 (2018).

217. Navarrete Alicia "Simultaneous online SPE–HPLC–MS/MS analysis of docetaxel, temsirolimus and sirolimus in whole blood and human plasma." *Journal of Chromatography B* 921 (2013).

218. Newcomer J.W., J.H. Krystal "NMDA receptor regulation of memory and behavior in humans." *Hippocampus* 11.5 (2001).

219. Newman D.J., G.M. Cragg "Natural products as sources of new drugs from 1981 to 2014." *Journal of natural products.* 79.3 (2016).

220. Nishimura Y. "Using zebrafish in systems toxicology for developmental toxicity testing." *Congenital Anomalies* 56.1 (2016).

221. Dahm R., C. Nüsslein-Volhard “Zebrafish: A practical approach”. *New York: Oxford University Press* (2002).

222. Ortiz-Villanueva E. "Metabolic disruption of zebrafish (Danio rerio) embryos by bisphenol A. An integrated metabolomic and transcriptomic approach." *Environmental pollution* 231 (2017).

223. Osorio Y. "Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system." *PLoS neglected tropical diseases* 5.2 (2011).

224. Ossato A. "JWH-018 impairs sensorimotor functions in mice." *Neuroscience* 300 (2015).

225. Ossato A. "Psychostimulant effect of the synthetic cannabinoid JWH-018 and AKB48: behavioral, neurochemical, and dopamine transporter scan imaging studies in mice." *Frontiers in psychiatry* 8 (2017).

226. Pamanji R. "Developmental toxic effects of monocrotophos, an organophosphorous pesticide, on zebrafish (Danio rerio) embryos." *Environmental Science and Pollution Research* 22.10 (2015).

227. Panas M.W. "Trace amine associated receptor 1 signaling in activated lymphocytes." *Journal of Neuroimmune Pharmacology* 7.4 (2012).

228. Panula P. "The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases." *Neurobiology of disease* 40.1 (2010).

229. Pathak P. "Small molecule inhibition of gut microbial choline trimethylamine lyase activity alters host cholesterol and bile acid metabolism." *American Journal of Physiology-Heart and Circulatory Physiology* 318.6 (2020).

230. Peterson R.T. "Small molecule developmental screens reveal the logic and timing of vertebrate development." *Proceedings of the national academy of sciences* 97.24 (2000).

231. Peterson R.T., M.C. Fishman. "Designing zebrafish chemical screens." *Methods in cell biology* 105 (2011).

232. Petkov V. "Effects of some benzodiazepines on the acetylcholine release in the anterior horn of the lateral cerebral ventricle of the cat." *Acta physiologica et pharmacologica Bulgarica* 8.3 (1982).

233. Pflanz N.C. "An intersubunit electrostatic interaction in the GABAA receptor facilitates its responses to benzodiazepines." *Journal of Biological Chemistry* 293.21 (2018).

234. Pickering M., S. Brown "Quantification and validation of HPLC-UV and LC-MS assays for therapeutic drug monitoring of ertapenem in human plasma." *Biomedical Chromatography* 27.5 (2013).

235. Pinna G. "Imidazenil and diazepam increase locomotor activity in mice exposed to protracted social isolation." *Proceedings of the National Academy of Sciences* 103.11 (2006).

236. Ponnayyan Sulochana S. "Review of the validated HPLC and LC-MS/MS methods for determination of drugs used in clinical practice for Alzheimer's disease." *Biomedical Chromatography* 28.11 (2014).

237. Pratt, J. A., P. Jenner, C. D. Marsden "Comparison of the effects of benzodiazepines and other anticonvulsant drugs on synthesis and utilization of 5-HT in mouse brain." *Neuropharmacology* 24.1 (1985).

238. Psychogios Nikolaos "The human serum metabolome." *PloS one* 6.2 (2011).

239. Qiao, R. "Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish." *Science of the Total Environment* 662 (2019).

240. Khan, F. R., S. Sulaiman Alhewairini. "Zebrafish (Danio rerio) as a model organism." *Current Trends in Cancer Management* (2018).

