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Automation and Analytical Techniques

Multianalyte Quantification of Vitamin B_6 and B_2 Species in the Nanomolar Range in Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry

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Background: **Homocysteine, a risk factor of cardiovascular disease, cognitive disorders, and pregnancy complications, exists at a point of metabolic convergence of several B vitamins, including vitamins B_6 and B_2 (riboflavin). Measurement of the various forms of these vitamins may be useful for the study of hyperhomocysteinemia as well as for the assessment of vitamin status.**
Methods: **Plasma (60 μL) was deproteinized by mixing with an equal volume of 50 g/L trichloroacetic acid that contained d_2-pyridoxal 5′-phosphate, d_3-pyridoxal, and d_8-riboflavin as internal standards. Pyridoxal (PL), pyridoxal 5′-phosphate (PLP), pyridoxine (PN), pyridoxine 5′-phosphate, pyridoxamine (PM), pyridoxamine 5′-phosphate, 4-pyridoxic acid (PA), riboflavin, flavin mononucleotide (FMN), and FAD were separated on a C_8 reversed-phase column, which was developed with an acetonitrile gradient in a buffer containing acetic acid and heptafluorobutyric acid. The analytes were detected by tandem mass spectrometry in the positive-ion mode.**
Results: **The chromatographic run lasted 8 min. Within- and between-day CVs were 3%–20% and 6%–22%, respectively, and recoveries were 78%–163%. Limits of detection (signal-to-noise ratio = 5) were in the range 0.1–4.0 nmol/L, and the response was linear over several orders of magnitude. In samples from 94 healthy persons, we obtained median concentrations (nmol/L) of 35.4 for PLP, 16.9 for PL, 22.4 for PA, 10.3 for riboflavin, 7.5 for FMN, and 63.1 for FAD. PN and PM were also detected in some cardiovascular patients taking B_6 supplements.**
Conclusions: **This method based on liquid chromatography–tandem mass spectrometry measures all known plasma forms of vitamins B_6 and B_2, which span a wide range of polarity. The assay is characterized by simple sample processing with no derivatization, low sample volume requirement, and a short run time.**
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Vitamins B_6 and B_2 are involved in the metabolism of homocysteine *(1)*, a risk factor for occlusive vascular disease, Alzheimer disease, and adverse pregnancy outcomes *(2)*. Vitamin B_6 serves as cofactor for cystathionine β-synthase and cystathionine γ-lyase, which convert homocysteine to cystathionine and then to cysteine *(1)*, whereas vitamin B_2 modifies the phenotypic expression of the commonly occurring methylenetetrahydrofolate reductase (*MTHFR*) 677C→T polymorphism and, thus, plasma concentrations of total homocysteine *(3, 4)*. The metabolic pathways involving vitamin B_6, vitamin B_2, and homocysteine are illustrated in Fig. S-1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue7/.

Vitamin B_6 exists in 7 forms: pyridoxine (PN),[1] pyridoxine 5′-phosphate (PNP), pyridoxal (PL), pyridoxal 5′-phosphate (PLP), pyridoxamine (PM), pyridoxamine 5′-phosphate (PMP), and the catabolite, 4-pyridoxic acid (PA; Fig. 1). PLP is the metabolically active form and is involved in ∼100 enzymatic reactions. At present, it has

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[1] Nonstandard abbreviations: PN, pyridoxine; PNP, pyridoxine 5′-phosphate; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PA, 4-pyridoxic acid; FMN, flavin mononucleotide; LC-MS/MS, liquid chromatography–tandem mass spectrometry; HFBA, heptafluorobutyric acid; TCA, trichloroacetic acid; MRM, multiple-reaction monitoring; LOD, limit(s) of detection; and S/N, signal-to-noise.

