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Short communication

An analytical method for the measurement of acid metabolites of tryptophan-NAD pathway and related acids in urine

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ABSTRACT

An analytical method has been developed for the measurements of five urinary acids namely, quinolinic acid, picolinic acid, nicotinic acid, 2-pyridylacetic acid and 3-pyridylacetic acid. The high performance liquid chromatograph–electrospray ionization mass spectrometry was operated in positive polarity under selected ion monitoring mode, with a column flow rate of 0.2 ml/min and an injection volume of 20 μl. The method used isotope-labelled picolinic acid (PA-d_4) and nicotinic acid (NA-d_4) as internal standards for the quantification. The sample preparation involved parallel use of two different types of mixed-mode solid phase extraction cartridges (Strata-X-AW for the extraction of quinolinic acid, and Strata-X-C for the remaining acids). Quantitative analysis of five target acids in several human and rat urine samples showed that the levels of acids were relatively uniform among rats while larger variations were observed for human samples.

1. Introduction

Rapid and sensitive measurements of acid metabolites and intermediates in the tryptophan-nicotinamide adenine dinucleotide (NAD) pathway have important contributions to make in clinical and biological research. Quinolinic acid (QA), a metabolite of tryptophan catabolism, is believed to be involved in the pathogenesis of several major inflammatory neurological diseases [1,2]. Picolinic acid (PA), another tryptophan metabolite, is a potent metal-binding ligand with a strong capacity to bind iron and other essential mineral elements [3]. The water soluble vitamin nicotinic acid (NA) is a primary substrate for the biosynthesis of NAD, a cofactor in energy metabolism, through the tryptophan-NAD pathway, and serves also as a therapeutic agent for its lipid-lowering properties [4]. Recent studies have reported that, in addition to elevation in fatty acid β-oxidation, activation of peroxisome proliferators activated receptor α (PPAR-α) also modulated the tryptophan-NAD pathway, probably through the inhibition of the key enzyme 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase [5,6]. As a result, chemicals that are PPAR-α agonists have been reported to induce marked increases in the excretion of QA, NAD and other tryptophan-NAD pathway metabolites [5–8]. The metabolites of tryptophan-NAD pathway including QA, PA and NA were primarily measured in urine to offer a continuous and non-invasive method to study PPAR-α agonist effects *in vivo* including kinetics and mechanisms of action of PPAR-α agonists and to screen chemicals with potential agonist activity.

Although several methods have been developed for the urinary metabolic acids in the tryptophan-NAD pathway [9–24], they are either limited to the analysis of a single acid in urine samples or to the instrumental separation and detection only. The objective of this study was to develop a LC/MS based method for the concurrent measurement of QA, PA and NA in urine samples. We also included 3-pyridylacetic acid (3-PAA) and 2-pyridylacetic acid (2-PAA) in the measurements. Although not being part of the tryptophan-NAD pathway metabolites, 3-PAA [25,26] has been identified as one of the urinary metabolites in rats following ingestion of nuts and grains rich in myosmine and can serve as an indicator of food consumption by rats during the animal experiment [27]. 2-PAA, a major metabolite of vestibular disorders drug betahistine [17], is an isomer of 3-PAA that elutes closely to 3-PAA on the liquid chromatogram. Monitoring 2-PAA in the method will ensure good separation of 3-PAA.

2. Material and methods

Quinolinic acid (QA, 99%), picolinic acid (PA, 99%), nicotinic acid (NA, 98%), 2-pyridylacetic acid hydrochloride (2-PAA, 99%), 3-pyridylacetic acid hydrochloride (3-PAA, 98%), trifluoroacetic acid (TFA) (99%), acetic acid (AA, 99.7%) and ammonium hydroxide

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(NH_3, 28–30%) were purchased from Sigma–Aldrich Company Ltd. (Oakville, ON, Canada). Internal standards PA-d_4 (98 atom %D), and NA-d_4 (98 atom %D) were from C/D/N Inc. (Montreal, QC, Canada). Formic acid (FA, 99%) was from ACROS Organics (New Jersey, USA). OmniSolv acetonitrile and methanol were HPLC grade and purchased from EMD® Chemicals Inc (New Jersey, USA). Water used in the study was in-house generated deionized (DI) water. The Strata-X-C cartridge (200 mg, 33 μm polymeric strong cation mixed-mode solid phase adsorbent) and Strata-X-AW cartridge (200 mg, 33 μm polymeric weak anion mixed-mode solid phase adsorbent) were both from Phenomenex (Torrance, CA, USA).

All stock standard solutions (1 mg/ml) were made in water. Subsequent calibration standard solutions were made from stock solution using 0.3% aqueous TFA. Spiking solution (100 ng/μl) was prepared by diluting 1 mg/ml stock solution with water. Blanks were made of 500 μl water. Lab control samples constituted either (a) 490 μl water and 10 μl of spiking solution for Strata-X-C cartridge for extracting PA, NA, 2-PAA and 3-PAA, or (b) 400 μl water and 100 μl of spiking solution for Strata-X-AW cartridge for extracting QA.

Human urine samples were donated by volunteers and rat urine samples were the control samples (rats that were fed with diet only) from an in-house animal study. The samples were thoroughly mixed by vortex, followed by centrifuging for 2 min at 1800 rpm. Finally 500 μl of the upper layer solution was pipetted into a 1.5-ml Eppendorf vial and mixed with 500 μl 3% aqueous FA by vortex.

