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Concurrent quantification of tryptophan and its major metabolites

Wojciech G. Lesniak [a,b,c,d], Amar Jyoti [c,d,e], Manoj K. Mishra [a,b,c,d], Nicolette Louissaint [b], Roberto Romero [c,d], Diane C. Chugani [f], Sujatha Kannan [e,f], Rangaramanujam M. Kannan [a,b,c,d,*]

[a] Department of Chemical Engineering and Material Science, Children's Hospital of Michigan, Wayne State University, Detroit, MI 48202, USA
[b] The Center for Nanomedicine, Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA
[c] Perinatology Research Branch, National Institute of Child Health and Human Development, NIH, DHHS, Detroit, MI 48201, USA
[d] Perinatology Research Branch, National Institute of Child Health and Human Development, NIH, DHHS, Bethesda, MD 20892, USA
[e] Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA
[f] Carman and Ann Adams Department of Pediatrics, Children's Hospital of Michigan, Wayne State University, Detroit, MI 48202, USA

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ABSTRACT

An imbalance in tryptophan (TRP) metabolites is associated with several neurological and inflammatory disorders. Therefore, analytical methods allowing for simultaneous quantification of TRP and its major metabolites would be highly desirable, and may be valuable as potential biomarkers. We have developed a HPLC method for concurrent quantitative determination of tryptophan, serotonin, 5-hydroxyindoleacetic acid, kynurenine, and kynurenic acid in tissue and fluids. The method utilizes the intrinsic spectroscopic properties of TRP and its metabolites that enable UV absorbance and fluorescence detection by HPLC, without additional labeling. The origin of the peaks related to analytes of interest was confirmed by UV–Vis spectral patterns using a PDA detector and mass spectrometry. The developed methods were validated in rabbit fetal brain and amniotic fluid at gestational day 29. Results are in excellent agreement with those reported in the literature for the same regions. This method allows for rapid quantification of tryptophan and four of its major metabolites concurrently. A change in the relative ratios of these metabolites can provide important insights in predicting the presence and progression of neuroinflammation in disorders such as cerebral palsy, autism, multiple sclerosis, Alzheimer disease, and schizophrenia.

Tryptophan (TRP)[1] is an essential amino acid necessary for protein biosynthesis and is also a precursor for metabolites such as serotonin, melatonin, and kynurenine. It is metabolized via several pathways, the major ones being the kynurenine and serotonin pathways (Scheme 1). A relative imbalance in TRP metabolites can cause neuronal damage and impairment of multiple behavioral, physiological, and neurological functions [1–3]. Serotonin is an important neurotransmitter, modulating numerous behavioral and physiological functions such as sleep, mood, appetite, learning, and memory [4]. Recent study has demonstrated decreased levels of cortical serotonin in neonatal rabbits with cerebral palsy induced by maternal-fetal inflammation [5]. 5-Hydroxyindoleacetic acid, the immediate metabolite of serotonin, is used as a biomarker in the diagnosis of malignant carcinoid, phenylketonuria, and migraine [6]. However, tryptophan is mainly metabolized via the kynurenine pathway (KP) by a cascade of enzymatic reactions involving the creation of multiple neuroactive species (Scheme 1). The KP is composed of two branches, leading to the formation of (1) kynurenic acid (KYNA) and (2) picolinic and quinolinic acids. Kynurenic acid is an *N*-methyl-D-aspartate receptor antagonist and is considered to be neuroprotective [2]. Higher concentrations of KYNA were found in the cerebrospinal fluid of patients with schizophrenia compared to age-matched healthy patients [2,7]. There is also evidence that elevated concentrations of KYNA can disrupt the learning and cognitive abilities in Alzheimer disease and result in impaired synaptogenesis when elevated in the developing brain [8,9]. Other kynurenine metabolites, including 3-hydroxykynurenine and quinolinic acid, are considered to be neurotoxic [1].

To date, a number of methods for analysis of TRP and its metabolites have been developed, mostly based on liquid or gas chromatography with various detection modalities, such as mass spectrometry, UV absorbance, and fluorescent, electrochemical, and evaporative light scattering [10–21]. Analysis by LC–MS or GC–MS involves synthesis of deuterated standards, somewhat complicated sample preparation procedures, and relatively sophisticated equipment. However, multiple species can be analyzed with high sensitivity at the femtomolar level, which is very useful when a small amount of sample is available, such as

* Corresponding author. Rangaramanujam M. Kannan, The Center for Nanomedicine, Department of Ophthalmology, Johns Hopkins School of Medicine, Baltimore, MD, USA. Fax: +1 410 614 6509.
E-mail address: krangar1@jhmi.edu (R.M. Kannan).

[1] *Abbreviations used:* KP, kynurenine pathway; TRP, tryptophan; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; KYN, kynurenine; KYNA, kynurenic acid.

