The application of HPLC methods for the preclinical and clinical assessment of neurological disorders

Summary of Ph.D. Thesis

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III. Török R, Salamon A, Sümegi E, Zádori D, **Veres G**, Molnár MF, Vécsei L, Klivényi P. Effect of MPTP on mRNA expression of PGC-1α in mouse brain. Brain Res. 2017 1660: 20-26. (original paper, **IF (2015): 2.561**)

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Török R, Kónya JA, Zádori D, **Veres G**, Szalárdy L, Vécsei L, Klivényi P. mRNA expression levels of PGC-1alpha in a transgenic and a toxin model of Huntington's disease. Cell Mol Neurobiol. 2015 35: 293-301. (original paper, **IF: 2.328**)

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Grozdics E, Berta L, Gyarmati B, **Veres G**, Zádori D, Szalárdy L, Vécsei L, Tulassay T, Toldi G. B7 Costimulation and intracellular indoleamine 2,3-dioxygenase expression in umbilical cord blood and adult peripheral blood. Biol Blood Marrow Transplant. 2014 20: 1659-1665. (original paper, **IF: 3.404**)

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Zádori D, **Veres G**, Szalárdy L, Klivényi P, Toldi J, Vécsei L. Glutamatergic dysfunctioning in Alzheimer's disease and related therapeutic targets. J Alzheimers Dis. 2014 42: S177-S187. (review, **IF: 4.151**)

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1 Introduction

According to the Global Burden of Disease Study, neurological disorders such as headache disorders (migraine, tension-type headache and medication-overuse headache), Alzheimer's disease (AD) and other dementias, multiple sclerosis (MS), Parkinson's disease (PD) and epilepsy are responsible for 3 percent of the worldwide burden of disease. The global prevalence of active headache disorders for the adult population is 46% for headache in general, 11% for migraine, 42% for tension-type headache and 3% for chronic daily headache. The treatment of primary headache disorders is challenging, requiring both acute and preventive therapeutic strategies. The efficacy of these treatments is not always satisfactory and the contraindications and side-effects often limit the options of the physician. Although neurodegenerative disorders, including AD, PD, Huntington's disease (HD) and amyotrophic lateral sclerosis, affect a smaller portion of the general population, there is no proven causative therapy, only symptomatic treatment is available. With the exception of PD, the efficacy of these medications is quite limited with regard to symptom management. In most cases, despite the well delineated cellular and molecular events such as mitochondrial dysfunction and increased formation of reactive species, glutamate excitotoxicity, deposition of aggregated proteins, inflammatory response and altered gene expression, the pathogenic process is hard to influence.

Animal and human studies suggest that glutamate receptors are present in various parts of the trigeminal system which is the system responsible for processing most of the pain originating from the head area. The stimulation of the trigeminal nerve results in elevated glutamate levels in the spinal trigeminal nucleus pars caudalis (TNC). These findings suggest that excitatory amino acid receptors (particularly N-methyl-D-aspartate receptors (NMDAR)), which are also present in migraineurs, play an important role in pain processing and the sensitization process. The kynurenine pathway (KP) of tryptophan (TRP) metabolism is extensively studied, mostly because of the well-established endogenous protective properties of kynurenic acid (KYNA) against the excitotoxic and oxidative stress inducing effects of other KP metabolites, such as quinolinic acid (QUIN) and 3-hydroxy-L-kynurenine (3-OHK) and this effect is mediated through a non-selective antagonism on the NMDA receptor. A well-known model of trigeminal

nociception is the orofacial formalin test, where a formalin solution is administered subcutaneously into the upper lip of rats, causing tissue injury, inflammation and nociception.

