A Simple Liquid-Chromatographic Method for Measuring Vitamin B₆ Compounds in Plasma

Paul Edwards, Peter K. S. Liu, and G. Alan Rose

This relatively simple high-performance liquid chromatographic (HPLC) method for measuring all seven known forms of vitamin B₆ in plasma from individuals supplemented with pyridoxine hydrochloride shows good analytical recovery (85–98%) and precision. Within-run and between-run CVs for plasmas supplemented with standards were 4% and 7%, respectively. The major forms of B₆ found in unsupplemented plasma from normal subjects were pyridoxal phosphate and 4-pyridoxic acid, with pyridoxal just detectable. The HPLC procedure correlated well (r = 0.94) with a modification of an enzymatic method involving apotryptophanase (Anal Biochem 1972;45:567–76) for measuring plasma pyridoxal phosphate, and also (r = 0.94) with a routine method for determining 4-pyridoxic acid in urine (Clin Chem 1984;10:479–89). Elimination of pyridoxine from the plasma of both normal and hyperoxaluric individuals was shown to be very rapid, with half-lives (t₁/₂) of 45 and 40 min, respectively. Finally, we present evidence for the existence of two other forms of B₆ and discuss the possibility of a new metabolic pathway in vitamin B₆ metabolism.

Additional Keyphrases: hyperoxaluria • urine • metabolism • pyridoxal phosphate • reference values

"Vitamin B₆" is the family name for a group of 3-hydroxy-2-methyl-pyridine derivatives exhibiting the same vitamin activity as pyridoxine. This family includes pyridoxal, pyridoxine, pyridoxamine, their respective 5'-phosphate esters, and 4-pyridoxic acid. Their chemical properties differ widely, from very acidic to very basic. Thus, especially when one considers the extremely low concentrations found in plasma (nanomoles per liter or less), the task of measuring these vitamins becomes quite formidable. Although microbiological assays, enzyme assays, radioimmunoassay, and various chemical assays are available (1–10 and reviewed in 11) for quantifying certain forms of the vitamin, none of these methods is really suitable for metabolic studies. Several high-performance liquid-chromatographic (HPLC) procedures for assay of vitamin B₆ have been reported, but they were either too insensitive for plasma assays or are rather complicated and non-reproducible (11–13).

Recently, Coburn and Mahuren (14) reported a cation-exchange HPLC procedure, involving gradient elution and post-column bisulfite derivatization followed by fluorimetric detection, that was suitable for quantifying B₆ compounds in plasma. This method was subsequently reproduced by Lui et al. (15), who used it to simultaneously measure six or more B₆ compounds in human plasma.

This HPLC method is substantially superior to previously reported methods and appears to work well with plasma, but it takes about 50 min to complete a run and it involves gradient elution. Here we report a much-simplified reversed-phase isocratic HPLC procedure based on the bisulfite derivatization technique of Coburn and Mahuren (14) for measuring all the seven known forms of vitamin B₆ in human plasma. Using this new methodology, we investigated the metabolic conversion of pyridoxine in human plasma after oral supplementation and present preliminary results here that suggest the presence of at least two other forms of vitamin B₆. We also compared the HPLC method with an enzymatic assay (apotryptophanase method) for determination of pyridoxal phosphate.

**Materials and Methods**

**Chemicals**

Pyridoxal phosphate, pyridoxal, pyridoxamine, pyridoxamine phosphate, pyridoxine, and pyridoxic acid were all obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Pyridoxine phosphate was kindly donated by Dr. Stephen Coburn (Fort Wayne State Development Center, Fort Wayne, IN 46815). Reagent grade potassium dihydrogen orthophosphate, perchloric acid, sodium metabisulphite, sodium perchlorate, triethanolamine, and methanol were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. "HPLC grade" acetonitrile was obtained from May & Baker Ltd., Dagenham, U.K.

**Preparation of Blood Samples**

For reference plasma PLP values, 10–12-mL specimens of blood were collected from 21 apparently healthy volunteers (16 men, five women), with potassium EDTA as anticoagulant. Blood was also sampled from normal and hyperoxaluric individuals who had been receiving pyridoxine supplementation (from 25 to 800 mg/day) for various periods of time.

