HPLC method development for the assessment of tryptophan metabolism and its application in a complete Freund's adjuvant model of orofacial pain

Summary of Ph.D. Thesis

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II. Nánási N., **Cseh E.K.,** Szentirmai M., Veres G., Klivényi P., Vécsei L., Zádori D. Development and validation of high performance liquid chromatography methods for vitamin e measurements. *Proceeding of the 23rd International Symposium on Analytical and Environmental Problems*. **2017**. ISBN 978-963-306-563-1 (conference proceeding).

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IV. Szalardy L., Molnar F.M., Zadori D., **Cseh E.K.,** Veres G., Kovacs G.G., Vecsei L., Klivenyi P. Non-motor behavioral alterations of PGC1α-deficient mice – a peculiar phenotype with slight male preponderance and no apparent progression. Front. Behav. Neurosci. **2018**, 12:180 (original paper IF: 3.104)

V. **Cseh E.K.,** Veres G., Danics K., Szalárdy L., Nánási N., Klivényi P., Vécsei L., Zádori D. Additional value of tau protein measurement in the diagnosis of Creutzfeldt-Jakob disease. Ideggy. Sz. **2019**, 72: 39 (original paper IF: 0.337)

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VII. Herédi J., **Cseh E.K.**, Magyariné B.A, Veres G., Zádori D., Toldi J., Kis Zs., Vécsei L., Ono E., Gellért L. Investigating KYNA production and kynurenergic manipulation on acute mouse brain slice preparations. Brain Res. Bull. **2019**, 146:185 (original paper IF: 3.370)

VIII. Tömösi F., Kecskeméti G., **Cseh E.K.**, Szabó E., Rajda C., Kormány R., Szabó Z., Vécsei L., Janáky T. A validated LC-MS method for tryptophan metabolites: Application in the diagnosis of multiple sclerosis. J. Pharm. Biomed. Anal. **2020**.185:113246 (original paper, IF(**2019**): 3.209)

IX. Zádor F., Nagy-Grócz G., Dvorácskó S., Bohár Z., **Cseh E.K.**, Zádori D., Párdutz Á., Szűcs E., Tömböly C., Borsodi A., Benyhe S., Vécsei L. Long-term sytemic administration of kynurenic acid brain region specifically elevates the abundance of functional CB1 receptors in rats. Neurochem. Int. **2020** (IF(**2019**): 3.881**).**

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1. Introduction

Neurological disorders are recognized as the leading cause of disability and second leading cause of death worldwide. Therefore, their contribution to the overall burden from all health conditions is increasing. These diseases, including headache disorders (e.g. migraine, tension-type headache and medication overuse headache), multiple sclerosis, epilepsy or neurodegenerative conditions, such as Alzheimer's disease (AD) or Parkinson's disease (PD) represent three percent of the worldwide burden of disease. Although it may seem to be a small rate, however, regarding disability-adjusted life years (DALYs) the numbers are increasing: migraine, stroke, epilepsy and dementia rank in the top 50 causes of DALYs. Moreover, from all neurological disorders, headache disorders have a considerably high prevalence, with a 46% of population suffering from headache in general for 1-year prevalence and 64% for lifetime prevalence, from which the majority are diagnosed with primary headache. The prevalence of this type is very high, with 14.4% from migraine, 26.1 % from tension-type headache and 3% to 5% from chronic daily headache. Secondary headaches are resulting from the traction or inflammation of pain-sensitive structures.

There are three neuronal levels involved in the pain signaling process. The first neurons, which have special receptors called nociceptors, have their cell bodies within the dorsal root or the sensory ganglia of the cranial nerves (e.g. trigeminal ganglia (TG)) and they are activated through various stimuli (chemical, thermal and mechanical), both external and internal. The signals are further conveyed to the dorsal horn of the spinal cord or to the trigeminal nucleus caudalis (TNC), recently also called as trigeminocervical complex (TCC), leading to the release of the neurotransmitters such as glutamate (Glu), calcitonin gene related peptide (CGRP), substance P, neurokinin A and pituitary adenylate cyclase activating peptide (PACAP). The co-release of Glu and CGRP is controlled by calcium influx via the P/Q-type channels, the latter leading to the activation of CGRP receptors, further evoking the release of Glu and Substance P. Moreover, CGRP receptors were identified presynaptically in the dorsal spinal horn on nerve terminals of glutaminergic neurons and their activation sensitizes the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, which may promote the release of Glu. The release of Glu subsequently increases NMDA receptor expression, further promoting and maintaining a sensitized state. The axons of second level neurons, the cell bodies of which are generally located in the spinal cord or brainstem, ascend further to the thalamus, from where the third level neurons project to the primary somatosensory cortex. At this level, there are several other neurotransmitters and neuromodulators, which are able to modulate the presented process of pain sensation, e.g. via