HD is an autosomal, dominantly inherited, progressive neurodegenerative disorder which results in cognitive, psychiatric and motor disturbances. HD is caused by an expansion of the cytosine-adenine-guanine (CAG) repeat in the gene coding for the *N*-terminal region of the huntingtin protein (Htt), which leads to the formation of a polyglutamine stretch. Although the exact mechanisms through which mutant Htt leads to the characteristic neuropathology are not fully understood, the potential roles of excitotoxicity and neuronal mitochondrial dysfunction are among the best-established concepts. Accordingly, striatal glutamatergic excitotoxicity is involved in the development of HD and predominantly mediated by the overactivation of NMDARs, and most specifically through NR2B subunit-containing NMDARs at the extrasynaptic sites. There is evidence indicating that excitotoxic injury caused by this overactivation is mediated, at least in part, by endogenous substances, including certain metabolites of the KP of TRP metabolism. Furthermore, decreased activity of the succinate dehydrogenase (SDH), complex II of the electron transport chain in post-mortem HD brains was one of the early findings suggestive of the role of mitochondrial dysfunction in the development of HD. In line with the decreased SDH activity, mitochondrial II complex inhibitors, such as 3-nitropropionic acid (3-NP), have been found to be useful in the investigation of HD through their utilization in animal toxin models.

Besides genetic predisposition, like in HD, certain environmental factors may also be responsible for the development of neurodegenerative processes. In case of PD, one of the well-established causes is the life-long cumulative low-dose exposure to mitochondrial toxins. The observation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinsonian symptoms yielded one of the first pieces of evidence for toxin-induced parkinsonism and since then, systemic MPTP administration has been widely used in various *in vivo* animal studies to investigate the pathogenesis of PD and to assess the therapeutic effect of potential neuroprotective agents. The most sensitive brain region to this toxin is the nigrostriatal system where neuronal loss and reduced DA levels, which is a characteristic trait in PD pathomechanism, are well-established by both histo- and biochemical methods in MPTP-treated animal models. According to the above-mentioned mitochondrial dysfunction in PD, several causative and susceptibility genes were identified, including peroxisome proliferator-

activated receptor-gamma (PPARγ) coactivator-1 alpha (PGC-1α). PGC-1α is a multifunctional transcriptional coactivator of nuclear respiratory factors 1 and 2, estrogen-related receptors and PPARs amongst others, and hereby regulates mitochondrial function and biogenesis.
Besides QUIN and 3-OHK there are numerous other substances that can facilitate the formation of reactive species (RS) which are responsible for most of the deleterious effects of pathological processes in the nervous system. The synthesis and toxic effects of RS are decreased by a complex system of antioxidant machinery, including enzymatic and non-enzymatic mechanisms. One of the most important non-enzymatic antioxidant is vitamin E, which is actually a group of molecules including four tocotrienols and four tocopherols as lipid soluble antioxidants. Severe tocopherol deficiency develops mainly as a result of malabsorption disorders, abetalipoproteinemia and ataxia with vitamin E deficiency (AVED). AVED is caused by the mutation of the TTPA gene resulting in the decreased activity of the α-tocopherol transfer protein. Decreased tocopherol levels are associated with several neurological symptoms, such as cerebellar ataxia, peripheral neuropathy and myopathy, as the nervous system is particularly sensitive to oxidative damage resulting from increased energy demand and reduced antioxidant capacity. Differential diagnosis sometimes can be difficult in certain conditions accompanied by neurological symptoms (especially in AVED), therefore the measurement of serum tocopherol levels is advised in cases of ataxia, myopathy or cognitive deficiency.

2 Aims

I., To study the possible pharmacokinetic explanation of the protective effects of two KYNA analogs (KA-1: N-(2-N,N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride and KA-2: N-(2-N-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride) in the orofacial formalin model of headache.

II., To examine the alterations in concentrations of some initial compounds in the KP of TRP metabolism following 3-NP administration as a toxin model of HD.

III., To determine the biochemical background to assess of the presence or the lack of MPTP-induced changes in tissue specific PGC1-α expression when grading nigrostriatal injury.

IV., To establish reference intervals (RI) for serum tocopherol concentrations in relation to the adult Hungarian population.

3 Materials and methods

The animals were housed under standard laboratory conditions (in an air-conditioned, humidity-controlled and ventilated room) and were allowed free access to drinking water and regular rodent chow on a 12 h−12 h dark-light cycle. The procedures used in this study were approved by the Committee of Animal Research at the University of Szeged and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board. The tocopherol study was approved by the Ethics Committee of the Faculty of Medicine, University of Szeged and all study participants gave their written informed consent, in accordance with the Declaration of Helsinki.