To determine the biological half-life of pyridoxine in blood, we collected and assayed blood specimens from normal and hyperoxaluric volunteers at various times after a single oral dose (200–400 mg) of pyridoxine hydrochloride. The blood specimens were centrifuged without delay and the plasmas were separated and either used promptly or quickly frozen and stored at −20 °C. Before the plasma samples were injected onto the HPLC column the proteins were precipitated by adding 4 mmol/L perchloric acid (50 μL per milliliter of plasma), then removed by filtering through HVP 01300 filters (Millipore Corp., Bedford, MA 01730).

**Analytical-Recovery Studies**

To determine the recovery of pyridoxal phosphate and other B₆ compounds from blood plasma, we added water or a known quantity of a standard mixture of the compounds to individual or pooled plasma samples before protein precipitation, then took these samples through the assay procedure.

Recovery of a particular compound was calculated as follows: [(compound in supplemented plasma − compound in unsupplemented plasma)/amount of compound added to the sample] × 100.
Apotryptophanase Assay

The apotryptophanase assay reported here is a modification of the procedure of Haskell and Snell (4). In this assay, pyruvate generated by the apotryptophanase reaction is coupled to NADH in the presence of lactate dehydrogenase (EC 1.1.1.27) to form lactate and NAD\(^+\). The decrease in absorbance at 340 nm as NADH is consumed is recorded directly in a reaction-rate analyzer (LKB, Bromma, Sweden). Full details of the modified apotryptophanase assay will appear elsewhere (16).

HPLC Instrumentation and Assay Procedure

For assays of vitamin B\(_6\) in plasma we used an LKB HPLC pump (Model 2150 with titanium parts) fitted with an inert injector (LKB Model 2154-002) and a homemade 500-\(\mu\)L sample loop. A 4.6 \(\times\) 250 mm LKB analytical column packed with 5-\(\mu\)m particles of reversed-phase material (TSK ODS-120T) was used in conjunction with a 3-cm guard column containing similar packing material (either LKB Spherisorb guard or Lichrosorb RP18). The eluent was 75 mmol/L Na\(_2\)HPO\(_4\) buffer containing 75 mmol of NaClO\(_4\), 8.5 mL of acetonitrile, and 0.5 mL of triethanolamine per liter and adjusted to pH 3.38 with concentrated HClO\(_4\). The flow rate was 1.2 mL/min, at a constant pressure of about 12 000 kPa. For postcolumn derivatization of pyridoxal phosphate with bisulfite ions as suggested by Coburn and Mahuren (24) we used a Braun syringe pump connected to a homemade tee junction and reaction coil. The postcolumn reagent consisted of Na\(_2\)HPO\(_4\) buffer (250 mmol/L, pH 11.7) containing 1 g of sodium metabisulfite per liter. The flow rate of the postcolumn reagent was kept at 6 mL/h. Column effluents were monitored with a Baird Atomic fluorometer (Fluorocord RC200; Baird & Tatlock Ltd., Romford, Essex RM1 1HA, U.K.) or a more sensitive Model L5-20 fluorometer (Perkin-Elmer Ltd., Beaconsfield, Bucks. HP9 1QA, U.K.) equipped with a 50-\(\mu\)L capillary flow cell. Excitation and emission wavelengths were set at 325 and 400 nm, respectively. Although as much as 200 \(\mu\)L of sample can be injected without significant peak broadening, we found that keeping the injection volume at \(\leq\)100 \(\mu\)L minimized baseline drifts and column contamination. Under the above chromatographic conditions, a complete profile of the vitamin B\(_6\) components of plasma can be produced every 25 min. At the end of the work day we first wash the column with de-ionized water, then reverse-flush it with methanol overnight to remove column contaminants.

For assays of 4-pyridoxic acid in urine we used Na\(_2\)HPO\(_4\) buffer (50 mmol/L, pH 7.00) containing 50 mmol of NaClO\(_4\), 0.05 mL of triethanolamine, and 56 mL of acetonitrile per liter. Excitation and emission wavelengths were 320 and 420 nm, respectively. Sample preparation only required dilution with eluent (from 100- to 1000-fold, depending on concentration).

Results

Analytical Variables

**HPLC separation of vitamin B\(_6\) standards.** With the HPLC procedure described above, all seven recognized forms of vitamin B\(_6\) were clearly resolved down to baseline (Figure 1).