the activation of γ-aminobutyric acid (GABA)-ergic or glycinergic inhibitory neurons. Not only GABA, but both serotonergic and noradrenergic axons, originating from different brainstem regions, such as the nucleus raphe magnus or locus coeruleus are involved in the descending inhibition that project to the spinal cord and brainstem.

Taken together, Glu and both its ionotropic and metabotropic receptors have pivotal role in the pathophysiology of headache and pain. The importance of the NMDA receptors culminates at the point where their activation becomes one of the most important steps in initiating and maintaining the central sensitization. The increase of Glu level is demonstrated in different animal models of headache and pain. GABA receptor agonists as well as inhibitors of GABA uptake and metabolism display significant antinociceptive activity in animal models of different pain conditions. The serotoninergic system is involved in the primary headaches, including migraine, in many ways.

Kynurenic acid (KYNA), a product of the kynurenine (KYN) pathway (KP) of tryptophan (TRP) metabolism, is also capable of influencing the glutamatergic neurotransmission in a complex way. The KP of the essential amino acid TRP accounts for 95% of its degradation, whereas the remaining 5% is degraded through the serotonin (5-HT) pathway. With its biologically active metabolites, including the above-mentioned KYNA with mostly neuroprotective properties the KP of TRP metabolism became of interest in different research fields. The antinociceptive properties of KYNA has been proved in different animal models of pain: in the study of chronic osteoarthritis-like joint pain, in carrageenan-induced thermal hyperalgesia or in a model of inflamed joint. Furthermore, TRP, KYN and KYNA have been related to migraine and other headache disorders, i.e., significant reductions in the serum levels of KYN and KYNA were demonstrated, whereas increased concentration levels of TRP were found in migraine and cluster headache.

These neurotransmitters can be detected with different high-performance liquid chromatography (HPLC) techniques, however, each of them has its own drawback.

Regarding the measurement of the main excitatory neurotransmitter, Glu, it is often detected alongside with GABA, to give a better picture on excitatory-inhibitory balance of the central nervous system (CNS). Some derivatization agents seem to provide a good sensitivity via the application of fluorescent light detector (FLD), a detection method that is simpler and more widely applied in a reverse-phase HPLC. One of the mainly used derivatization agents is the o-phthalaldehyde (OPA), in the presence of 3-mercaptopropionic acid (3MP), as it furnishes fast reactions, its derivates can be obtained in aqueous solutions at ambient temperature and they are fluorescent compounds with high selectivity and sensitivity. Furthermore, it does not break

down or react further to form byproducts if it is added in excess. Although its disadvantage may be its unstable character and sensitive reaction to the change of pH in sample preparation, OPA can be used pre-column, yielding a relatively short running time resulting in a relatively easy simultaneous measurement of Glu and GABA.

The determination of the concentrations of various TRP metabolites, including KYNA, from biological matrices represents a great challenge due to their distinct chemical properties or their different concentrations in samples. The detection with FLD can yield lower limit of detection (LOD) value, which may have a special importance especially in light of low sample amount in several cases (e.g. mouse CNS samples). Accordingly, the partial assessment of the KP is a widely applied approach using simple HPLC methods with different detection techniques, including the UV detector (UVD), diode array detector, FLD, or electrochemical detector. In case of the above-mentioned partial assessment of TRP metabolites, TRP, 5-HT and KYNA are detected by FLD, whereas KYN by UVD, and accordingly, at least 2 internal standards (ISs) should be applied during their detection. In light of these requirements, 3-nitro-L-tyrosine (3NLT) is appropriate for the UVD, whereas a newly synthetized compound, 4-hydroxyquinazoline-2-carboxylic acid (HCA) was utilized for FLD.

Regarding all the above-mentioned analytical procedures, a detailed validation process, including at least selectivity, linearity, LOD, limit of quantification (LOQ), precision and recovery, is essential to be able to determine the robustness of the developed method in harmonization with the official guidelines.