The extraction of QA from urine samples was carried out using Strata-X-AW cartridges. The cartridge was conditioned with 4 ml of methanol and 4 ml of water prior to sample loading. After sample loading, the cartridge was washed with 4 ml of 1% aqueous FA, followed by 4 ml of methanol. QA was then eluted with 2.5 ml of 3 M aqueous ammonia. The eluate was concentrated to 0.5 ml under a nitrogen stream. After mixing with 50 μl of TFA, 50 μl of 100 ng/μl of the internal standards (PA-d_4 and NA-d_4) and 4.4 ml of water, the resulting solution with a final volume of 5 ml was ready for HPLC–MS analysis.

The extraction of PA, NA, 2-PAA and 3-PAA from urine samples was performed using Strata-X-C cartridges. The cartridge was conditioned with 2 ml of methanol, 2 ml of 1:1 mixture of methanol and 50% aqueous AA, and 2 ml of 50% aqueous AA prior to sample loading. The loaded cartridge was washed with 4 ml of water, followed by 4 ml of methanol. The acids were then eluted by 2.5 ml of 3 M aqueous ammonia. The eluate was concentrated to 0.5 ml under a nitrogen stream. After mixing with 20 μl of TFA and 5 μl of 100 ng/μl of the internal standards (PA-d_4 and NA-d_4), the resulting solution with a final volume of 0.5 ml was ready for HPLC–MS analysis. After the determination of PA and NA, the extracts were diluted 10 times to determine the levels of PAAs.

Table 1
Time profile of HPLC mobile phase.

Time (min)	A (0.3% TFA in water) (%)	B (0.3% TFA in acetonitrile) (%)	C (water) (%)
0	100	0	0
7	95	5	0
7.5	61	5	34
14	61	5	34
14.5	0	100	0
25	0	100	0
26	100	0	0
46	100	0	0

An Agilent 1100 series LC/MS (Agilent Technologies Inc., Waldbronn, Germany) equipped with a Primesep® 100 analytical column (5 μm, 150 mm × 2.1 mm) (SIELC Technologies, Inc., Prospect Heights, IL, USA) was used for the analysis. Column temperature was set at 30 °C. Injection volume was 20 μl. Elution was performed at a flow rate of 0.20 ml/min using a mobile system consisting of solutions A (0.3% TFA in water), B (0.3% TFA in acetonitrile) and C (water). The time profile of the HPLC mobile phase is listed in Table 1.

The MS was operated in positive ion mode in selected ion monitoring (SIM) modes (m/z: 168 for QA, 124 for NA and PA, 128 for NA-d_4 and PA-d_4, and 138 for 2-PAA and 3-PAA). The values of the fragmentor, step size, and gain, were set at 50, 0.1 and 3.0, respectively. The MS capillary voltage was maintained at 3500 V. Nitrogen was used as the nebulizer gas at 30 psi and the drying gas was delivered at 10 l/min.

Daily instrument calibration (from 0.02 to 62.5 ng/μl) was run at the beginning of each batch. The calibration curve was derived from the concentration ratios of standard over internal standard versus the peak area ratios according to the following equation: $C_s/C_i = a \times (A_s/A_i)^b$, where C_s is the concentration of the standard and C_i the internal standard; A_s and A_i are peak areas of the standard and the internal standard, respectively; a and b are constants determined from the regression. PA-d_4 was used for quantifying QA and PA while NA-d_4 for the rest of the three acids. For samples, the concentration of the target analyte (C_s) can be calculated from its peak area (A_s), response of the inter-

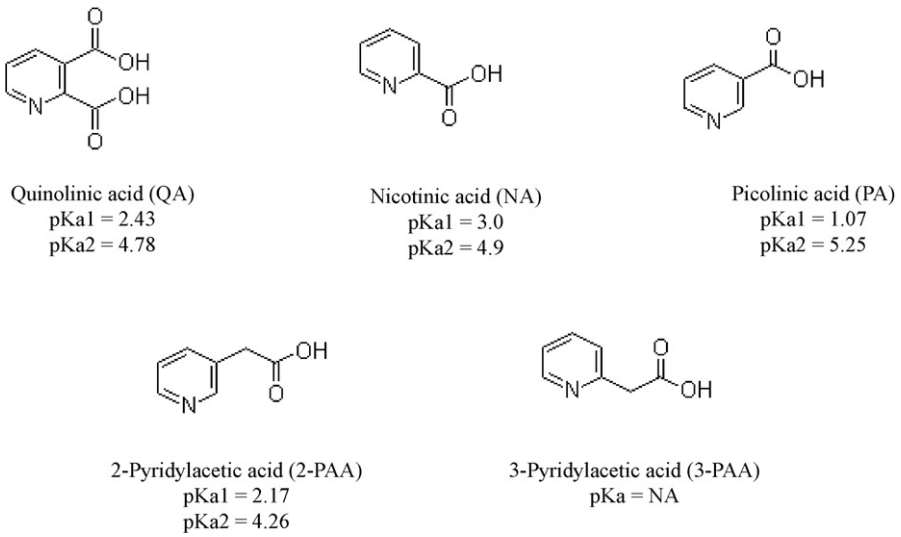

Fig. 1. Structures and pKa values of target acids.