**Calibration.** The HPLC system is calibrated by injecting known quantities of vitamin B\(_6\) and measuring the resulting peak area, either by triangulation or with a Merck Hitachi D-2000 chromato-integrator (BDH Ltd., Dagenham, Essex, U.K.). The detection limit of the procedure, as performed with the Baird RC-200 fluorimeter, for the various B\(_6\) compounds is about 0.5 ng injected. Using a series of single injections for a range of B\(_6\) vitamins (2.5-15 ng injected), we obtained the following information on assay linearity: for 4-pyridoxic acid, \(r = 0.999\) (slope = 0.25, y-intercept = 0.32); for pyridoxine, \(r = 0.998\) (slope = 0.09, y-intercept = 0.63); for pyridoxal phosphate, \(r = 0.974\) (slope = 0.04, y-intercept = 0.99); for pyridoxal, \(r = 0.995\) (slope = 0.07, y-intercept = 0.61); for pyridoxine phosphate, \(r = 0.993\) (slope = 0.09, y-intercept = 0.24); for pyridoxine, \(r = 0.996\) (slope = 0.12, y-intercept = 0.40), and for pyridoxamine phosphate, \(r = 0.996\) (slope = 0.12, y-intercept = 0.24). The units for the y-axis were integrated peak areas in arbitrary units.

**Recovery of B\(_6\) vitamers from plasma.** Table 1 shows recovery data for the individual B\(_6\) compounds. Mean recoveries ranged from 85% to 98%. Concentrations of pyridoxal phosphate, pyridoxamine, pyridoxine phosphate, and pyridoxine in normal plasma were below the limit of detection, so for purposes of calculation their concentrations in unsupplemented plasma were assumed to be zero. Although recoveries were good for plasma pyridoxal phosphate in this study and that of others (5), its extraction by perchloric acid proved more variable than that of other B\(_6\) forms, probably because of the protein-binding properties of pyridoxal phosphate. Therefore, to calculate pyridoxal phosphate concentrations, we must correct results for the recovery in each sample.

**Precision.** The within-run CV of this HPLC assay for the

---

**Table 1. Analytical Recoveries (Percent) of B\(_6\) Compounds Added to Pooled Specimen of Plasma (Nine Replicates)**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>PL</th>
<th>PLP</th>
<th>PM</th>
<th>PMP</th>
<th>PN</th>
<th>PNP</th>
<th>4-PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>87.0</td>
<td>89.3</td>
<td>97.3</td>
<td>100.0</td>
<td>87.0</td>
<td>97.6</td>
<td>87.1</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>97.6</td>
<td>87.1</td>
<td>91.3</td>
<td>90.2</td>
<td>87.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>87.0</td>
<td>96.4</td>
<td>97.3</td>
<td>100.0</td>
<td>91.3</td>
<td>97.6</td>
<td>90.3</td>
<td></td>
</tr>
<tr>
<td>82.6</td>
<td>92.9</td>
<td>97.3</td>
<td>100.0</td>
<td>85.7</td>
<td>85.1</td>
<td>90.3</td>
<td></td>
</tr>
<tr>
<td>82.6</td>
<td>92.9</td>
<td>94.6</td>
<td>95.4</td>
<td>91.3</td>
<td>92.7</td>
<td>87.1</td>
<td></td>
</tr>
<tr>
<td>87.0</td>
<td>98.3</td>
<td>94.6</td>
<td>100.0</td>
<td>91.3</td>
<td>92.7</td>
<td>90.3</td>
<td></td>
</tr>
<tr>
<td>87.0</td>
<td>98.3</td>
<td>94.6</td>
<td>97.7</td>
<td>91.3</td>
<td>92.7</td>
<td>87.1</td>
<td></td>
</tr>
<tr>
<td>87.0</td>
<td>96.4</td>
<td>97.3</td>
<td>97.7</td>
<td>91.3</td>
<td>95.1</td>
<td>89.9</td>
<td></td>
</tr>
<tr>
<td>87.0</td>
<td>96.4</td>
<td>94.6</td>
<td>97.7</td>
<td>91.3</td>
<td>97.8</td>
<td>83.9</td>
<td></td>
</tr>
</tbody>
</table>

Mean 85.1 92.5 95.8 96.2 91.3 94.6 87.5

*Abbreviations as in legend in Figure 1.*
various B₈ compounds was 4% for plasmas supplemented with standards, the between-run CV about 7% (n = 6 in each case).