2. Aims

The aims of our study were as follows:

(i) To explore the neurochemical profile of CFA-induced orofacial pain in rats, including the assessment of Glu, GABA, TRP, 5-HT, KYN and KYNA, and finding the shift point regarding small molecule neurotransmitter concentration changes versus that of the previously described pain-related neuropeptides.

(ii) To optimize and validate a HPLC-UVD/FLD method for the determination of TRP, 5-HT, and that of the neuroprotective branch of the KP from several different biological matrices, including mouse and rat CNS and plasma, and human cerebrospinal fluid (CSF) and plasma, by using two ISs, one for each detector.

3. Materials and methods

3.1 CFA model of orofacial pain

During experiments, the 10-12 weeks old, Sprague-Dawley rats were bred and maintained under standard laboratory conditions with 12 h-12 h light/dark cycle at 24 ± 1ºC and 50% relative humidity. The rats had free access to standard rat chow and water. All experimental procedures performed in this study complied fully with the guidelines of Act 1998/XXVIII of the Hungarian Parliament on Animal Experiments (243/1988) and with the recommendations of the International Association for the Study of Pain and European Communities Council (86/609/ECC). The studies were in harmony with the Ethical Codex of Animal Experiments and were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged, with a permission number of XI./1102/2018. 50 µl of Complete Freund's adjuvant (CFA) was administered per animal. The rats were anesthetized with intraperitoneal 4% chloral hydrate solution mainly based on its safe application in the morning and 50 µl of CFA was injected into the right whisker pad. Control rats were injected with an equal volume of saline. CSF was taken from the suboccipital cistern, applying the above-described anesthetic procedure after injection, and following that the animals were perfused transcardially with 200 ml phosphate-buffered saline (PBS). Blood samples were taken from the left ventricle into ice-cold glass tubes containing disodium ethylenediaminetetraacetate dihydrate, and the plasma was separated by centrifugation (3500 RPM for 10 min at 4°C). Following decapitation, two different brain structures were dissected, the TNC and the somatosensory cortex (ssCX). Both right- and left-sided samples were separately removed on ice and stored at -80°C until further use in each case. Prior to all measurements, during the tissue weighting or plasma/CSF precipitation process, all samples were relabeled, and a blind study was conducted. Validated HPLC measurements were performed during the experiment.

All chromatographic analyses described below were performed using an Agilent 1100 HPLC system (Santa Clara, CA, USA) with UVD and FLD attached. Chromatographic separations were performed on a Kinetex C18 150×4.6 i.d. 5 µm particle size column (Phenomenex Inc., Torrance, CA, USA) after passage through a SecurityGuard pre-column C18, 4×3 mm i.d., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA).

First, the brain samples were homogenized in 0.5 M perchloric acid (PCA), at 1:5 w/v containing the ISs (3NLT and HCA), applied in the measurement of TRP metabolites as detailed below. Then, supernatants were aliquoted and kept at -80°C until the bioanalytical procedure. Regarding Glu and GABA measurements, 100 µl of the brain supernatant was diluted to 1:100 v/v with distilled water and 100 µl of this dilution was derivatized with 100 µl

solution (2 ml OPA, 7.94 ml 0.2 M borate puffer (pH = 9.9) and 60 µl 3MP and further diluted with 50 µl distilled water containing the corresponding IS, the homoserine. For the separation, gradient elution was applied. Mobile phase 'A' was 95:5 v/v 0.05 M sodium acetate (pH = 5.5):methanol, whereas mobile phase 'B' was 45:45:10 v/v methanol:acetonitrile (ACN):water. The elution started with 95% 'A' decreasing linearly to 50% then staying there for 2 min and re-equilibrating to 95% in 1 min for a total 16 min runtime. The flow rate was 1 ml/min, injection volume was 10 µl and the FLD was set to 230/440 nm for excitation/emission wavelengths. Regarding the Glu and GABA measurements from CFS samples, the initial amount of mobile phase 'A' applied for the brain samples was 95%, but for CSF samples it was changed to 93%, as coelution was observed under the initial circumstances. The ratios applied for the CSF sample preparation (1:1:0.5 = sample: derivatization solution: IS) remained the same, similar to brain supernatants.

For the TRP, 5-HT, KYN and KYNA measurements from brain samples, the mobile phase consisted of 200 mM zinc-acetate (ZnAc) solution at pH 5.8, adjusted with acetic acid. The organic component (ACN) in the mobile phase was 5%. The flow rate was 1.2 ml/min and the injection volume was 50 µl. During the measurement of CSF and plasma samples, TRP, KYN and KYNA was separated by a mobile phase similar to the one used for the CNS methods, except that pH was set at 6.2. The injection volume was 50 µl and 20 µl, respectively.

