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Quantitative analysis of phenylalanine, tyrosine, tryptophan and kynurenine in rat model for tauopathies by ultra-high performance liquid chromatography with fluorescence and mass spectrometry detection

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ABSTRACT

We developed and validated a simple and sensitive ultra-high performance liquid chromatography (UHPLC) method for the analysis of phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and kynurenine (Kyn) in rat plasma. Analytes were separated on Acquity UPLC HSS T3 column (2.1 mm × 50 mm, 1.8 μm particle size) using a 4 min ammonium acetate (pH 5) gradient and detected by fluorescence and positive ESI mass spectrometry. Sample preparation involved dilution of plasma, deproteinization by trichloroacetic acid and centrifugation. The procedure was validated in compliance with the FDA guideline. The limits of quantification (LOQ) were 0.3 μM for Kyn and from 1.5 to 3 μM for Phe, Tyr, Trp. The method showed excellent linearity with regression coefficients higher than 0.99. The accuracy was within the range of 86–108%. The inter-day precision (n = 5 days), expressed as % RSD, was in the range 1–13%. The benefit of using UHPLC is a short analysis period and thus, a very good sample throughput. Using this method, we analyzed plasma samples and detected significant changes of Kyn and Phe in transgenic rat model for tauopathies.

1. Introduction

Inflammatory reactions often accompany various human diseases including neurodegeneration. In tauopathies, from which Alzheimer's disease is the most prevalent, the inflammatory reactions include activation of microglia, production of inflammatory mediators such as cytokines, chemokines, metabolites, reactive oxygen species and infiltration of brain by monocytes/macrophages [1]. Phenylalanine (Phe) is an essential aromatic amino acid that is an important precursor of catecholamines. Phe is converted into tyrosine (Tyr) by phenylalanine hydroxylase. Tetrahydrobiopterin (BH_4) is an essential co-factor in this process. The determination of the Phe/Tyr ratio may therefore serve as an indirect measure of BH_4 availability [2]. Increased inflammation induces nitric oxide production that contributes to BH_4 oxidation thus limiting its availability for enzymatic conversions. Tryptophan (Tryp) is an important amino acid that is converted either to neurotransmitter serotonine by tryptophan hydroxylase or to the NAD+, kynurenic acid and xanthurenic acid in the tryptophan–kynurenine (Kyn) pathway. More than 95% of tryptophan is metabolized through Try–Kyn pathway [3]. Most metabolites in the Kyn pathway are neuroactive, either neurotoxic like quinilinic acid (NMDA receptor agonist) or neuroprotective like kynurenic acid (α7 nicotinic acetylcholine receptor agonist) [4]. Evidence indicate that Phe–Tyr and Try–Kyn pathways are affected in inflammation and neurodegenerative disorders, including Huntington's disease, Parkinson's disease and Alzheimer's disease (AD).

Several methods have been developed and used for the analysis of Phe, Tyr, Tryp and Kyn in plasma. The most common method for the analysis of Phe and Tyr is HPLC with fluorescence detection [5]. With little modification, the same method can be used also for tryptophan. Kynurenine has been analyzed either by HPLC/UV [6]

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or together with other Tryp metabolites by liquid chromatography-tandem mass spectrometry [7].

Here we developed a rapid and sensitive method for analysis of phenylalanine, tyrosine, tryptophan and kynurenine in rat plasma. All analytes were separated and quantified within a single chromatographic run. The use of UHPLC in combination with fluorescence (FLD) and mass spectrometry detection (MS) offered an advantage of a short analysis time and therefore a very good sample throughput, applicability for all analytes of interest and selectivity. The method was subsequently used for the analysis of plasma samples from the transgenic rat model for tauopathies.

2. Experimental

2.1. Materials and methods

Chemicals and reagents L-phenylalanine, L-tyrosine, L-tryptophane and L-kynurenine, bovine serum albumin (BSA) and LC/MS grade acetonitrile were obtained from Sigma–Aldrich (Lambda Life, Bratislava), D4 L-kynurenine was obtained from Buchem (Apeldoorn, The Netherlands), trichloroacetic acid was from Merck (Darmstadt, Germany).

Deionized water used in this work was prepared from the water purification system Milli-Q (Millipore, Molsheim, France). Ammonium acetate and acetic acid were purchased from Fluka (Chemika, Switzerland).

2.2. HPLC-FLD/MS conditions

The chromatographic apparatus consisted of an ACQUITY UPLC H-Class chromatographic system with a quaternary gradient pump, auto sampler, column thermostat, fluorescence detector and mass single-quadrupole detector (QDa) with an electrospray ionization source (Waters, Praha, CZ). Data were acquired, calibrated and quantified by Empower 3 software.