During the pharmacokinetic study of KYNA amides adult, male Sprague-Dawley rats received intraperitoneal (i.p.) injection with the KYNA amides (1 mmol/kg) at set time points (15, 30, 60, 120 and 300 min). Blood samples were collected from vena cava caudalis and the CNS samples containing the medullary segment of the TNC were then removed and stored at - 80°C until measurements. The KYNA concentrations of the serum and CNS samples were quantified by an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with fluorescence and a UV detector; the former was applied at excitation and emission wavelengths of 344 nm and 398 nm for the determination of KYNA and the latter was set at 365 nm for the determination of the internal standard (3-NLT). For the determination of KYNA amides, a Thermo LCQFleet ion trap mass spectrometer was used equipped with an ESI ion source combined with an HPLC system.

For the measurement of TRP metabolites in the 3-NP toxin model of HD, we used 30 5-month-old male C57Bl/6 mice. On the 18th day of the experiment (12 days after the last 3-NP injection), the mice were deeply anesthetized and venous blood was obtained from the right ventricle by intracardial puncture. After perfusion with artificial cerebrospinal fluid, the brains were rapidly removed on ice and stored at - 80°C until analysis. The TRP, KYN and KYNA concentrations of the samples were quantified with the same equipment and method as described above where

the fluorescence detector was set at excitation and emission wavelengths of 254 nm and 398 nm for TRP, and the UV detector was set at 365 nm for the determination of KYN. For determination of the concentrations of 3-OHK and its internal standard isoproterenol, we applied an electrochemical detector.

With regard to the MPTP toxin model of PD, 12-week-old C57Bl/6 male mice were used. The first and second group received i.p. injection of 15 mg/kg body weight MPTP 5 times at 2 h intervals. The animals in the first group were deeply anesthetized with isoflurane and the brains were dissected 90 min following the last MPTP injection (acute treatment – acute assessment), while animals in the second group were deeply anesthetized with isoflurane and the brains were dissected one week later (acute treatment - subacute assessment). The mice in the third group were injected i.p. with 15 mg/kg body weight MPTP once a day for 12 days (chronic treatment). DA and its metabolites, DOPAC and HVA were measured with an HPLC system combined with an electrochemical detector.

For the tocopherol measurements, the sample population comprised of 30 male and 30 female volunteer individuals without any major chronic illness and 30 male and 30 female patients with other neurological diseases (OND; presence of ataxia, myopathy or cognitive dysfunction was excluding criteria). The distribution of the age of the subjects was Gaussian in all groups and there was no significant difference between the groups. The recent regular intake of antihyperlipidemic agents or any kind of drugs or food supplements containing antioxidants was exclusion criteria as well in all groups. All participating individuals were of Hungarian origin and were enrolled in the Department of Neurology at the University of Szeged. Blood was collected by venipuncture and the serum was shot into a solution containing ascorbic acid and butylated hydroxytoluene and the resulting solution was stored on - 80°C until measurement. Total cholesterol and triglyceride levels were determined by commercially available kits. The concentrations of α-, β/γ-, δ-tocopherol and rac-tocol internal standard were quantified with an HPLC system equipped with an UV/VIS diode array detector. The separation of β- and γ-tocopherol is challenging because they only differ in the position of a methyl group. With the use of a C18 column these two compounds have almost the same retention time, accordingly, only γ-tocopherol was applied as standard compound for the establishment of the calibration curve in this study, and the concentrations at the corresponding retention time includes both substances and reported as β/γ-tocopherol.

For all our measurements a partial validation method was applied according to the International Conference on Harmonization guidelines. Calibrants were prepared at 6 different concentration levels and the peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method. The selectivity was checked by comparing the chromatograms for a blank serum and CNS sample and those for a spiked sample. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined via the signal-to-noise ratio with a threshold of 3 and 10. Precision was determined by 6 replicate HPLC analysis and the relative recoveries were estimated by measuring spiked samples at 2 concentrations with 3 replicates of each.

