



Validation of HPLC-UV Method for Analysis of 4-Pyridoxic Acid in Human Urine

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ABSTRACT

We present a simple and validated HPLC method for measuring Vitamin B6 urinary metabolite 4-pyridoxic acid (4-PA). Urine samples were treated with 6% perchloric acid. HPLC separation was achieved using Waters column (Symmetry® C18 250 mm × 4.6 mm i.d (5 μM). The mobile phase was a mixture of methanol and 35 mM sodium phosphate buffer containing 2.5 mM sodium heptane polysulfonate at pH=3.5 with 85% O-phosphoric acid. Retention times were 8.0 min for 4-pyridoxic acid. Detection (UV at 302 nm) of analyte was linear in a range from 0.0125 to 0.8 μM. This method is stable, reproducible and has practical advantages such as ease and low cost.

Keywords: Vitamin B6, Validation, 4-pyridoxic acid, HPLC, Urine

INTRODUCTION

Vitamin B6 is a water-soluble vitamin that exists predominantly in the chemical form of pyridoxine. The biologically active form is its metabolite pyridoxal 5'-phosphate (P5P). The end catabolic product of vitamin B6 is the urinary 4-pyridoxic acid (4-PA).

Vitamin B6 once converted by pyridoxal kinase to its active metabolite (P5P) is involved in more than 140 biochemical reactions. P5P is referred to as the activity precursor of vitamin B6. Pyridoxal 5 phosphate is highly protein bound and ultimately converted to inactive 4-pyridoxic acid that is excreted into urine.

Different analytical methods are reported in the literature for the assay of vitamin B6 metabolites in plasma and urine including spectrophotometry, fluorimetry, HPLC. Several analytical methods were reported utilizing fluorimetry detection either directly or through chemical modification to improve sensitivity of detection. The methods varied between pre-column or post column derivatization [1-5]. Other methods utilized the different coulometric detection [6].

The aim of this work was to develop new and validated, simple and reproducible method allowing the estimation of the major urinary metabolite of vitamin B6 in human urine.

MATERIALS AND METHODS

Reagents

The compound 4-pyridoxic acid (4-PA) was purchased from Sigma (St. Louis, MO, USA). Methanol, sodium heptane sulfonate, and orthophosphoric acid 85%, were purchased from Fluka. Sodium phosphate monobasic, sodium phosphate dibasic, animal charcoal and perchloric acid were purchased from Fischer. Reagent-grade water obtained from a Millipore Milli-Q system was used throughout the experiments. All other reagents were of analytical grade.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a Waters 2695 separation module and Waters 2998 photodiode array detector. A reversed-phase column Waters Symmetry® C18 250 mm × 4.6 mm i.d (5 μM) was used to separate analytes. The gradient mobile phase consists of A: methanol and B: 35 mM sodium phosphate buffer containing 2.5 mM ion-pairing

sodium heptane polysulfonate at pH=3.5 with orthophosphoric acid 85%. The buffer was sonicated, filtered and degassed prior to run. The flow-rate was 1 ml/min for a 10-minute run time. The temperatures of column were held at 25°C. Injection volume was 50 μ L. The detector was set at 302nm.

Preparation of Solutions

Stock solution of 4-pyridoxic acid (1 mM) was prepared dissolving the drug in water. Stock solution was further diluted with charcoal pre-treated urine to working standard solutions ranging from 0.0125 μ M to 0.8 μ M. All solutions were stored at -80°C to simulate the storage conditions of the study samples and were stable for at least 2 months. Quality control samples were prepared in a separate weighing of 4-PA.

Sample Preparation

All urine samples were treated by 1:1 6% perchloric acid to precipitate proteins and impurities. After mixing (30s on a vortex mixer) and centrifugation (5 min at 10000 \times g), the supernatant was transferred to another 5 min at 10000 \times g. Then, 100- μ l aliquots of supernatant were transferred to HPLC vials and 50 μ l of this mixture was injected into the HPLC system.

RESULTS AND DISCUSSION

Validation of the method was performed according to latest FDA guidelines [7].

Selectivity and Sensitivity

Six blank urine obtained from six different sources were analyzed according to the procedure previously described, in order to evaluate method specificity.