241. Rappaport S. M. "The blood exposome and its role in discovering causes of disease." *Environmental health perspectives* 122.8 (2014).

242. Rastogi R. B. "Effects of acute diazepam and clobazam on spontaneous locomotor activity and central amine metabolism in rats." *European journal of pharmacology* 43.2 (1977).

243. Rees D.A., J. C. Alcolado "Animal models of diabetes mellitus." *Diabetic medicine* 22.4 (2005).

244. Rex, A., C. A. Marsden, H. Fink "Effect of diazepam on cortical 5-HT release and behaviour in the guinea-pig on exposure to the elevated plus maze." *Psychopharmacology* 110.4 (1993).

245. Richter Lars "Diazepam-bound GABA A receptor models identify new benzodiazepine binding-site ligands." *Nature chemical biology* 8.5 (2012).

246. Roberts L.D. "Targeted metabolomics." *Current protocols in molecular biology* 98.1 (2012).

247. Robinson N. Bryce "The current state of animal models in research: A review." *International Journal of Surgery* 72 (2019).

248. de Fonseca F. R. "Role of the endogenous cannabinoid system in the regulation of motor activity." *Neurobiology of disease* 5.6 (1998).

249. Rohde L.A., Carl-Philipp Heisenberg "Zebrafish gastrulation: cell movements, signals, and mechanisms." *International review of cytology* 261 (2007).

250. Rohle D. "An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells." *Science* 340.6132 (2013).

251. Rosenkrantz H. "Effects of cannabis on fetal development of rodents." *Marihuana Biological Effects* (1979).

252. Rosenkrantz H. "Marihuana-induced embryotoxicity in the rabbit." *Fundamental and applied Toxicology* 7.2 (1986).

253. Rosenthal N., S. Brown "The mouse ascending: perspectives for human-disease models." *Nature cell biology* 9.9 (2007).

254. Ross A. C., M. E. Ternus "Vitamin A as a hormone: recent advances in understanding the actions of retinol, retinoic acid, and beta carotene." *Journal of the American Dietetic Association* 93.11 (1993).

255. Rotroff D. M. "Metabolomic signatures of drug response phenotypes for ketamine and esketamine in subjects with refractory major depressive disorder: new mechanistic insights for rapid acting antidepressants." *Translational psychiatry* 6.9 (2016).

256. Rubic T. "Triggering the succinate receptor GPR91 on dendritic cells enhances immunity." *Nature immunology* 9.11 (2008).

257. Saleem S., R. R. Kannan. "Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospecific drug discovery." *Cell death discovery* 4.1 (2018).

258. Sallustio B. C. "LC–MS/MS for immunosuppressant therapeutic drug monitoring." *Bioanalysis* 2.6 (2010).

259. Sánchez-Blázquez, Pilar, M. Rodríguez-Muñoz, J. Garzón. "The cannabinoid receptor 1 associates with NMDA receptors to produce glutamatergic hypofunction: implications in psychosis and schizophrenia." *Frontiers in pharmacology* 4 (2014).

260. Sántha M.. "Biologia futura: animal testing in drug development—the past, the present and the future." *Biologia Futura* (2020).

261. Santoriello C., L. I. Zon "Hooked! Modeling human disease in zebrafish." *The Journal of clinical investigation* 122.7 (2012).

262. Sañudo-Peña, M. Clara, K. Tsou, J. M. Walker. "Motor actions of cannabinoids in the basal ganglia output nuclei." *Life sciences* 65.6-7 (1999).

263. Sarath Babu N. "1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine induced Parkinson's disease in zebrafish." *Proteomics* 16.9 (2016).

264. Sato E. "Metabolic alterations by indoxyl sulfate in skeletal muscle induce uremic sarcopenia in chronic kidney disease." *Scientific reports* 6.1 (2016).

265. Savchuk S. "In vivo metabolism of the new synthetic cannabinoid APINAC in rats by GC–MS and LC–QTOF-MS." *Forensic Toxicology* 35.2 (2017).

266. Savitz J. "The kynurenine pathway: a finger in every pie." *Molecular psychiatry* 25.1 (2020).

267. Schrimpe-Rutledge A.C. "Untargeted metabolomics strategies—challenges and emerging directions." *Journal of the American Society for Mass Spectrometry* 27.12 (2016).