Chromatographic separation was performed on an Acquity UPLC HSS T3 column (2.1 × 50 mm; 1.8 μm particles) using a gradient of ammonium acetate buffer and acetonitrile.

Mobile phase A consisted of ammonium acetate in water (20 mM, pH adjusted to 5.0 with acetic acid). Mobile phase B consisted of 100% acetonitrile. The elution started at 0% B (0–0.5 min), increasing to 40% B (0.5–2 min), to 90% B (2.1–3.0 min), returning to 0% B and re-equilibrating at 3.1 to 4.0 min. The column temperature was 40 °C. The flow rate was 0.5 mL/min and the injection volume was 10 μL.

Trp was detected by a fluorescence detector at an excitation wavelength of 285 nm and emission wavelength of 365 nm. Excitation and emission wavelengths for Tyr and Phe were 248/308 nm, 240/287 nm respectively.

Positive electrospray ionisation mode (ESI+) was used for the MS detection and the Kyn (m/z 209.1) and D4 Kyn (m/z 213.1) ions were monitored in the single ion-monitoring (SIM) mode. The following MS conditions were applied. The capillary voltage was set at 0.8 kV, the source block and probe temperatures were 120 °C and 600 °C, respectively. Nitrogen was used as the desolvation and nebuliser gas. The cone voltage was set to 15 V.

2.3. Standards and sample preparation

The mixed working solution (5 M for Tyr, Phe, Trp and 0.5 M Kyn) was prepared from freshly thawed stock solutions by adding each standard into deionized water. The calibration standards were prepared from the working solution by dilution with deionized water.

The calibration line was prepared from the calibration standards added to the BSA solution (70 g/L). One-hundred microliters of standards in BSA or plasma was diluted with 100 μL of deionized water that contains D4 Kyn and proteins were precipitated with addition of 25 μL of trichloroacetic acid (2 M). The capped tubes with the precipitate were immediately vortexed and centrifuged for 10 min at 30,000 × g. One-hundred microliters of the supernatants were transferred into microvials and analyzed.

2.4. Method validation

The method was validated for linearity, specificity, sensitivity, limit of quantification (LOQ) and limit of detection (LOD). The inter-day precision and accuracy of the method were determined by analyzing the 3 different QC samples over 5 days. Intra-day accuracy and precision was calculated from 6 repeat injections. The LOD and the LOQ were calculated from the calibration line at low concentrations. The extraction recovery was assessed using pre-extraction addition method. Autosampler stability was tested by analyzing the samples stored in autosampler at 10 °C for up to 24 h.

2.5. Animals

The generation and characterization of a transgenic rat model for tauopathy expressing human truncated tau (aa 151-391/3R) is described in details elsewhere [8]. For this study, heterozygous transgenic rats (14–16 months old) and non-transgenic SHR age-matched controls were used. All animals were housed under standard laboratory conditions with free access to water and food and were kept under diurnal lighting conditions (12 h light/dark cycles with light starting at 7:00 a.m.). All experiments on animals were carried out according to the institutional animal care guidelines conforming to international standards and were approved by the State Veterinary and Food Committee of Slovak Republic and by the Ethics Committee of Institute of Neuroimmunology. Efforts were made to minimize the number of animals utilized and to limit discomfort, pain or any other suffering of the experimental animals used in this study.

3. Results and discussion

3.1. Method development

The samples were prepared by protein precipitation without any additional derivatization or purification step. Ammonium acetate buffer, which is compatible with mass spectrometry detection, was used as an aqueous mobile phase. The use of UHPLC allows for a rapid throughput with a 4 min run time. All three aromatic amino acids (Tyr, Phe and Tryp) contain chromophores, hence we used fluorescence detector for their analysis. The HPLC/UV is the most common method for analysis of kynurenine in biological matrices. However, the method is not specific and not sensitive enough to be used for analysis of rat plasma. Therefore we used single quadrupole MS detector QDa for quantification of Kyn. QDa is robust, reliable and requires almost no sample adjustments. It is very easy to operate—in comparison to more sophisticated mass spectrometers, it can be switched on and off in 30 min. Kyn, together with deuterated IS, were detected in positive electrospray mode. For the optimal separation, the gradient elution program was established. The retention time under these conditions was 0.66 min for Tyr, 1.52 min for Phe, 1.93 min for Tryp and 1.47 min for Kyn. D4 kynurenine displays a slight shift (1.42 min) of retention time in comparison to Kyn, which can be explained by deuterium isotope effect [9]. Figs. 1 and 2 display typical chromatograms of analytes in BSA standard solution and rat plasma.