The between-run CVs for B₈ vitamers in normal plasma was 7% for pyridoxal phosphate, about 16% for 4-pyridoxic acid, and 22% for pyridoxal (n = 6 for each compound). The high CVs for 4-pyridoxic acid and pyridoxal in normal plasma samples are attributable to their very low concentrations (usually <30 nmol/L).

Correlation studies. Concentrations of pyridoxal phosphate in plasma from pyridoxine-supplemented and unsupplemented individuals by the HPLC and apotryptophanase methods were compared (Figure 2). Results agree well over a wide concentration range (r = 0.94, n = 52).

In a similar correlation study, concentrations of urinary 4-pyridoxic acid were measured by both the HPLC and a routine fluorimetric method (19). Again, values for 4-pyridoxic acid in urine agreed well (Figure 2, r = 0.97, n = 37).

Clinical Studies

Plasma B₈ profile for a normal subject. Figure 3 depicts a typical B₈ chromatogram obtained for plasma from an apparently healthy volunteer who was taking no vitamin supplement. The major peaks observed in normal plasma were pyridoxal phosphate and 4-pyridoxic acid, with pyridoxal sometimes detectable and no other B₈ forms detected. Little or no interference was seen in these deproteinized samples.

Reference values for plasma pyridoxal phosphate. For 29 plasma samples from a group of 21 apparently healthy individuals, as determined by the present method, the reference range for pyridoxal phosphate was 5–33 μg/L (20–134 nmol/L), the mean 17 μg/L (69 nmol/L), and the SD 7 μg/L (28 nmol/L).

For seven samples from five women the range was 12–30 μg/L (49–121 nmol/L), and the mean ± 19 (SD 7) μg/L (65, SD 28 nmol/L). The differences between the sexes was non-significant by unpaired, two-tailed Student's t-test.

The values agree well with both the apotryptophanase assay and those already reported in the literature (3, 5, 14, 17).

Elimination of pyridoxine from the plasma of normal and hyperoxaluric individuals. In a preliminary study, we compared the rates of elimination of pyridoxine from blood plasma of hyperoxaluric and normal individuals after an oral dose of 200 to 400 mg of pyridoxine hydrochloride. Figure 4 shows the plasma elimination curve for pyridoxine for an apparently healthy volunteer after a single 400-mg oral dose of pyridoxine hydrochloride. As shown, once the pyridoxine enters the circulation it is eliminated very rapidly, with a half-life of about 45 min. We repeated the experiment on another normal volunteer who received a single 200-mg oral dose of pyridoxine hydrochloride, finding the half-life to be just less than 40 min. Similar examination of plasma from a patient with mild metabolic hyperoxaluria (17) after a single 200-mg oral dose (Figure 4) showed a similar rate of elimination (t½ = 40 min). This patient was taking 20 mg of pyridoxine hydrochloride per day before this experiment.

Plasma B₈ profiles for individuals on pyridoxine supplementation. Figure 5A shows the plasma B₈ profile for a patient with primary hyperoxaluria who was taking 800 mg of pyridoxine hydrochloride per day, orally. In addition to the major metabolites pyridoxal, pyridoxine, 4-pyridoxic acid, and to a much lesser extent pyridoxal phosphate, two other additional peaks, designated 1 and 2, were seen. They appeared about an hour after the last vitamin supplement but had disappeared from samples collected 2 h later. We believe these two peaks to be previously unrecognized metabolites of pyridoxine.

Figure 5B shows a plasma B₈ profile for a normal volunteer who took 800 mg of pyridoxine hydrochloride per day for one week. This blood sample was taken about 4 h after the last dose. Again, the putative B₈ metabolite (peak 1) appeared, with peak 2 absent on this occasion, probably owing to its complete conversion to other metabolic forms.

Peaks corresponding to these two new B₈ metabolites were consistently seen in the profiles of plasma from normal and hyperoxaluric individuals who were taking 400 mg of pyridoxine hydrochloride or more per day.

A separate HPLC experiment demonstrated that the pyridoxine hydrochloride tablets used in this study were about 99% pure, so it is highly unlikely that peaks 1 and 2 are ascribable to impurities in the tablets.

Discussion

Until recently there were no satisfactory methods for simultaneously measuring all the seven known forms of
Our CV for all the B6 compounds was <4% and ~7% between runs for plasma samples supplemented with B6 standards. The between-run CV for the B6 vitamers at concentrations found in normal plasma was 7% for pyridoxal phosphate, higher for 4-pyridoxic acid and pyridoxidal.

