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Quantitation of the niacin metabolites 1-methylnicotinamide and l-methyl-2-pyridone-5-carboxamide in random spot urine samples, by ion-pairing reverse-phase HPLC with UV detection, and the implications for the use of spot urine samples in the assessment of niacin status

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Abstract

A simple ion-pairing reverse-phase HPLC method, with UV diode array detection, was developed and validated for quantitation of the urinary niacin metabolites 1-methylnicotinamide and l-methyl-2-pyridone-5-carboxamide in a single run. Urine samples were purified using a polymer-based mixed mode anion exchange reverse-phase cartridge. Analysis was performed on a reverse-phase C18 column, using a methanol gradient elution system, containing phosphate buffer pH 7.0, 1-heptanesulphonic acid as the ion-pairing agent and trimethylamine as a modifier. The assay was applied to the measurement of the niacin status of two subjects using spot urine samples. The samples were collected over 4 consecutive days and at four time points during 1 day. Status, expressed as the concentration ratios (2-PYR or 1-MN)/creatinine and 2-PYR/l-MN, varied within and between days and was least for fasting samples. This work illustrates the potential of spot urine sampling for niacin status assessment, but highlights the need for further validation prior to its use in field nutritional surveys.

Keywords: Niacin; Metabolism; Human; Urine; Spot sampling; 1-Methylnicotinamide; l-Methyl-2-pyridone-5-carboxamide; HPLC

1. Introduction

Pellagra is a disease caused by a diet deficient in niacin (the generic name for nicotinic acid and nicotinamide) and tryptophan, or by an inability to absorb and process these nutrients. Metabolism of dietary tryptophan, via the kynurenine pathway [1], leads to the formation of niacin. The process is not very efficient (a conversion rate of 60 mg tryptophan to 1 mg niacin is usually assumed), however given the amounts of tryptophan found in some foods; it can be an important source. Zinc, iron, riboflavin and vitamin B6 dependent enzymes are involved in the pathway and deficiencies in these may also contribute to pellagra. Although pellagra is rare nowadays, mainly due to awareness and food fortification, it remains an issue in situations where food is not fortified and diets are restricted, for example in disaster situations [2,3]. In addition, there is increasing interest in niacin status due to its possible association with HIV related conditions [4] and cancer [5]. Clinical signs are only apparent when deficiency is well advanced, are initially difficult to identify, and include changes to the skin, gastrointestinal tract and nervous system. As a consequence, when individuals with pellagra are identified in a population, they usually represent a far greater underlying public health problem and prompt action is required. In order to determine the extent and severity of deficiency, suitable biochemical methods are required to survey at-risk populations. Niacin status is currently assessed by quantification of the major urinary metabolites 1-methylnicotinamide (1-

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MN) and l-methyl-2-pyridone-5-carboxamide (2-PYR) and sometimes the more minor metabolite 1-methyl-4-pyridone-3-carboxamide [3,6,7]. Status is usually expressed as the concentration ratio 2-PYR/l-MN or as the concentrations of the individual metabolites relative to creatinine. Both 2 and 24 h urine collections [8] have been used, however in field studies these are often not practical due to compliance issues, the difficulty of re-finding individuals and households, and the costs associated with solving these problems. Random spot urine sampling, together with the measurement of 1-MN and 2-PYR concentrations, has been suggested as an alternative, as it avoids these issues, and would provide a guide to status [2,7]. However, no studies have actually used random spot sampling for niacin status assessment in populations. In order to investigate the utility of spot urine sampling, a simple HPLC method was developed and validated for the analysis of 2-PYR and 1-MN in urine. The literature contains a number of methods for the analysis of the separate niacin metabolites; however there are very few straightforward assays in which both metabolites are analysed in the same run. The advantages of this assay are that it is simple, uses commercially available purification cartridges and gives baseline resolution for both metabolites. The method was applied to the analysis of spot urine samples collected from two subjects on consecutive days and at regular intervals during 1 day. The relationship between the metabolites and niacin status is discussed.