268. Séguret A., B. Collignon, J. Halloy "Strain differences in the collective behaviour of zebrafish (Danio rerio) in heterogeneous environment." *Royal Society open science* 3.10 (2016).

269. Sehnert A. J. "Cardiac troponin T is essential in sarcomere assembly and cardiac contractility." *Nature genetics* 31.1 (2002).

270. Shaw W. "Increased urinary excretion of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), an abnormal phenylalanine metabolite of Clostridia spp. in the gastrointestinal tract, in urine samples from patients with autism and schizophrenia." *Nutritional neuroscience* 13.3 (2010).

271. Sigel E. "The major central endocannabinoid directly acts at GABAA receptors." *Proceedings of the National Academy of Sciences* 108.44 (2011).

272. Smith W. L. "The eicosanoids and their biochemical mechanisms of action." *Biochemical Journal* 259.2 (1989).

273. Sobanska M. "Applicability of the fish embryo acute toxicity (FET) test (OECD 236) in the regulatory context of registration, evaluation, authorisation, and restriction of chemicals (REACH)." *Environmental toxicology and chemistry* 37.3 (2018).

274. Söderpalm B. "Evidence for a role for dopamine in the diazepam locomotor stimulating effect." *Psychopharmacology* 104.1 (1991).

275. Sosa M.A. Gama, R. De Gasperi, G. A. Elder. "Modeling human neurodegenerative diseases in transgenic systems." *Human genetics* 131.4 (2012).

276. Speciale, C. "High-affinity uptake of L-kynurenine by a Na+-independent transporter of neutral amino acids in astrocytes." *Journal of Neuroscience* 9.6 (1989).

277. Stein, Eytan M. "Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia." *Blood, The Journal of the American Society of Hematology* 130.6 (2017).

278. Stein, L., C. D. Wise, J. D. Belluzzi "Effects of benzodiazepines on central serotonergic mechanisms." *Advances in biochemical psychopharmacology* 14 (1975).

279. Stengel D., S. Wahby, T. Braunbeck "In search of a comprehensible set of endpoints for the routine monitoring of neurotoxicity in vertebrates: sensory perception and nerve transmission in zebrafish (Danio rerio) embryos." *Environmental Science and Pollution Research* 25.5 (2018).

280. Steppuhn K. G., L. Turski. "Diazepam dependence prevented by glutamate antagonists." *Proceedings of the National Academy of Sciences* 90.14 (1993).

281. Stewart A.M., A. V. Kalueff "The behavioral effects of acute Δ9-tetrahydrocannabinol and heroin (diacetylmorphine) exposure in adult zebrafish." *Brain research* 1543 (2014).

282. Stork O., T. Hashimoto, K. Obata. "Increase of tyrosine hydroxylase and its mRNA in the rat substantia nigra pars reticulata by diazepam and picrotoxin." *Neuroscience research* 19.1 (1994).

283. Strähle Uwe "Zebrafish embryos as an alternative to animal experiments—a commentary on the definition of the onset of protected life stages in animal welfare regulations." *Reproductive Toxicology* 33.2 (2012).

284. Stubbs J. R. "Serum trimethylamine-N-oxide is elevated in CKD and correlates with coronary atherosclerosis burden." *Journal of the American Society of Nephrology* 27.1 (2016).

285. Sulcova E., R. Mechoulam, E. Fride. "Biphasic effects of anandamide." *Pharmacology Biochemistry and Behavior* 59.2 (1998).

286. Sullivan J. P. "The oxidation of tryptamine by the two forms of monoamine oxidase in human tissues." *Biochemical pharmacology* 35.19 (1986).

287. Engler B. "Effects of exogenous and endogenous cannabinoids on GABAergic neurotransmission between the caudate-putamen and the globus pallidus in the mouse." *Journal of Pharmacology and Experimental Therapeutics* 316.2 (2006).

288. Tain Y.L., C.N. Hsu. "Toxic dimethylarginines: asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)." *Toxins* 9.3 (2017).