The concentration of the various components and the pH of the elution buffer are factors critical to the effective separation of all the known B6 compounds, as well as the two unknowns. A pH difference of 0.05 unit will determine whether unknown peak 1 is resolved from the pyridoxal phosphate peak. The concentration of the ion-pairing reagent, sodium perchlorate, is also important and should be kept the same as that of the dihydrogen phosphate buffer for effective separation of the fast-eluting compounds (pyridoxamine and pyridoxamine phosphate). It is also necessary to readjust the pH of the eluent for optimum resolution when a new column is used.

We have shown here that, normally, the predominant B6 metabolites in plasma are pyridoxal phosphate, 4-pyridoxic acid, and, to a lesser extent, pyridoxidal, with no other forms being detected. This agrees with other reports (11, 14, 15).

The normal reference interval for plasma pyridoxal phosphate obtained in this study agrees well with values obtained by various other methods (3, 5, 11, 14) and with an enzymatic method developed in this laboratory for measurement of pyridoxal phosphate in plasma (16).

Our observation that the rate of pyridoxine elimination from the blood of a patient with mild metabolic hyperoxaluria was almost identical to that found in two normal subjects suggests that the link between an increased vitamin B6 requirement and hyperoxaluria (17, 18) is not attributable to an abnormality in the handling of this compound.

An abnormality in the interconversion or rate of removal of other B6 metabolites in hyperoxaluric individuals is currently being investigated.

Pyridoxal-3-sulfate, pyridoxine-3-sulfate, and N-methylpyridoxine have been identified in the urine of cats (20), and other minor metabolites are present in human urine (21). Our finding of the two new metabolites of pyridoxine in human plasma raises the question of what they might be. Pyridoxine has methanol groups attached to two adjoining points on the heterocyclic ring. Only one is known to undergo phosphorylation; the other is oxidized to the aldehyde or aminated. Could the roles of these two methanol groups be reversed, so giving rise to a series of metabolites,

---

**Fig. 3. Vitamin B6 profile for plasma from a normal volunteer who was on a non-vitamin-supplemented diet**

**Fig. 4. Plasma elimination curves for pyridoxine in plasma from (top) a normal volunteer after a single 400-mg oral dose of the vitamin, and (bottom) mild hyperoxaluric patient after a single 200-mg oral dose of vitamin B6 in biological fluids. The method of Coburn and Mahuren (14), an ion-exchange HPLC method involving gradient elution and bisulfite derivatization, for measuring these compounds in human serum is very useful, but it has obvious disadvantages.**

The HPLC system described here is isocratic and is therefore much simpler than previous reported gradient systems (14, 15). The method has been successfully applied to plasma, giving good sensitivity and excellent resolution with negligible interference in plasma extracts. With a running time of 20–25 min per B6 sample, up to 20 B6 analyses can easily be performed during a working day, even without the use of an autosampler.

Our analytical recovery of the various B6 vitamers from plasma is comparable with that for earlier methods involving deproteination with trichloroacetic acid (14, 15), but the present method is more convenient because the perchloric acid used in deproteination need not be removed. Like other investigators (5, 14), we have found on some occasions that recovery of added pyridoxal phosphate from plasma is incomplete; binding to protein may be the cause.

---

244 CLINICAL CHEMISTRY, Vol. 35, No. 2, 1989
perhaps including an isomer of pyridoxic acid? If so, instead of seven $B_6$ compounds there could be 13, although some of them might be too transient to be found. These new metabolites were only seen in individuals who were receiving doses of pyridoxine hydrochloride exceeding 200 mg/day, whether they were normal subjects or patients with primary hyperoxaluria and mild metabolic hyperoxaluria.

We have successfully applied the present procedure to urine for the measurement of 4-pyridoxic acid. One recent report (15) had shown urine to pose a difficult analytical problem for measurement of other $B_6$ metabolites, owing to interference from other compounds. We have also noticed this to be a problem for urine, especially with the earlier-eluting compounds.

We thank Dr. Stephen Coburn (Fort Wayne State Hospital and Training Center, Fort Wayne, IN) for a sample of pure pyridoxine phosphate. The HPLC equipment was provided by the St. Peter's Research Trust.

References