HPLC method for the assessment of tryptophan metabolism utilizing separate internal standard for each detector

Edina Katalin Cseh[a], Gábor Veres[a,b], Márton Szentirmai[a], Nikolett Nánási[a], István Szatmári[c], Ferenc Fülöp[c,d], László Vécsei[a,b], Dénes Zádori[a,*]

[a]Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Szeged, Hungary

[b]MTA-SZTE Neuroscience Research Group, Szeged, Hungary

[c]Institute of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary

[d]MTA-SZTE Stereochemistry Research Group, Szeged, Hungary

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***Corresponding author:**

Dénes Zádori MD, PhD
Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center
University of Szeged, Semmelweis street 6, H-6725 Szeged, Hungary
Phone: +36 62 545351
Fax: +36 62 545597
E-mail: zadori.denes@med.u-szeged.hu

Abbreviations: *3NLT* 3-nitro-L-tyrosine, *5-HT* serotonin, *ACN* acetonitrile, *CS(s)* calibration standard(s), *CSF* cerebrospinal fluid, *FLD* fluorescence detector, *HCA* 4-hydroxyquinazoline-2-carboxylic acid, *HPLC* high-performance liquid chromatography, *IS(s)* internal standard(s), *IQR* interquartile range, *KP* kynurenine pathway, *KYN* kynurenine, *KYNA* kynurenic acid, *LOD* limit of detection, *LOQ* limit of quantification, *SD* standard deviation, *PCA* perchloric acid, *TRP* tryptophan, *UVD* UV detector, *ZnAc* zinc acetate, *WS* working solution, *ww* wet weight

Abstract

The development of a validated method, applicable for the measurement of tryptophan (TRP) and serotonin (5-HT), and that of the neuroprotective branch of the kynurenine pathway from several different biological matrices, including mouse brain, is described. Following the spectral analysis of the metabolites, they were quantified with reversed-phase high-performance liquid chromatography (HPLC), using separate internal standards (ISs) for UV (3-nitro-L-tyrosine) and fluorescent (the newly utilized 4-hydroxyquinazoline-2-carboxylic acid) detectors. With regard to validation parameters, selectivity, linearity, limit of detection, limit of quantification, precision and recovery were determined. Although the linearity ranges were different for the assessed matrices, the correlation coefficient was > 0.999 in each case. Furthermore, good intra- and inter-day precision values were obtained with coefficient of variation < 5%, and bias < 6.5% (except the 5-HT level in brain samples), respectively. The recoveries varied between 82.5% and 116%. The currently developed methods yield opportunities for the assessment of concentration changes in the TRP metabolism from a wide range of biological matrices, therefore they may well be utilized in future clinical and preclinical studies, especially in view that so many metabolites with the application of ISs have not been detected from mouse brain with such a simple HPLC method before.