4 Results

The time-course profile of the KYNA amides in the rat serum revealed that, after a steep increase in the concentration, a subsequent steep decrease occurred in the first hour, followed by a prolonged further gradual decrease. Although the serum concentration of KA-2 did not show such a high level as that of KA-1, a slightly slower decrease in concentration was observed. In the rat CNS samples the KA-1 concentration was under LOD, KA-2 was present in detectable amounts reaching its maximum concentration after an hour which subsequently gradually decreased. The CNS pharmacokinetics of KYNA following KA-1 and KA-2 administration showed quite similar profiles, characterized by an approximately maximal 10-fold increase in basal concentration within the first hour.

With regard to the 3-NP toxin treated mice, in the case of KYN, a significant difference was detected in the cortex relative to the control value and significantly decreased TRP levels were found in the striatum, cortex, hippocampus, cerebellum and brainstem as compared with the controls. In 3-OHK levels, a significant decrease was observed in the cerebellum in the 3-NP-treated mice in comparison with the control value, but not in the other brain regions. The observed decreases in TRP level were generally associated with an increased KYN/TRP ratio, an index widely used to assess the metabolic activity of KP.

The MPTP administration caused significant reductions in the striatal DA, DOPAC and HVA levels compared to control values 90 min following its last administration in the acute treatment regimen. Moreover, a significant reduction in metabolite levels was also observed one week

after the last injection in the acute treatment regimen in the DA, DOPAC and HVA values in the striatum of the MPTP-treated mice compared to the control animals. However, chronic MPTP treatment resulted in significant reductions of only striatal HVA levels. Seven days following the acute treatment regimen the DA levels significantly decreased compared to those data from samples obtained 90 min following the last MPTP injection in the acute treatment regimen.

The group-wise comparisons failed to detect any significant difference between groups regarding the concentrations of α-tocopherol, β/γ-tocopherol or δ-tocopherol. Accordingly, in order to establish RI with appropriate subject numbers, the values for each measured compounds were pooled and the minimum required sample size (n = 120) was achieved. For the determination of lower (2.5%) and upper (97.5%) RI with the corresponding confidence intervals and standard errors, the bootstrap method was applied. To obtain cholesterol-corrected tocopherol values as well, serum cholesterol concentrations were determined for each subject and the tocopherol/cholesterol ratios were calculated. The bootstrap method was applied again for the lipid corrected values. The cholesterol levels positively correlated with the age of subjects and in case of uncorrected α- and β/γ-tocopherol concentrations this correlation with age was present as well. When tocopherol levels were normalized to cholesterol levels, all the correlations with age were eliminated.

5 Discussion

Bioanalytical measurements play an essential role in differential diagnostics and may have prognostic value as well. Accordingly, numerous preclinical and clinical studies aim to discover novel biomarkers to monitor various pathological processes or the therapeutic effects of novel drugs. Amongst analytical methods, HPLC is one of the most popular options because it provides fast and robust determination of a wide range of compounds. However, these methods require substantial development of procedures in order to obtain valid, i.e., replicable, results.

For the investigation of the potential beneficial effects of KYNA amides in headache, a well-known model of trigeminal nociception, the orofacial formalin test, was applied. Our aim was to give a pharmacokinetic explanation for the observed beneficial effects of two KYNA amides (KA-1 and KA-2) in the above-mentioned formalin model, which involves both peripheral and

central components of pain processing. With regard to the serum concentrations of the analogs following their i.p. administration, the levels of KA-2 were considerably lower than those of KA-1, in contrast, KA-1 could not be detected in the examined CNS region, and the concentration of KA-2 was likewise relatively low. On the other hand the serum pharmacokinetic data revealed that KA-2 decays into KYNA in larger amounts than KA-1, but nevertheless, in the examined CNS region, there is no major difference between KYNA levels following the treatments with KA-1 or KA-2. In view of these findings, the difference in the observed effects in behavioral and immunohistochemical studies may be explained by the differences in the serum KYNA levels. These findings suggest that the difference in the beneficial effects of the two analogs may be explained by the peripheral effect of elevated KYNA concentrations on formalin-induced pathological alterations. The molecular background would be the inhibition of NMDAR-mediated neurotransmission at the strychnine-insensitive glycine-binding site which is present on the peripheral process of the trigeminal nociceptors.