2. Experimental

2.1. Reagents and standards

Standard 1-methylnicotinamide chloride and 1-methylnicotinamide iodide were purchased from Sigma–Aldrich Company Ltd. (Poole, UK). Standard 2-PYR, was synthesized (Appendix B) by the oxidation of 1-methylnicotinamide iodide [9]. Ion-pairing agent, 1-heptanesulphonic acid sodium salt, was purchased from VWR Int. Ltd. (Lutterworth, UK). All other chemicals were of analytical grade and were purchased from a variety of suppliers.

2.2. Instrumentation and chromatographic conditions

HPLC was performed on a Jasco LC-1500 series system [Jasco (UK) Ltd., Great Dunmow, Essex, UK] incorporating two PU-1580 pumps, an AS-1555-10 autosampler, a CO-1560 column thermostat, a HG-980-30 high pressure mixer, a MD-1510 diode array detector and an in-line DG-980-50 degasser. Analysis was performed using a Luna C18(2) (particle size 5 μm, pore diameter 100 Å) 150 mm × 4.6 mm column, protected with a C18 (ODS) 4(L) × 3.0(D) mm guard cartridge (Phenomenex UK Ltd., Macclesfield, UK) and maintained at 25 °C. All buffers were filtered under reduced pressure, through a 0.2 μm filter (Anodisc 47, Whatman, Maidstone, UK), prior to use. Purified urine sample or standard (25 μL) was injected onto the column. Elution was performed at a flow rate of 1 mL/min using a binary system consisting of solution (A) (10 mmol/L pH 7.0 sodium phosphate buffer, containing 7.00 mmol/L sodium 1-heptanesulphonic acid and 4.00 mmol/L trimethylamine hydrochloride) and solution (B) [20%, v/v methanol in solution (A)]. The column was eluted isocratically with 5% B for 10 min and with a linear gradient from 5 to 40% B over a further 15 min. The column was cleaned by increasing B to 100% over 5 min and re-equilibrated by decreasing B to 0% over 5 min and holding at 0% B for 10 min. The total run time was 45 min. Eluate was monitored at 195–650 nm and integrated at 265 nm.

2.3. Standard curve preparation

Concentrated standard solutions of 2-PYR (6.6 mmol/L) and 1-methylnicotinamide chloride (1-MNC1; 5.8 mmol/L) were prepared in water, stored at −80 °C, and diluted in mobile phase (A) prior to analysis. Standard curves were prepared for 1-MN and 2-PYR over the range from 0.3 to 579 μmol/L and 0.3 to 657 μmol/L, respectively. Curves were fitted using the Lorentzian robust minimization (Table Curve 2D, Systat Software Inc., Richmond, CA, USA) procedure.

2.4. Urine sample collection

Urine samples were provided by subjects in good health, who consumed western style omnivorous diets and some of whom took vitamin supplements. Samples were collected in mid-flow, in sterile containers without preservative, aliquoted and stored frozen at −80 °C. A pooled urine sample from seven subjects was used for developmental and validation studies. The pool was generated by combining equal volumes of urine from each subject. Stability studies were conducted with urine samples from three subjects. To monitor changes in the output of niacin metabolites with time, urine samples were collected from two subjects over 4 consecutive days. On each day, an overnight fasting urine sample was collected early in the morning and on day 4, additional non-fasting urine samples were collected at 3-h intervals over 9 h (total of three additional samples). Any use of vitamin supplements was noted.

2.5. Purification of urine samples for analysis

Each urine sample was vortex mixed, an aliquot (1.00 mL) taken and adjusted to pH 7.4–8.5 by addition of microlitre aliquots of 1 mol/L NaOH. Following each addition, the sample was remixed and the pH measured by addition of 5 μL to a pH indicator strip (VWR International Ltd., Lutterworth, Leicestershire, UK). The sample was transferred to a preconditioned [methanol (2 mL) and water (3 mL)] mixed-mode anion exchange-reversed phase 3 mL 60 mg Oasis MAX extraction cartridge (Waters Ltd., Elstree, Herts, UK), eluted (0.5 mL/min) under reduced pressure and the eluate collected. The cartridge was eluted with water (1 mL), to ensure that all