1. Introduction

Tryptophan (TRP), the essential amino acid obtained from diet, is mainly metabolized through the kynurenine (KYN) pathway (KP; Fig. 1), whereas only a small proportion of it is catabolized to the neurotransmitter serotonin (5-HT) [1]. Recently, a special attention has been paid to the KP in neuroscience research, especially in light of the well documented alterations of the pathway in numerous neurological disorders, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis and schizophrenia [2–6]. Accordingly, several methods have been developed for the quantification of TRP and its metabolites, including those with multiple-step sample preparation, pre- or post-column derivatization or those needing complex instrumental background (e.g., high-performance liquid chromatography (HPLC) mass spectrometry, gas chromatography mass spectrometry) [7]. The assessment of TRP, KYN and kynurenic acid (KYNA), usually designated as the neuroprotective branch of the KP, may yield meaningful information in several preclinical and clinical studies [1]. The detection and quantification of these metabolites can be achieved in an easier way compared to the other compounds of the KP [7–9]. The partial assessment of the KP has been described with different HPLC methods, using several detection techniques, including UV detector (UVD) and fluorescence detector (FLD) [9,10], diode array detector [11], electrochemical detector [12] or mass spectrometry [13,14], targeting different biological matrices: human serum or plasma, and cerebrospinal fluid (CSF) [5,13–16], murine serum or plasma [9,14], and brain [9,17]. The heterogeneity of the methods is further increased by the application of internal standards (ISs), although only some (37.7%) of the articles of interest (Supplementary file, Table S1) applied ISs at all, and none of them utilized ISs separately for each detector. This especially makes sense when in addition to the obviously necessary features of ISs (stability, pure form, absence in native sample, or no interference with another compound) [18,19], two often neglected characteristics are also taken into account: compatibility with the detector response and similarity in structure and properties with the analyzed compounds. Accordingly, the application of different ISs is required for each detector as the concentrations are calculated from a calibration plot where the concentration values are plotted against the response ratios. In light of these requirements, 3-nitro-L-tyrosine (3NLT) is not appropriate as an IS for the fluorescent detectable compounds TRP, 5-HT and KYNA. Consequently, the application of 4-hydroxyquinazoline-2-carboxylic acid (HCA) emerged as a new IS for the measurement of TRP, 5-HT and KYNA, with a special relevance to the latter one due to the similarities in their structure, which probably enables the detection of HCA at the same wavelength as KYNA, without affecting the running time of the sample.

3NLT has already been applied widely as an IS for UVD, as its structure is similar to that of KYN (Fig. 1).

The aim of the current study was to present a simple, rapid, precise, robust and economical method (95% water in the mobile phase) for the simultaneous quantification of TRP, 5-HT (present in detectable amounts only in the mouse brain), KYN and KYNA by HPLC-UVD and FLD, using ISs for each detector (3NLT for the UVD, and HCA for the FLD), following a complete spectral analyses of each compound. To demonstrate the robustness of the method, the validation process was completed on four different biological matrices (mouse plasma and brain, human plasma and CSF) according to the ICH and FDA guidelines [20, 21]. Furthermore, to verify the applicability of the currently developed methods, all of the metabolites of interest were quantified from the above-mentioned matrices, and the obtained concentration values were compared with the available literature data.

2. Material and methods

2.1. Instrumentation and reagents

The chromatographic analyses were performed using an Agilent 1100 HPLC system (Santa Clara, CA, USA) with Agilent G1314A UVD and G1321A FLD. The spectral analyses of the UV-detected compounds were made with an Agilent 8453 UV-Vis Spectroscopy System (Santa Clara, CA, USA). The reference compounds TRP, 5-HT, KYN, KYNA, 3NLT; perchloric acid (PCA), zinc acetate (ZnAc) and phosphoric acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile (ACN) was obtained from Scharlau (Barcelona, Spain) and acetic acid from VWR International (Radnor, PA, USA). The di-sodium-hydrogen phosphate dihydrate was obtained from VWR International (Radnor, PA, USA) and potassium dihydrogen phosphate from Applichem Panreac (Darmstadt, Germany). The IS used for the FLD (HCA) was synthesized at the Department of Pharmaceutical Chemistry, University of Szeged, involving the ring closure of anthranilamide with diethyl oxalate, followed by the hydrolysis of the ester function [33].

2.2. Collection and preparation of biological samples

Mouse plasma samples and mouse brain tissues were obtained from 3-4 months old C57Bl/6 mice. The blood samples were collected into Na-EDTA-containing tubes and centrifuged at 3500 RPM for 10 min and the resulting plasma samples were stored at -80°C until analysis. The

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. 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. Regarding the freshly prepared mouse brain 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. For the validation process, pooled brain homogenates were applied, whereas the applicability of the method was tested on 8 independent striatum, cortex and hippocampus, obtained from the same mice as used for plasma sample measurements. The animal experiments were authorized by the local ethical committee of University of Szeged with adherence to the NIH guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. Human plasma samples were obtained from 26-39 years old healthy subjects following obtaining written informed consent. 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). The assessment of the applicability of the method was also carried out on 8 independent samples. The CSF samples were taken from 17-71 years old patients with headache who were initially suspected to have subarachnoid hemorrhage and underwent a spinal tap, but the CSF analysis was negative. Written informed consent was also obtained in each case. 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. The applicability of the method was also tested on 8 independent CSF samples. All the human samples were obtained with the approval of the local Ethical Committee of the University of Szeged (46/2014), adhering to the tenets of the most recent revision of the Declaration of Helsinki.