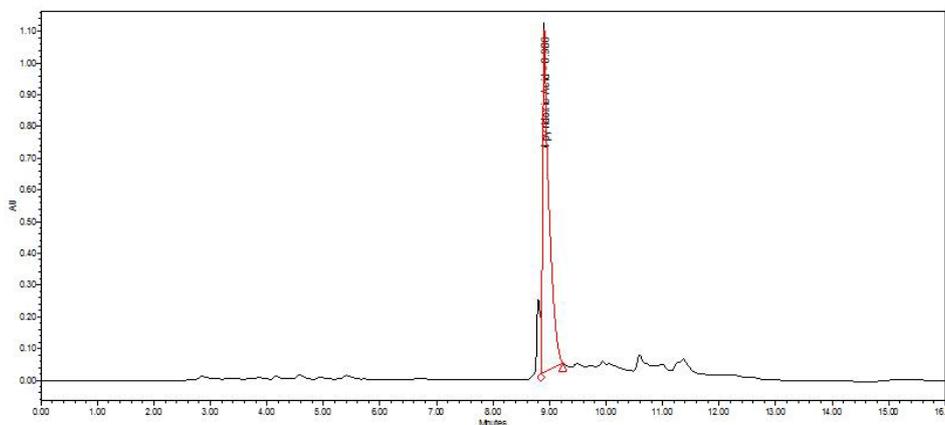


Figure 1 Chromatogram of 4-pyridoxic acid urine (0.8 μ M)

HPLC-UV peak areas detected at the retention times of the analytes of interest were measured; calibration curves equations were used to determine 'virtual' mean analyte concentrations (Figure 1).

Linearity

Matrix-based calibration standards, in the range of 0.0125 μ M - 0.8 μ M, were independently prepared and analyzed, in three different days (lowest standard in triplicate). For each curve, the absolute peak areas of the analyte (4-pyridoxic acid) were plotted against the nominal concentrations. Calibration curves were generated by plotting Area (y-axis) with concentration (x-axis) (Figure 2).

Correlation coefficient of linear fit curve for 4-pyridoxic acid obtained from data was greater than 0.99. The accuracy and precision data from back calculated calibration standards demonstrate suitability of the calibration method. Deviations from the nominal concentrations and CV values were from 1 to 16% and 3.8 to 17.5% for all concentration levels respectively.

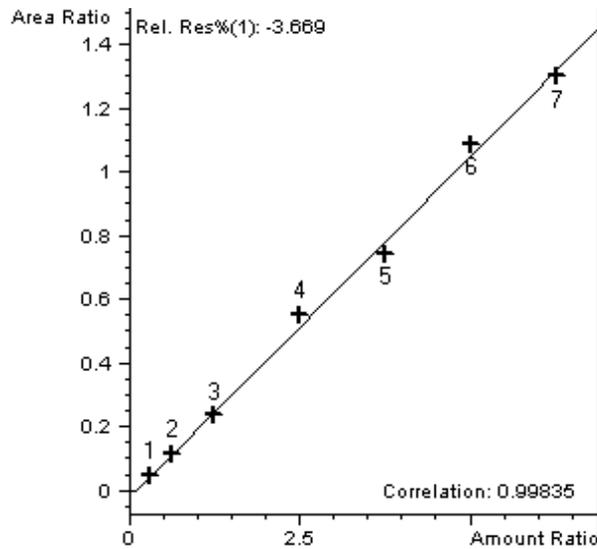


Figure 2 Calibration curve of 4-pyridoxic acid in urine

The accuracy and precision of the analytical method were evaluated by analyzing quality control samples at three concentration levels (QCH 0.5 μM, QCM 0.25 μM, QCL 0.05 μM). Six of both QCH and QCM level and twelve of QCL level were analyzed daily for three runs. The calculated mean concentration was used to express accuracy (% deviation):

$$\% \text{ deviation} = \frac{(\text{Calculated concentration value} - \text{Reference concentration})}{\text{Reference Concentration}} \times 100$$

Means, standard deviations and coefficients of variation were calculated from QC values and used to estimate the intra- and inter-day precision:

$$\% \text{ Coefficient of Variation} = \frac{\text{Standard deviation of calculated Concentrations}}{\text{Mean of calculated concentrations}} \times 100$$

The obtained values for accuracy and precision were within FDA requirements. The mean intra- and inter-day accuracy and precision for the back calculated concentrations for QCM and QCH samples were within 15% and for QCL samples were within 20% (Tables 1 and 2).

