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A single-run liquid chromatography mass spectrometry method to quantify neuroactive kynurenine pathway metabolites in rat plasma

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ABSTRACT

Neuroactive metabolites in the kynurenine pathway of tryptophan catabolism are associated with neurodegenerative disorders. Tryptophan is transported across the blood–brain barrier and converted via the kynurenine pathway to N-formyl-L-kynurenine, which is further degraded to L-kynurenine. This metabolite can then generate a group of metabolites called kynurenines, most of which have neuroactive properties.

The association of tryptophan catabolic pathway alterations with various central nervous system (CNS) pathologies has raised interest in analytical methods to accurately quantify kynurenines in body fluids. We here describe a rapid and sensitive reverse-phase HPLC–MS/MS method to quantify L-kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxy-L-kynurenine (3HK) and anthranilic acid (AA) in rat plasma. Our goal was to quantify these metabolites in a single run; given their different physico-chemical properties, major efforts were devoted to develop a chromatography suitable for all metabolites that involves plasma protein precipitation with acetonitrile followed by chromatographic separation by C18 RP chromatography, detected by electrospray mass spectrometry. Quantitation range was 0.098–100 ng/ml for 3HK, 9.8–20,000 ng/ml for KYN, 0.49–1000 ng/ml for KYNA and AA. The method was linear ($r > 0.9963$) and validation parameters were within acceptance range (calibration standards and QC accuracy within ±30%).

1. Introduction

Tryptophan metabolism via the kynurenine pathway plays a key role in several physiological processes and its alteration has been implicated in the pathophysiology of a wide range of disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, AIDS dementia complex, malaria, cancer, depression and schizophrenia [1]. The kynurenine pathway also plays a key modulatory role in the immune response and mediates interactions between immunological and neuronal functions. Study of these (patho)physiological mechanisms, including potential therapeutic approaches, requires accurate quantification of kynurenine and its major degradative metabolites in human and rodent samples.

HPLC methods employing ultraviolet (UV) [2,3], fluorescence [4–9], electrochemical [10] and mass spectrometric [11–13] detection have been described. Among these, HPLC–MS proved to be the most valuable for the specificity and sensitivity of the mass spectrometry detector. Moreover, since a single HPLC–MS method can measure more metabolites together, lower sample

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volume is required for analysis. For example, an automated on-line solid-phase extraction HPLC tandem mass spectrometry method has been described for the determination of tryptophan (Trp), kynurenine (KYN) and 3-hydroxy-L-kynurenine (3HK) in human plasma [14]. A similar method based on solid-phase extraction HPLC–tandem mass spectrometry can quantify Trp, KYN, KYNA, 3-hydroxyanthranilic acid (3OHAA), anthranilic acid (AA), quinolinic acid (QA) and picolinic acid (PA) in rat plasma [15].

Different physico-chemical properties and different levels of metabolites in biological fluids represent the major challenges faced during development of pool quantitation methods.

We describe here a sensitive, rapid and specific LC–MS/MS method that enables simultaneous extraction, separation and quantitation of KYN, KYNA, 3HK and AA in rat plasma. Our goal was to quantify these metabolites in a single run, improving the limit of quantitation of previously published methods for 3HK and extending the upper limit of quantitation for KYN to 20,000 ng/ml, avoiding sample concentration or dilution after extraction. Major efforts were devoted to develop a chromatography suitable for all metabolites. The method involves plasma protein precipitation with acetonitrile followed by chromatographic separation by C18 RP chromatography. Quantitation range was 0.098–100 ng/ml for 3HK, 9.8–20,000 ng/ml for KYN, 0.49–1000 ng/ml for KYNA and AA. Method qualification was repeated on four different days to assess sensitivity, linearity, accuracy and precision.

2. Materials and methods

2.1. Materials

HPLC–MS grade acetonitrile, water and formic acid were obtained from Fluka. L-kynurenine (CHDI-00314491-0000-002), 3-hydroxy-DL-kynurenine (CHDI-00124628-0000-002), kynurenic acid (CHDI-00051482-0000-003) and anthranilic acid (CHDI-00314643-0000-001), were synthesized at Evotec (UK) Ltd. (Abingdon, UK) (Saint Louis, MO, USA). Labeled internal standard L-kynurenine sulfate:H_2O (ring-d4, 3,3-d2) was from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) while anthranilic acid-ring-13C6 was from Sigma–Aldrich (Schnelldorf, Germany). 3-Hydroxy-DL-kynurenine 15N, 13C2 was from AMRI (Albany, NY, USA) and (3,5,6,7,8-d5-KYNA) was from Qmx Laboratories (Thaxted, UK). Pooled Sprague-Dawley rat plasma was from Sera Laboratories International Ltd. (Haywards Health, UK).