3.2 Quantification of TRP, 5-HT, KYN and KYNA from different biological samples

Due to the importance of TRP and its metabolites in different neurological disorders and their animal model as well, paying a special attention to pain models, their analyses may be of interest, not only in rats, but in human and mouse samples too. Before the method optimization process, the spectral analyses of the UV-detected compounds were made with an Agilent 8453 UV-Vis Spectroscopy System (Santa Clara, CA, USA). Regarding the mobile phase, it consisted of 200 mM ZnAc solution at pH of 6.2 for human and murine plasma and CSF samples, and at pH of 5.8 for murine CNS samples; the pH value was adjusted with acetic acid. The organic component (ACN) in the mobile phase was 5%. The flow rate was 1.2 ml/min and 20 µl of the plasma supernatants were injected, whereas in case of CSF and CNS homogenate the injection volume was 50 µl. In each case, two ISs were applied: 3NLT for the UVD, and HCA for the FLD, both chosen by their similarities with one of the detected metabolites. During the validation process, for the calibration curve, which is further used for LOD and LOQ value determination, six calibration standards were prepared by spiking the respective working solutions into blank biological matrices, i.e., blank human and rat plasma and CSF, and blank

mouse plasma, respecting the same dilution ratios as the ones applied in the sample preparation as well. Due to hard sampling and contamination issues, the amount of the obtained rat CSF samples was enough only for the linearity study, along with the LOD and LOQ determination. During sample preparation, regarding murine samples, frozen plasma was thawed at room temperature, then deproteinized with 0.5 M PCA solution (1:1 v/v), containing both ISs at final concentration of 100 nM HCA and 2 μM 3NLT, and centrifuged for 10 min at 12000 RPM at 4°C. Regarding the freshly prepared mouse brain and rat CNS samples, the tissues were weighed and then sonicated for 90 s in an ice-cooled solution, 1:5 w/v, comprising 0.5 M PCA and the 2 ISs in an Eppendorf tube. The content of the Eppendorf tube was centrifuged for 10 min at 12000 RPM at 4°C. Human plasma sample handling was almost the same as in case of mouse plasma samples, only the deproteinization process differed somewhat (the ratio of plasma and 0.5 M PCA solution was 1:3 v/v). For the CSF samples, the same preparation procedure was applied as in cases of plasma samples, except using a dilution of 5:6 v/v.

For the validation process, the individual samples were pooled, whereas for the demonstration of the applicability of the method and comparison of the obtained results with those from the literature, the metabolites of interest were measured from 8 independent samples.

3.3 Statistics

All statistical calculations were performed with the use of the freely available R software 3.5.3 (R Development Core Team). During the method validation and concentration calculation steps, the peak area response ratios were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method.

In the CFA study, the distribution of our data population was not determined as the applied statistical tests do not need assumptions regarding the distribution of underlying data. Accordingly, first we performed the Levene test to assess the homogeneity of variances. As the variances were equal, we performed a general independence test for two sets of variables measured on arbitrary scales, where the reference distribution was approximative based on the Monte-Carlo method. Afterwards, we carried out permutation t-tests as post hoc analysis for pairwise comparison. Permutations were applied via the Monte-Carlo method (10 000 random permutations) and Type I errors from multiple comparisons were controlled with false discovery rate. No test for outliers was conducted. With the key aspects of 3Rs in mind we tried to keep the sample size as low as we can based on experiences from previous experiments. For every statistically significant result, we calculated the corresponding effect size (Cohen’s d in

this case) and based on its value, we decided whether the increase of sample size is necessary or not.