2.3. Chromatographic conditions

Chromatographic separations were performed on a reversed-phase C18 column (Kinetex, 150 x 4.6 mm I.D., 5 μm particle size; Phenomenex Inc., Torrance, CA, USA) after passage through a pre-column (SecurityGuard, 4 x 3.0 mm I.D., Phenomenex Inc., Torrance, CA, USA) with a mobile phase composition of 200 mM ZnAc solution at pH of 6.2 for plasma and CSF samples, and at pH of 5.8 for brain samples, adjusted with acetic acid. The organic component (ACN) in the mobile phase was 5%, and the solution was filtered through a cellulose membrane with 0.2

μm pore size. The flow rate was 1.2 ml/min and 20 μl of the plasma supernatants were injected, whereas in case of CSF and brain homogenate the injection volume was 50 μl. The application of ZnAc at such high concentration as 200 nM was necessary – focusing on the parallel prevention of precipitation as well – in light of the considerable increase of the fluorescence intensity of KYNA, which seems essential for its detection above limit of quantification (LOQ; Supplementary file, Fig. S3A). With the careful use of ZnAc at 200 nM, we did not experience precipitation and the lifespan of the applied column was not affected as well. The UV-Vis spectra data were collected from 200-800 nm in cases of KYN and 3NLT to determine the optimal wavelengths for measurements (Supplementary file Fig. S1).

The determination of optimal wavelengths in case of FLD was carried out via the collection of spectral data in the ranges of 220–380 nm (excitation) and 300–495 nm (emission) for each fluorescent compound, i.e., TRP, 5-HT, KYNA, and HCA (Supplementary file, Fig. S2A, B, C, D).

2.4. Method validation

The investigated validation parameters were selectivity, linearity, limit of detection (LOD), LOQ, precision and recovery, respecting the ICH [20] and FDA [21] guidelines. As insufficient amount of sample was obtained from one animal (especially in case of mouse plasma), following the recommendations provided by the FDA [21], pooled samples were used in each case, for constancy of validation process.

2.4.1. Calibration curve and linearity

Stock solutions with the concentration of 100 μM were prepared by dissolving accurately weighed standard compounds in 0.5 M PCA solution, except the KYNA, which was dissolved in phosphorous buffer, with pH set at 6.2 with 85% phosphoric acid, due to solubility issues. A series of working solutions (WS) of the analytes with different concentration ranges for each matrix was prepared containing the ISs at final concentration of 2 μM for 3NLT and 100 nM for HCA. For the calibration curve, LOD and LOQ values, six calibration standards (CSs) were prepared by spiking the respective WSs into blank biological matrices, i.e., blank human plasma and CSF, and blank mouse plasma, respecting the dilution ratios presented above. With regard to mouse brain samples, the first step was the homogenization of the respective brain regions, and thereafter the supernatant was added the appropriate amount of the WSs. TRP, 5-HT, KYN and KYNA were prepared in the presented final concentration ranges (Table 1). The peak area

response ratios were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R software (R Development Core Team, 2002). The same procedure was applied for the preparation of the quality controls (QCs), i.e., spiking the blank biological matrices with the appropriate solutions, containing the analytes in three different concentration levels (low (LOQ), medium and high; Table 3) for performing the accuracy assays. Both CSs and QCs were prepared freshly, on the day of the measurements, whereas stock solutions and WSs were stored at –80ºC.