In another study, regarding the KP of TRP metabolism, we used the 3-NP toxin model of HD. Our findings indicated a decreased TRP level in association with an increased KYN/TRP ratio in most of the examined brain regions of C57Bl/6 mice treated with 3-NP, and also a reduced concentration of 3-OHK in the cerebellum. It is noteworthy that we did not detect any significant difference in serum samples, which suggests that the observed alterations are specific for the examined brain regions and are not affected by a systemic change and/or an altered permeability of the blood-brain barrier. Our results on 5-month-old 3-NP-treated animals resemble those from studies of 3-month-old transgenic YAC128 animals, which is known to be one of the best animal strains for the modeling of the alterations in human HD. The elevated KYN/TRP ratio is comparable with the previous finding of increased indolamine dioxigenase-1 activity in the brain (but not in the serum) of YAC128 animals, an alteration reflecting that observed in several neurodegenerative diseases and their animal models, and which is suggested to contribute to the neurodegenerative process. We presume that the KP alterations observed in transgenic animals might be secondary, at least partially, to a mitochondrial dysfunction, a well-known phenomenon in the pathogenesis of HD, and that 3-NP toxicity may comprise a useful and cheap tool for the screening of the efficacy of potential drug candidates before the application of more demanding genetic models.

With regard to DA measurements, our aim was to confirm that the applied acute MPTP treatment regimen was effective in decreasing striatal DA levels, while the chronic low dose administration of the toxin was not. Ninety min after the last MPTP injection of the high-dose acute treatment (75 mg/kg/day total dose) the concentration of DA and its metabolites, DOPAC and HVA, were significantly decreased compared to the controls. Seven days after the last injection this difference could still be observed. Additionally, DA levels were notably lower compared to the animals processed after ninety min. With regard to the chronic low dose administration of MPTP (15 mg/kg/day), it did not induce significant DA depletion (i.e., neurotoxic effect at biochemical level), a meaningful decrease in concentration was observed only in the case of HVA. These results demonstrated the presence of significant neurotoxicity in the case of the acute treatment regimen, associated with a sudden, but temporary, increase in PGC-1α expression, while the chronic low dose regimen failed to evoke any significant changes either in striatal DA concentration or in PGC-1α expression, suggesting that chronic low dose intoxication did not induce protective mechanisms with the involvement of PGC-1α.

The determination of exact serum tocopherol concentrations may be substantial for the diagnosis and therapeutic monitoring of certain conditions usually accompanied with neurological symptoms, such as ataxia, myopathy or cognitive deficiency. For proper evaluation physicians need a well-established RI, which can vary considerably between populations. The aim of our tocopherol measurements was to establish RIs for the adult Hungarian population and to compare the method of patient selection and the analytical procedure with that of previously published studies. The selection of a homogeneous study population may have a special importance, because the distribution of age can considerably influence reference values in light of the fact that the levels of certain tocopherols significantly increase with age. An another qualitative aspect of the composition of the study population, in addition to the involvement of subjects without any chronic illness, the group of assessed individuals also comprised patients with different neurological disorders where tocopherol levels were not previously reported to be abnormal. This study setup may ensure the absence of significant alterations of tocopherol levels in neurological cases lacking the symptoms of ataxia, myopathy and cognitive deficiency, which may be important for future screening studies. Thorough statistical assessment resulted in no significant differences, thus subgroups became suitable for pooling, i.e., the number of individuals in the reference population could

be easily increased to the desired level. With regard to adults, in light of the fact that lipid status, which has a close relationship with tocopherol levels, can vary with age, the application of lipid corrected values may be necessary for the characterization of vitamin E status.

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