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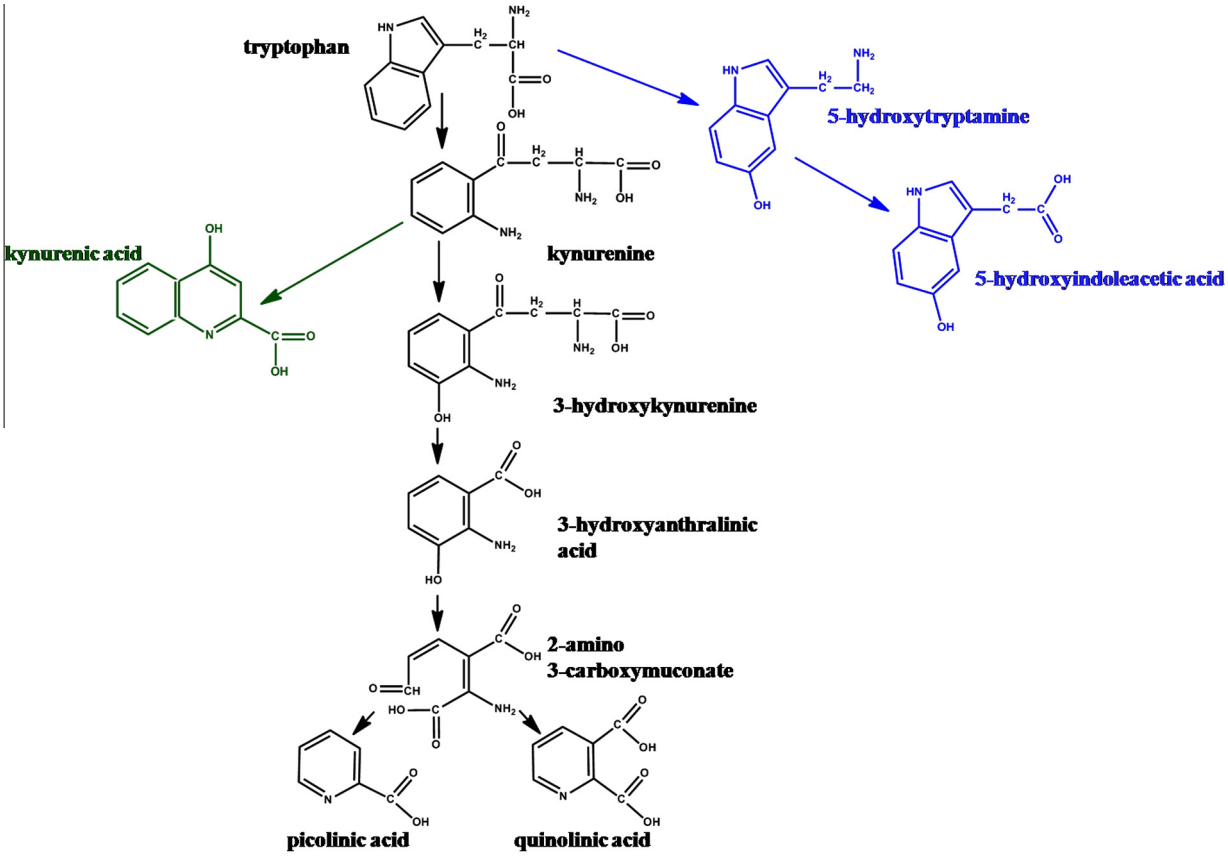

Scheme 1. Schematic depiction of the major tryptophan metabolic pathways.

samples of cerebral spinal fluid or from neonatal and pediatric patients [13].

The goal of this study was to develop a simple and rapid HPLC method for the concurrent detection and quantification of tryptophan and its major metabolites, serotonin, 5-hydroxyindoleacetic acid, kynurenine, and kynurenic acid. The developed method was assessed for accuracy, linearity, limit of detection, and reproducibility. Feasibility of using this method was demonstrated in tissue obtained from rabbit fetal brains (gestation day 29) and amniotic fluid. In comparison with other published methods, the present method has the advantage of simultaneous detection and robust quantification of TRP and four of its metabolites using rather simple sample preparation and HPLC setup equipped with two pumps and photodiode array (PDA) and fluorescence detectors for quantification of analytes in tissue and body fluids. However, the HPLC system can be simplified to the use of one pump and one detector, since isocratic elution was used and the results obtained based on absorbance and fluorescence are in good agreement. Run time and elution conditions can be adjusted according to the analytes of interest.

Materials and methods

Materials and reagents

TRP, serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), KYNA, acetonitrile (ACN), trifluoroacetic acid (TFA), acetic acid (AA), and chloroform were purchased from Sigma–Aldrich. TSK-Gel ODS-80 Ts (250 × 4.6 mm i.d., 5 μm) and TSK gel guard column were bought from Tosoh Bioscience LLC. Purified water with resistivity of 18.2 M (symbol) cm^{-1} obtained using a Branstead Nanopure Diamond Lab water purification system was used in all the experiments. All other reagents and HPLC solvents were used as received without further purification.

Preparation of brain and amniotic fluid samples

All procedures were approved by the institutional animal care and use committee. New Zealand White rabbits with timed pregnancies (Charles River Laboratories International, Wilmington, MA, USA) were used. Amniotic fluid and fetal brains were collected on gestation day 29. Samples were snap-frozen on dry ice and stored at −80 °C for quantification study.

Brain tissue

For brain sample preparation, the periventricular region (PVR) was microdissected and ~200 mg tissue was homogenized in 1 ml of deionized water. Resulting suspensions were extensively vortexed and centrifuged at 21,130*g* (15,000 rpm) for 10 min using a 5424 R Eppendorf centrifuge (Eppendorf, Hauppauge, NY, USA). Then, 1 ml of the supernatant was collected and vortexed with 3 ml of chloroform to extract fatty acids and lipids. Obtained mixtures were then centrifuged at 3007*g* (4000 rpm) for 10 min using a Thermo Electron CR3i multifunction centrifuge (Thermo Fisher Scientific, Pittsburgh, PA, USA). The aqueous fraction (0.8 ml) was mixed with acetonitrile (3 ml) for protein precipitation. Resulting samples were left in ice for 30 min for protein coagulation, followed by centrifugation at 3007*g* (4000 rpm) for 10 min at 4 °C. The supernatant was divided into three aliquots of 1.2 ml each and lyophilized. For HPLC analysis, residues were dissolved in 1 ml of the mobile phase (0.14% TFA in H_2O:ACN 90:10 v/v) and three injections of 200 μl for each sample were performed.