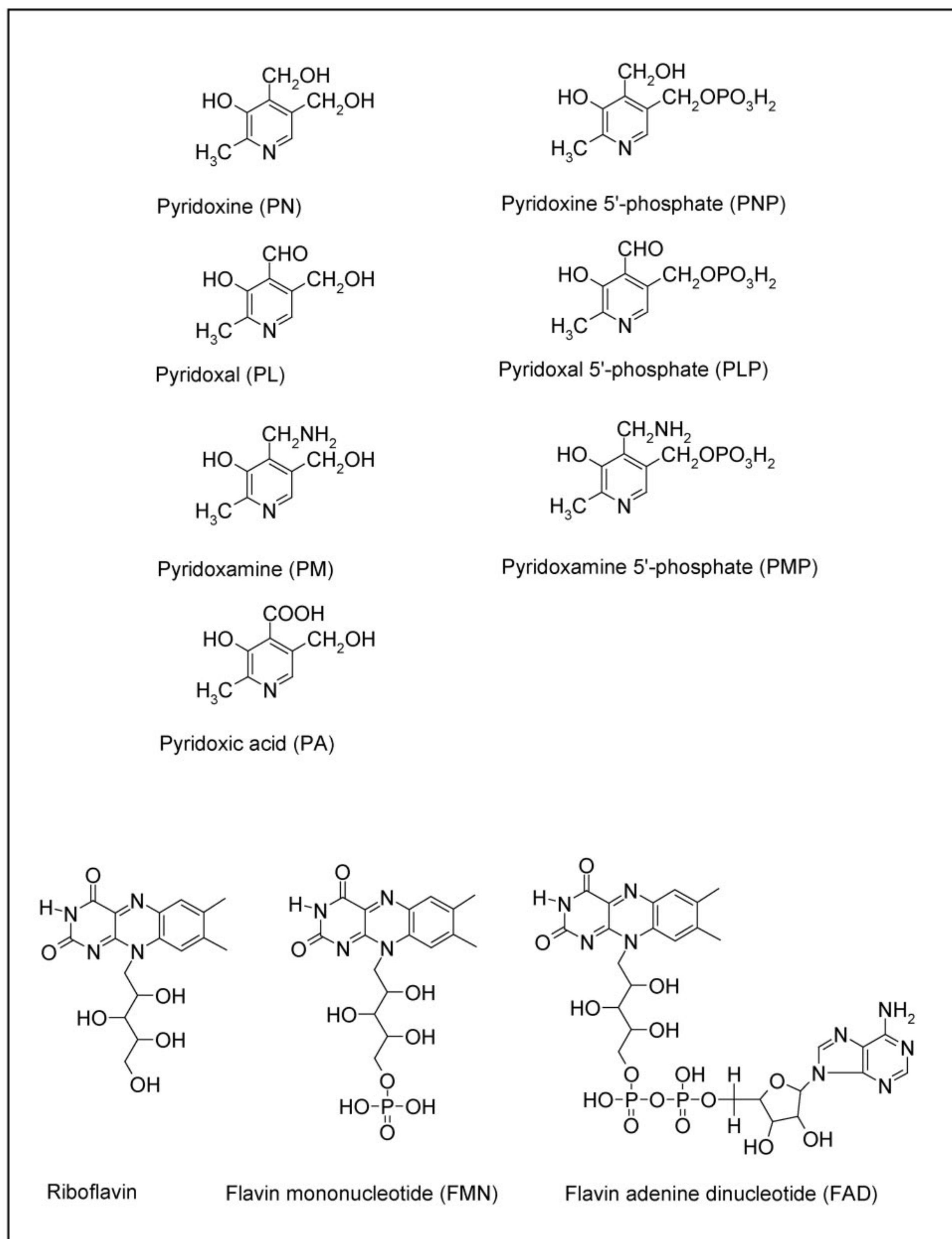

Fig. 1. Chemical structures of vitamin B_6 and B_2 species.

not been established which B_6 vitamer(s) should be used as indicator of B_6 status *(5)*, but several indices have been suggested, including plasma PLP *(6, 7)*, the combinations plasma PLP and PL *(8)*, or plasma PLP plus PA *(5, 9, 10)*.

Riboflavin serves as a precursor for flavin mononucleotide (FMN) and FAD *(11)*. These coenzymes are involved in numerous reduction–oxidation reactions and take part in the metabolism of other vitamins, e.g., vitamin B_6 and folate (Fig. S-1 of the online Data Supplement). Plasma riboflavin appears to be a better indicator of vitamin B_2 status than are plasma FMN and FAD *(12)*.