289. Takada A., F. Shimizu, J. Masuda. "Measurement of plasma tryptophan metabolites: Clinical and experimental application for depression and stress states assessment." *Melatonin—Molecular Biology, Clinical and Pharmaceutical Approaches, IntechOpen, London* (2018).

290. Taliani S. "Exploiting the indole scaffold to design compounds binding to different pharmacological targets." *Molecules* 25.10 (2020).

291. Teng M. "Acute exposure of zebrafish embryo (Danio rerio) to flutolanil reveals its developmental mechanism of toxicity via disrupting the thyroid system and metabolism." *Environmental Pollution* 242 (2018).

292. Teng M. "Metabolomics and transcriptomics reveal the toxicity of difenoconazole to the early life stages of zebrafish (Danio rerio)." *Aquatic Toxicology* 194 (2018).

293. Thau L., J. Gandhi, S. Sharma. "Physiology, cortisol." (2019).

294. Thomaidis N.S., A. G. Asimakopoulos, A.A. Bletsou "Emerging contaminants: a tutorial mini-review." *Global NEST Journal* 14.1 (2012).

295. Thorburn A. N. "Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites." *Nature communications* 6.1 (2015).

296. Tkáč I. "In vivo 1H NMR spectroscopy of the human brain at high magnetic fields: metabolite quantification at 4T vs. 7T." *Magnetic Resonance in Medicine: An Official Journal of the International Society for Magnetic Resonance in Medicine* 62.4 (2009).

297. Tolhurst G. "Glutamine triggers and potentiates glucagon-like peptide-1 secretion by raising cytosolic Ca2+ and cAMP." *Endocrinology* 152.2 (2011).

298. Treviño-Becerra A. "Uric Acid: The unknown uremic toxin." *Uric Acid in Chronic Kidney Disease* 192 (2018).

299. Tripathi H. L. "Effects of cannabinoids on levels of acetylcholine and choline and on turnover rate of acetylcholine in various regions of the mouse brain." *Alcohol and drug research* 7.5-6 (1987).

300. Truong L., M. T. Simonich, R. L. Tanguay. "Better, faster, cheaper: Getting the most out of high-throughput screening with zebrafish." *High-Throughput Screening Assays in Toxicology*. Humana Press, New York, NY, (2016).

301. Tu, Z., R. H. Mach. "C-11 radiochemistry in cancer imaging applications." *Current topics in medicinal chemistry* 10.11 (2010).

302. Turner R.M. "From the Lab to the Prescription Pad: Genetics, CYP 450 Analysis, and Medication Response." *Journal of Child and Adolescent Psychiatric Nursing* 26.2 (2013).

303. Tzavara E. T., M. Wade, G. G. Nomikos "Biphasic effects of cannabinoids on acetylcholine release in the hippocampus: site and mechanism of action." *Journal of Neuroscience* 23.28 (2003).

304. Usenko C. Y. "PBDE developmental effects on embryonic zebrafish." *Environmental toxicology and chemistry* 30.8 (2011).

305. Vasilopoulou C. G., M. Margarity, M. I. Klapa. "Metabolomic analysis in brain research: opportunities and challenges." *Frontiers in physiology* 7 (2016).

306. Viant M.R. "Recent developments in environmental metabolomics." *Molecular Biosystems* 4.10 (2008).

307. Vondroušová J. "Monitoring of kynurenine pathway metabolites, neurotransmitters and their metabolites in blood plasma and brain tissue of individuals with latent toxoplasmosis." *Journal of pharmaceutical and biomedical analysis* 170 (2019).

308. Walker G. S. "Biosynthesis of drug metabolites and quantitation using NMR spectroscopy for use in pharmacologic and drug metabolism studies." *Drug Metabolism and Disposition* 42.10 (2014).

309. Wang, Pengcheng, Amina I. Shehu, and Xiaochao Ma "The opportunities of metabolomics in drug safety evaluation." *Current pharmacology reports* 3.1 (2017).

310. Wang Y. "1H NMR-based metabolomics analysis of adult zebrafish (Danio rerio) after exposure to diniconazole as well as its bioaccumulation behavior." *Chemosphere* 168 (2017).