3. Results and discussion

3.1. Selection of the excitation and emission wavelengths

As a result of absorbance analyses for KYN and 3NLT (Supplementary file, Fig. S1) the wavelength of the UVD was set to 365 nm when pH was 6.2, whereas a slight maximum absorbance shift was observed at pH 5.8, therefore 360 nm was applied in this case. Following spectral analyses in case of fluorescent detection, the excitation and emission wavelengths at pH 6.2 were set to 246 and 396 nm for the determination of KYNA and HCA, and to 220 and 410 nm for the determination of TRP, whereas at pH 5.8 the excitation and emission wavelengths were set to 239 and 400 nm for the determination of 5-HT, KYNA and HCA, and to 220 and 355 nm for the determination of TRP (Supplementary file, Fig. S2).

3.2. Utilization of two internal standards and selectivity of the applied methods

Several methods have already been published for the quantification of TRP and some of its metabolites (Supplementary file, Table S1), but from the published articles, only our research group reported the use of the 3NLT, when quantifying the analytes of interest from brain samples via the application of an Onyx Monolithic C18 column (100 mm× 4.6 mm I.D., Phenomenex Inc., Torrance, CA, USA) [9]. Indeed, the monolith column provided a good running time (7 min, [9]), but the introduction of the novel IS (HCA) for the FLD led to co-elution on the monolithic column (Supplementary file, Fig. S4) which resulted in the necessary change of the column. Accordingly, Kinetex C18 column was chosen with the aim of the parallel improvement of resolution. Although this novel setup with the optimization of flow rate and detection wavelengths was found to be suitable for measurements from plasma and CSF samples with appropriate selectivity and signal amplitude (Fig. 2), in case of mouse brain samples, an interfering peak was detected causing co-elution with both UVD and FLD (Fig. 3A,

B). Accordingly, a further adjustment (i.e., the change of pH value of the mobile phase from 6.2 to 5.8, Fig. 3A, B) should be carried out to regain the appropriate selectivity (Supplementary file, Fig. S4). The further reduction of the pH considerably decreases the signal amplitude of KYNA, so it should be avoided (Supplementary file, Fig. S3B).

3.3. Method validation

3.3.1. Linearity

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. The applied ranges mentioned in the Table 1 were confirmed to be linear in all cases, with a correlation coefficient > 0.999 for each compound when either FLD or UVD was applied.

3.3.2. LOD and LOQ

LOD and LOQ were determined based on the guidelines [20, 21] calculating by the Equation 1, where σ is the standard error of the intercept and S' is the slope of the calibration curve of the analyte, presented in Table 1.

$$\text{LOD} = 3.3 \cdot \sigma / S' \text{ and LOQ} = 10 \cdot \sigma / S' \quad (1)$$

The obtained values were in line with literature data in each case (Supplementary file, Table S1).

3.3.3. Precision

The precision of the method was determined for each analyte in all matrices (Table 2). Intra-assay precision, expressed as CV%, was evaluated by running six replicates, with values ranging between 1.14–4.25%, i.e., all of them were below 5%, in line with the values expected by the FDA [21]. Inter-assay precision was calculated by measuring the same samples used for the intra-assay precision with separate calibrations curves, after three days. The calculated values ranged between 1.11–6.37%, except for the 5-HT in the mouse brain sample, where a decrease of 52% was observed. This bias is higher than the maximum recommended value (15%) [21]. Due to the heterogeneity of the bioanalytical studies [35], there are many cases where the FDA proposed limits 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 brain samples show stable concentration values (4.25 CV%).

3.3.4. Accuracy

Recovery studies were performed using spiked samples at three different concentration levels (LOQ, medium and high), representing the entire range of values used for the calibration curve, with three replicates for each concentration. Recovery percentages were calculated as $R = 100 \times [(C_{ss}-C_{ns})/C_{spike}]$, where C_{ss} is the concentration in the spiked homogenate sample, whereas C_{ns} is the concentration of the homogenate native sample (without spiking) and C_{spike} is the added concentration. The obtained values ranged between 82.5-116% (Table 3), which are within 15% of the nominal value, except the LOQ-spiked recovery values, which did not deviate by more than 20%, as recommended by the official guidelines [20, 21].