4. Results

4.1 CFA model of orofacial pain

Both contralateral and ipsilateral CNS regions were measured separately, but we did not find significant differences in concentrations of any of the metabolites between the two sides, so the coherent data were pooled for further analysis. Regarding TNC, pairwise permutation t-tests following the independence tests revealed a significant elevation in the concentration of Glu ($p = 0.0319$, Cohen's $d = 1.49$), KYN ($p = 0.0123$, Cohen's $d = 1.58$) and KYNA ($p = 0.0098$, Cohen's $d = 1.92$) 24 h following CFA injection compared to the controls and a significant decrease could be observed in Glu ($p = 0.0357$, Cohen's $d = 1.29$), KYN ($p = 0.0123$, Cohen's $d = 1.85$) and KYNA ($p = 0.0263$, Cohen's $d = 1.39$) levels by 48 h compared to the 24 h group, whereas there was no difference between the control and 48 h groups. Regarding ssCX samples, an elevation in KYNA concentration ($p = 0.0237$, Cohen's $d = 1.36$) could be observed 24 h following CFA administration, followed by a significant decrease by 48 h ($p = 0.0173$, Cohen's $d = 1.80$) and there was no difference between control and 48 h groups. Furthermore, in the ssCX, there was a significant increase in 5-HT levels in the 48 h group compared to the controls ($p = 0.0479$, Cohen's $d = 1.21$) and to the 24 h group ($p = 0.0479$, Cohen's $d = 1.20$). We calculated the KYN/TRP and KYNA/KYN ratios as well. The KYN/TRP ratio was significantly elevated in the 24 h group compared to the controls ($p = 0.0419$, Cohen's $d = 1.19$) or to the 48 h group ($p = 0.0419$, Cohen's $d = 1.35$). Regarding CSF samples, TRP metabolites, Glu and GABA were measured. We found no significant alterations in the CSF, however, the power of the statistical tests in this case is low due to low case number ($n = 5, 5, 4$ for control, 24 h and 48 h groups, respectively). The concentration values of KYN in the control and CFA treated 48 h groups were below LOD (0.107 μM), except one case from each group (due to the low amount of 5-HT in the CSF samples, we could not quantify it, as the values were below LOD, LOD = 0.0274 μM). In case of plasma samples, only the TRP metabolites were measured, and no significant differences were observed

4.2 Quantification of TRP, 5-HT, KYN and KYNA from different biological samples

For the validation process, in addition to the linear equation calculated according to the applied concentration range, LOD, LOQ, intra- and inter-day precision were given as well, along with the recovery values. With regard to the ranges for external standards, it was kept in mind that

under pathological or treatment conditions, a considerably large alteration may occur compared to the physiological values detected in different biological matrices. Accordingly, we tried to set up a relatively wide concentration range for external standards focusing at carrying out measurements with good linearity as well, i.e. the correlation coefficient was higher than 0.999 for each compound when either FLD or UVD was applied. LOD and LOQ were determined by the equation LOD = $3.3 \cdot \sigma / S'$ and LOQ = $10 \cdot \sigma / S$, where σ is the standard error of the intercept and S' is the slope of the calibration curve of the analyte and in both LOD and LOQ values were in line with the literature data using the same instruments, i.e., during the measurement of human and murine plasma samples, when 20 µl was injected, the LOD values were above 0.557 µM, 0.025 µM and 1.23 nM, regarding TRP, KYN and KYNA, respectively. Furthermore, in case of murine CNS samples and human and rat CSF samples, when 50 µl was injected, the LOD values were above 0.08 µM, 0.009 µM, 0.0274 µM and 0.456 nM, regarding TRP, 5-HT, KYN and KYNA, respectively. Intra-day precision, expressed as CV%, ranged between 1.03%−4.25% in each case, whereas all inter-assay values were below 15%, ranging between 1.11%−10.6%, except that for the light and air sensitive 5-HT in the mouse brain and rat CNS samples, where a decrease of 52% and 19% was observed, respectively. The recovery values ranged between 79.6 and 116%, which are within 15% of the nominal value, except the LOQ spiked recovery values, which did not deviate by more than 20%, except one occasion. As a last step, we successfully proved the applicability of the validated methods on six different biological matrices.

5. Discussion

5.1 CFA model of orofacial pain

Headache is one of the most common neurological disorders and it is one of the leading causes of health-related problems worldwide. In 2010, tension type headache and migraine were the second and third most prevalent conditions in the world, respectively, according to the Global Burden of Disease (GBD) study. The activation of the peripheral terminals of nociceptors is responsible for Glu release at central sites with the activation of ionotropic and metabotropic Glu receptors. This process was demonstrated not only in preclinical studies, but in migraine patients as well. Accordingly, the role of glutamatergic pathways in association with different types of pain is well established and several antagonists of ionotropic glutamate receptors were investigated and found to be effective to decrease nociceptive transmission. However, they had severe side effects, and therefore, the interest in this direction of research diminished Nevertheless, ketamine, an NMDA receptor antagonist, is so far the only promising option in