Table 1 Recovery, accuracy, and intra-day precision

Theoretical concentration (ng/ml)	Calculated concentration ng/ml ± SD	Precision CV%	Accuracy %	Recovery % ± SD
25	26.286 (1.73)	7.12	5.1	86.1 (3.29)
50	51.543 (1.70)	3.18	3.1	94.7 (2.91)
100	107.33 (0.33)	0.3	7.3	99.9 (1.80)
200	196.75 (2.30)	1.18	-1.6	90.2 (5.61)
300	297.57 (5.01)	1.69	-0.8	91.3 (1.40)
400	384.47 (3.88)	1.02	-3.9	81.1 (0.90)
500	518.50 (7.82)	1.49	3.7	89.8 (1.87)

Table 2 Recovery, accuracy, and inter-day precision

Theoretical concentration (ng/ml)	Calculated concentration ng/ml ± SD	Precision CV %	Accuracy %	Recovery % ± SD
25	23.959 (1.68)	7.12	-4.2	92.6 (3.32)
50	54.536 (1.75)	3.18	9.1	98.6 (2.99)
100	107.72 (0.38)	0.3	7.7	99.1 (1.92)
200	193.61 (2.42)	1.18	-3.2	98.3 (5.58)
300	301.16 (5.11)	1.69	0.4	92.4 (1.25)
400	382.65 (3.98)	1.02	-4.3	82.2 (1.12)
500	528.94 (7.91)	1.49	5.8	90.6 (1.83)

Carry-over

Carry-over was evaluated by placing vials of blank mobile phase at different locations in the analysis set specially after the highest standard and QC. There was no carry-over evident in any of the blank reagent samples proved by absence of the peaks in chromatogram.

Recovery

Matrix recovery of 4-PA from urine was assessed by spiking additional analyte into water at the same concentrations of the quality controls. Two spiked samples of each concentration (QCL and QCH) were analyzed using standard curves generated from urine based standards as described previously. Matrix recovery after extraction was compared with original concentration, which was defined as 100%. Means, standard deviations and coefficients of variation were calculated. QCL recovery from urine was about 76% vs 104% from water and QCH recovery was about the same from both water and urine samples.

Stability

Bench-top short-term stability after 1 h and 4 h at room temperature was studied to verify if 4-pyridoxic acid degrade over the course of analyses. Short-term stability was evaluated by analyzing triplicate of both QCL and QCH kept at room temperature before the extraction step.

The autosampler stability was evaluated by leaving triplicates of both QCL and QCH samples for 24 h.

Long-term stability was studied in order to ensure that 4-pyridoxic acid in urine samples does not undergo degradation in the storage conditions before being analyzed. Hence, long-term stability was studied on triplicates of matrix-based QC's (QCL and QCH) stored at -80°C for 3 months.

In addition, Triplicate samples of both QCL and QCH after 3 thaw-freeze cycles were analyzed to evaluate stability.

The stability of analytes (expressed as percentage recovery) at room temperature for 1 and 4 hours, in the autosampler for 24 hours, in the storage conditions after 3 months, and after 3-thaw-freeze cycles was evaluated by comparing the back calculated concentration to that of the nominal concentration.

The obtained results, show that 4-pyridoxic acid is stable under tested conditions without a relevant loss. All recovery values were within acceptable range.

Dilution Analysis

Individual samples were prepared for the linearity of dilution analysis at a concentration of 2x HQC. Aliquots of the samples were then diluted 1:1, 1:2, and 1:4 with blank urine prior to analysis. Each dilution level was processed in triplicate and back calculated against the reference concentration. The mean accuracy and precision obtained for all dilution levels was within $\pm 15\%$ according to FDA guidelines.

CONCLUSION

An analytical methodology for quantification of 4-pyridoxic acid in urine is described. Full validation according to the FDA guidelines was performed, and the limit of quantification level of $0.0125\ \mu\text{M}$ was reached for 4-pyridoxic acid, with accuracy and precision levels within FDA requirements.

This method is suitable for the estimation of 4-pyridoxic acid in urine samples as a precursor of vitamin B6 status. It can be useful as a non-invasive technique to monitor vitamin B6 metabolites especially in patients who may experience deficiency due to high rate of degradation like in small bowel transplantation.

DECLARATIONS**Conflict of Interest**

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

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