3.4 Application of the developed method on different biological matrices

The results of the measurements of the metabolites of interest from the assessed biological matrices (mouse plasma and brain, human plasma and CSF) with the developed and validated method are summarized in Table 4. All the reported data in the current study are in line with those obtained from the scientific literature [9,10,15,16,23–40].

4. Conclusion

In summary, in this paper we report an improved HPLC-UVD and FLD method for the quantification of TRP and some of its metabolites (5-HT, KYN, and KYNA). The novelty of this study is the utilization of two different ISs, a widely applied one for the UVD and a novel one for the FLD, proved to be adaptable for measurements from all the four different biological matrices. Although the developed method, suitable for measurements from mouse plasma and human plasma and cerebrospinal fluid was not appropriate for measurements from the mouse brain samples, the method could be further improved with slight modifications (changing the pH from 6.2 to 5.8) to become applicable for the assessment of all the 4 above-mentioned compounds from mouse brain samples as well with a single run, which has not been published

before with HPLC-UVD and FLD (Supplementary file, Table S1). With regard to validation process, in addition to the achievement of appropriate selectivity, the linearity, LOD, LOQ, recovery and intra-assay precision values were all within the acceptable ranges provided by FDA and ICH [20, 21] and were in line with literature data proving the robustness of the method. The considerably high inter-assay value for 5-HT draws attention to the necessity of brain homogenization right before the measurement. Moreover, to demonstrate the applicability of the developed method, the above-mentioned metabolites were quantified in different biological matrices and all of the reported concentration values were within or near the ranges obtained from the scientific literature. In conclusion, a fit-for-purpose, simple and economical method with the simultaneous application of two ISs was developed with one-step sample preparation, acceptable running time and with applicability in either human or animal model studies.

Declaration of interest

The authors confirm that there were no conflicts of interest in performing this study.

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Figure legends

Fig. 1. The partial metabolism of tryptophan through the kynurenine and serotonin pathways and the structure of the two internal standards used for the UV and fluorescence detectors.
5-HT serotonin, *IDO*/*TDO* indoleamine 2,3-dioxygenase/ tryptophan 2,3-dioxygenase, *KAT* kynurenine aminotransferase, NAD^+ nicotineamide adenine dinucleotide, *TRP* tryptophan.

Fig. 2. Chromatograms of pooled mouse brain (A) and plasma (B), and human plasma (C) and cerebrospinal fluid (D) samples. UV chromatograms were obtained at 365 nm (B, C, D) and 360 nm (A), whereas for the fluorescence chromatograms, we applied Ex/Em.: 246/396 nm for the first 7 minutes and 220/410 nm for the remaining time (running time: 11 min) (B, C, D). For the brain samples (A) 239/400 nm and 220/335 nm were applied (running time: 9 min).
3NLT 3-nitro-L-tyrosine, *5-HT* serotonin, *HCA* 4-hydroxyquinazoline-2-carboxylic acid, *IS* internal standard, *KYN* kynurenine, *KYNA* kynurenic acid

Fig. 3. The UVD (A) and FLD (B) chromatograms of some pooled mouse brain samples, in different tested conditions. The X axis represents the running time of a sample, whereas the Y axis the detector response (mAU for the UVD, LU for the FLD). *3NLT* 3-nitro-L-tyrosine, *ACN* acetonitrile, *FLD* fluorescence detector, *HCA* 4-hydroxyquinazoline-2-carboxylic acid, *IS* internal standard, *KYN* kynurenine, *KYNA* kynurenic acid, *mAU* mili absorbance unit, *LU* luminescence, *UVD* UV detector