2.2. Stock solutions

Stock solutions for calibration standards and quality controls were prepared from independent weight of compounds and stored at −20 °C. KYN, d6-KYN, AA and 13C6-AA were prepared in water/DMSO (1/1, v/v) at 2.5, 1.5, 2.5 and 5.0 mg/ml respectively. KYNA, d5-KYNA, 3HK and 15N,13C2-3HK were prepared in water/DMSO (1/19, v/v) at 0.5, 0.5, 0.25 and 2.0 mg/ml respectively.

2.3. Preparation of calibration standards

A working-stock calibration standard solution (WS-CS) at 500 μg/ml KYN, 25 μg/ml KYNA, 25 μg/ml AA, 2.5 μg/ml 3HK in water/acetonitrile (9/1, v/v) was freshly prepared from stock solutions.

Calibration standards were prepared by serial dilution of WS-CS solution with water/acetonitrile (9/1, v/v) 0.1% formic acid containing 0.08% ascorbic acid (final concentrations: 9.77, 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500, 5000, 10,000, 20,000 ng/ml KYN: 0.098, 0.195, 0.391, 0.781, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 ng/ml 3HK: 0.488, 0.977, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000 ng/ml KYNA and AA).

Calibration curves were constructed using the respective analyte/IS peak area ratios against analyte concentrations with a weighted ($1/C^2$) least-squared linear regression.

2.4. Preparation of quality controls

A working-stock quality control solution (WS-QC) at 1000 μg/ml KYN, 50 μg/ml KYNA, 50 μg/ml AA, 5 μg/ml 3HK in water/acetonitrile (9/1) was freshly prepared from stock solutions. QC spiking solutions were prepared by serial dilution of the WS-QC solution in water/acetonitrile (9/1, v/v) with 0.1% formic acid. Nine quality control (QC) standards at low, medium and high concentration of the linearity range were prepared (triplicate at each level) by spiking plasma kept in ice with the QC spiking solutions. QC final concentration in plasma was: 200, 2000, 10,000 ng/ml KYN; 1, 10, 50 ng/ml 3HK; 10, 100, 500 ng/ml KYNA and AA. QC standards were frozen for at least 24 h before analysis.

2.5. Sample preparation

Aliquots of 50 μl of calibration standards or QCs were mixed with 200 μl of acetonitrile containing IS (ISWS: ACN with 0.1% formic acid with d5-KYNA (25 ng/ml), d6-KYN (50 ng/ml), 13C6-AA (25 ng/ml) and 15N,13C2-3HK (50 ng/ml)), vortexed, centrifuged (16,000 × *g*, 10 min) and the supernatants were gently evaporated under nitrogen in a heater block at 25 °C. Dried samples were reconstituted with a volume of water containing 0.08% ascorbic acid and after vortex and sonication samples were analyzed by LC–MS/MS.

Sample preparation was repeated on four different days using different pools of rat plasma and was performed manually or with a Hamilton Microlab Star Plus robotic liquid handling system (Hamilton Bonaduz AG, Bonaduz GR, Switzerland).

2.6. Instrumentation

LC–MS/MS was performed using a Waters Acquity UPLC (Waters Corporation, Milford, MA, USA) equipped with a Acquity Binary Solvent Manager, Acquity Sample Manager with a 10 μl sample loop. The LC system was interfaced with an API-6500 triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with a TurboIonSpray ionization source operating in positive ion mode. Analyst™ software version 1.6 (AB Sciex, Toronto, Canada) was used for data acquisition and processing. Metabolites were separated using an Atlantis dC18 column (2.1 mm × 100 mm; 3 μm particle size, Waters), column temperature was maintained at 43 °C and flow rate was 0.5 ml/min. Injection volume was 10 μl. The mobile phases consisted of 0.2% formic acid in water (mobile phase A) and 0.1% formic acid acetonitrile (mobile phase B). Gradient elution was performed using a linear gradient starting at 2% B, holding at 2% B until 0.35 min, increasing linearly to 50% B at 1.6 min, increasing to 90% B at 2.85 min, holding at 90% B until 3.35 min, returning to 2% B at 3.36 min and holding at 2% B until 4.30 min.

Fine tune of the mass spectrometer for each metabolite was performed. After optimization the ion source parameters were: ionspray voltage (IS) 5500 V, source temperature (TEM) 550 °C, curtain gas (CUR) 35 psi, nebulizer gas (GS1) 55 psi, heater gas (GS2) 50 psi, collision gas (CAD) 9 psi. For each analyte the most intense precursor/product transition was selected. Precursor ions and MRM transitions used were: KYN *m/z* 209.1 → 146.2, d6-KYN *m/z* 215.0 → 152.0, KYNA *m/z* 189.9 → 116.1, d5-KYNA *m/z* 194.9 → 121.1, 3HK *m/z* 225.0 → 208.1, 15N,13C2-3HK *m/z* 228.0 → 209.9, AA *m/z* 137.9 → 91.9, 13C6-AA *m/z* 143.9 → 97.9.

Dwell time was 25 ms for 3HK and 15N,13C2-3HK and 10 ms for all the other metabolites and IS.