Measurement of vitamins B_6 and B_2 has become important for the assessment of homocysteine status *(2)*. Overt vitamin B_6 deficiency is regarded as uncommon *(6)*, but marginal deficiency may be related to an increased risk of cardiovascular disease *(13, 14)*. Vitamin B_2 deficiency may be more common than previously recognized, particularly in developing countries *(15–17)*, among the elderly, in children, and in women during pregnancy *(11)*.

Several methods for the determination of various B_6 vitamers in human plasma or serum have been developed *(9, 18–29)*, but at present no rapid and simple method exists for the determination of all 7 forms of vitamin B_6. Vitamin B_6 methods are usually based on HPLC with fluorescence detection, which requires derivatization of PLP and complete chromatographic separation of the analytes. This often gives relatively long analysis times of ~30 min or longer *(18, 19, 23, 25–27)*. In studies involving vitamin B_6 in plasma, usually only PLP was determined *(20, 30–34)*, but in some cases *(5, 10, 29, 35, 36)*, several B_6 vitamers were measured.

Analytical techniques for the determination of vitamin B_2 concentrations include fluorometric *(37)*, liquid chromatographic *(38, 39)*, and capillary electrophoretic methods *(40)*. Most HPLC methods have been designed for the detection of high concentrations of vitamin B_2 in food, pharmaceutical preparations, and urine *(38)*. Some methods have been used for measurement of B_2 in whole blood *(41–44)*, serum *(42, 43, 45)*, or plasma *(39, 40, 46–48)*.

We here describe a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the simultaneous measurement of 7 vitamin B_6 species and 3 vitamin B_2 species in 60 μL of plasma. The assay is sufficiently sensitive to quantify nanomolar concentrations of these vitamers.

Materials and Methods

MATERIALS

PL hydrochloride (99%), PLP (98%), PN hydrochloride (>99%), PM dihydrochloride (98%), PA (>99%), riboflavin (>98%), FMN (sodium salt; ~95%), and FAD (disodium dihydrate salt; >95%) were purchased from Sigma-Aldrich. PMP (98%) and heptafluorobutyric acid (HFBA; 99.5%) were purchased from Fluka, and PNP was kindly supplied as a gift from C.J. Argoudelis, Department of Food Science and Human Nutrition, University of Illinois-Urbana Champaign (Urbana, IL). Acetic acid (99.8%) and trichloroacetic acid (TCA; 99.5%) were from Merck, and acetonitrile (99.8%) was from SDS. Three deuterated species were used as internal standards. d_3-PL (99%) and d_8-riboflavin (98.3%) were synthesized by Beta Chem (Leawood, KS), and d_2-PLP (95%) was kindly supplied as a gift by S.P. Coburn, Department of Chemistry, Indiana University–Purdue University (Fort Wayne, IN). Doubly deionized water was used.

All vitamer calibrators were checked (using this assay) for the presence of other vitamin B_6 or vitamin B_2 forms. None were detected except in FAD, which contained ~3% FMN. Likewise, no nondeuterated vitamers were detected in the deuterated internal standards d_2-PLP, d_3-PL, and d_8-riboflavin.

For routine assay calibration, we used plasma pooled from 4 persons (not taking B-vitamin supplements). The vitamer concentrations (nmol/L), as determined by this assay, were <4 for PMP, 87 for PLP, <0.2 for PNP, 35 for PA, 15 for PL, <0.1 for PN, <0.4 for PM, 71 for FAD, 8.3 for FMN, and 9.7 for riboflavin. To a fraction of this plasma pool we added 20 nmol/L each of PL, PN, PM, PNP, and riboflavin; 50 nmol/L each of PMP, PA, FMN, and FAD; and 100 nmol/L PLP. The calibration samples were aliquoted and stored at −80 °C. The calibrators with added vitamins were prepared freshly at regular intervals of 14 days over 2 years. A constant ratio between endogenous and added vitamers indicated stability of the endogenous vitamins during storage.

For the matrix effect experiments, we obtained plasma from 9 healthy persons. For the recovery and precision experiments, we used plasma from a healthy blood donor, obtained at the blood bank at Haukeland University Hospital. Endogenous vitamer concentrations (nmol/L) were determined by this assay to be 59 for PLP, 14 for PA, 11 for PL, 60 for FAD, 8.4 for FMN, and 14 for riboflavin. The other vitamers were not detected in this plasma.