the treatment of severe or long-lasting migraine aura, and tezampanel, which acts on the AMPA and kainate subtypes of ionotropic Glu receptors, has also shown promising results in acute migraine therapy. Furthermore, it has been already demonstrated that the level of KYNA and some other KP metabolites are altered in migraine and cluster headache patients as well. KYNA as an endogenous NMDA receptor antagonist, is a molecule of interest for CNS drug development in case of several neurological conditions, but due to its poor ability to cross the blood-brain barrier (BBB) and its rapid clearance from the body, its application for most CNS-related alterations is limited, and therefore, several KYNA analogs were synthetized. However, the first order neuron of pain processing is located outside the BBB, so KYNA itself may have therapeutic potential as well. Accordingly, the antinociceptive properties of KYNA were proved in animal models of pain. Furthermore, some of the developed analogs also displayed promising results in different animal models of headache. In an earlier study we investigated two KYNA analogs where both of them proved to be effective in the formalin model of trigeminal pain. However, one of them was more effective than the other and according to our analyses the better performing compound caused a more pronounced elevation of KYNA concentration on the periphery, whereas in the CNS the concentrations of KYNA were similar. Based on these results we hypothesized that the peripheral elevation of KYNA may be enough to exert beneficial effects on pain processing and targeting this component could provide an option to pharmaceutical drug design without the obligation of good penetration through the BBB. Elevated Glu concentration in the TNC of CFA-treated rats, demonstrated by the current study, is accompanied by increased KYN and KYNA levels, which may serve as a feedback mechanism to the sensitization process caused by Glu. This hypothesis is supported by the other findings that decreased KP metabolite levels are associated with those headache disorders, where increased NMDA receptor activation may play a crucial role. These results may have a great importance especially in light of the finding that the slightly, but not significantly elevated GABA level may not be enough to counterbalance the effects of increased Glu levels. With regard to 5-HT, its cortical elevation by 48 h may serve as a feedback inhibitory response as well to ameliorate the activation of the trigeminovascular pathway.

The results of this study draw attention to the limited time interval for therapies targeting glutamatergic pathways as well, as based on our previous experiments, a clear shift to dominantly peptide-mediated pain processing can be seen even from 24 h after CFA application. This time point corresponds to the onset of peripheral and central sensitization of the TS as well in this model. At this stage, mainly novel antibody-based therapies may come into account. With regard to these novel therapies, the focus of attention is on monoclonal

antibodies targeting the CGRP pathway for the prophylactic treatment of migraine. Currently, four of these antibodies are in clinical trials (eptinezumab, galcanezumab, fremanezumab, erenumab) with promising results. However, the cost of these therapies is considerably higher than that of acute phase treatments.

5.2 Quantification of TRP, 5-HT, KYN and KYNA from different biological samples

During the method validation, all the calculated parameters (LOD, LOQ, intra- and interassay precision, and recovery) were in line with literature data. Intra-day precision, expressed with CV% was below 5% in each case. Inter-assay values were all below 15%, the maximum limit recommended by guidelines, except that for the 5-HT in the mouse brain and rat CNS samples, where a decrease of 52% and 19% were observed, respectively. This bias is higher than the maximum recommended value, but it can be easily explained, as due to the heterogeneity of the bioanalytical studies, there are many cases where the limits proposed by the official guidelines may not be applicable. In case of the brain samples of the current study, the inter-assay precision measurements were done from the already homogenized samples, as we considered that brain sample regions cannot be divided into two homogenous parts compared to the supernatant samples. Therefore, the bias value draws attention to the necessity of brain homogenization right before the measurement in line with our currently applied laboratory practice. Accordingly, the freshly homogenized mouse brain and rat CNS samples show stable concentration values, all below the recommended 5%, i.e., 4.25 CV%, and 1.91 CV%, respectively. During the recovery study, the values should be within 15% of the nominal value, except the LOQ spiked recovery values, which should not deviate by more than 20%, as recommended by the official guidelines. Regarding metabolite concentration values all of them were presented to be in the ranges from literature data. Recovery studies were performed using spiked samples at three different concentration levels (LOQ, medium and high), with three replicates for each concentration. Recovery percentages were calculated as R=100 x [(Css-Cns)/Cspike], where Css is the concentration in the spiked homogenate sample, whereas Cns is the concentration of the homogenate native sample (without spiking) and Cspike is the added concentration. The obtained values ranged between were within 15% of the nominal value, except the LOQ spiked recovery values, which did not deviate by more than 20%, as recommended by the guidelines.

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