311. Wang Z. "Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease." *Nature* 472.7341 (2011).

312. Ward P. S. "The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting α-ketoglutarate to 2-hydroxyglutarate." *Cancer cell* 17.3 (2010).

313. Watson F. L. "Organophosphate pesticides induce morphological abnormalities and decrease locomotor activity and heart rate in Danio rerio and Xenopus laevis." *Environmental toxicology and chemistry* 33.6 (2014).

314. de Weerth C. "Do bacteria shape our development? Crosstalk between intestinal microbiota and HPA axis." *Neuroscience & Biobehavioral Reviews* 83 (2017).

315. Wermuth C. G. "Strategies in the search for new lead compounds or original working hypotheses." *The practice of medicinal chemistry*. Academic Press, (2015).

316. Wermuth C.G. "Search for new lead compounds: the example of the chemical and pharmacological dissection of aminopyridazines." *Journal of heterocyclic chemistry* 35.5 (1998).

317. Wishart D. S. "HMDB: the human metabolome database." *Nucleic acids research* 35 (2007).

318. Wishart D. S. "Applications of metabolomics in drug discovery and development." *Drugs in R & D* 9.5 (2008).

319. Wishart D.S. "Quantitative metabolomics using NMR." *TrAC trends in analytical chemistry* 27.3 (2008).

320. Wishart D. S. "Advances in metabolite identification." *Bioanalysis* 3.15 (2011).

321. Wishart D. S. "Emerging applications of metabolomics in drug discovery and precision medicine." *Nature reviews Drug discovery* 15.7 (2016).

322. Wishart D.S. "Metabolomics for investigating physiological and pathophysiological processes." *Physiological reviews* 99.4 (2019).

323. Xia J."Translational biomarker discovery in clinical metabolomics: an introductory tutorial." *Metabolomics* 9.2 (2013).

324. Yan L. "Perturbation of metabonome of embryo/larvae zebrafish after exposure to fipronil." *Environmental toxicology and pharmacology* 48 (2016).

325. Yen K. "AG-221, a first-in-class therapy targeting acute myeloid leukemia harboring oncogenic IDH2 mutations." *Cancer discovery* 7.5 (2017).

326. Yerges-Armstrong L. M. "Purine pathway implicated in mechanism of resistance to aspirin therapy: pharmacometabolomics-informed pharmacogenomics." *Clinical pharmacology & Therapeutics* 94.4 (2013).

327. Young H.S., L.G. Herbette, V. Skita "α-bungarotoxin binding to acetylcholine receptor membranes studied by low angle X-ray diffraction." *Biophysical journal* 85.2 (2003).

328. Yu H. "Widespread expression of arginase I in mouse tissues: biochemical and physiological implications." *Journal of Histochemistry & Cytochemistry* 51.9 (2003).

329. Yu X. "Erythropoietin receptor signalling is required for normal brain development." (2002).

330. Zaitsu K. "Metabolic profiling of urine and blood plasma in rat models of drug addiction on the basis of morphine, methamphetamine, and cocaine-induced conditioned place preference." *Analytical and bioanalytical chemistry* 406.5 (2014).

331. Zhang N., P. Liu, X. He "Effect of single-use versus combined-use moschus and diazepam on expression of amino acid neurotransmitters in the rat corpus striatum." *Neural regeneration research* 7.3 (2012).

332. Zhao X. "High mobility group box-1 (HMGB1; amphoterin) is required for zebrafish brain development." *Journal of Biological Chemistry* 286.26 (2011).

333. Zheng T. "Metabolic phenotype of rats exposed to heroin and potential markers of heroin abuse." *Drug and alcohol dependence* 127.1-3 (2013).

334. Zhou Y., N. C. Danbolt. "Glutamate as a neurotransmitter in the healthy brain." *Journal of neural transmission* 121.8 (2014).

335. Zhu H. "Pharmacometabolomics of response to sertraline and to placebo in major depressive disorder–possible role for methoxyindole pathway." *PLoS One* 8.7 (2013).