SAMPLE COLLECTION AND PROCESSING

EDTA plasma was obtained by collecting blood into Vacutainer Tubes (Becton Dickinson); the final EDTA concentration was 4 mmol/L. The samples were immediately put on ice and centrifuged (at 2000*g* for 10 min at 4 °C) within 60 min. The plasma was then processed further or stored at −80 °C until use.

Proteins were precipitated by mixing 60 μL of plasma with an equal volume of TCA in water (50 g/L) containing the deuterated internal standards (100 nmol/L each of d_2-PLP and d_3-PL and 200 nmol/L d_8-riboflavin). The precipitation step was carried out by a robotic workstation (Plato 7; RoSyst Anthos). The solution was mixed for 30 s by the robot, left for 60 min on ice, and finally centrifuged at 6000*g* for 12 min. The supernatant (65 μL) was transferred to a new vial, which was placed in a cooled (4 °C) autosampler. The samples were protected from light.

instrumentation

A series 1100 HPLC system (Agilent Technologies) equipped with a thermostated autosampler and a degasser was used for solvent delivery and sample introduction. The HPLC system was coupled to an API 4000 triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX) outfitted with an electrospray ion source. A column switcher from Valco (type VMHA) was used to divert the flow to waste during the first 2.0 min and the last 2.2 min of the run. Analyst (Ver. 1.3.1; Applied Biosystems/MDS SCIEX) was used for data acquisition and analysis.

lc-ms/ms

We injected 50 μL of deproteinized plasma into an Agilent stable-bond C_8 reversed-phase column [150 × 4.6 (i.d.) mm; particle size, 3.5 μm] equipped with an Agilent stable-bond C_8 guard column [12.5 × 4.6 (i.d.) mm; particle size, 5 μm]. The guard and analytical columns were mounted in a thermostated column compartment set at 20 °C. The flow rate was 1 mL/min.

The mobile phase consisted of 3 components, solution A (650 mmol/L acetic acid), solution B (100 mmol/L HFBA in A), and solution C (900 mL/L acetonitrile in water).

The column was developed with gradient elution according to the following timetable: 0–0.2 min (97.5% A and 2.5% B), 6 min (28% A, 2.5% B, and 69.5% C), 6.1–6.6 min (2.5% B and 97.5% C), 6.7–8 min (97.5% A and 2.5% B). All gradient steps were linear.

Samples were injected every 8.75 min. The column effluent was delivered to the mass spectrometer with no split.

Acquisition settings were optimized by infusion of a 10 μmol/L solution of each vitamer at a rate of 1 μL/min. Before entering the mass spectrometer, this solution was mixed at a T-junction with mobile phase delivered at a rate of 1 mL/min and with a composition corresponding to the time of elution of the actual analyte. The optimized conditions were adjusted according to 3 scan segments to acquire a sufficient signal quality, which is related to the number of data points across each chromatographic peak. The settings are detailed in Table 1. Curtain gas (10 psig), collision gas (4 psig), ion source temperature (490 °C), ion source gases 1 and 2 (10 psig), and activated interface heater were identical for all segments.

The mass spectrometer was operated in the positive-ion mode. The vitamers were detected in the multiple-reaction monitoring (MRM) mode with unit resolution at both Q1 and Q3. The ion transitions for all analytes are shown in Table 1, which lists the protonated molecular ion $[M+H]^+$ and the dominant product ion. Table 1 also shows the division of the chromatographic cycle into 3 scan segments, each of which encompassed 2–8 ion pairs.

For quantification, we included d_2-PLP, d_3-PL, and d_8-riboflavin as internal standards. The responses of PMP, PLP, and PNP were calculated as the ratios between the signal intensity areas for the vitamers divided by the area for d_2-PLP. Likewise, d_3-PL was used for calculating the area ratios for PA, PL, PN, and PM, and d_8-riboflavin for the vitamin B_2 species.

assessment of matrix effects

We investigated the matrix effects *(49)*, which can be observed as ion enhancement or ion suppression *(50)*, in plasma by measuring the peak intensities of all B_6 and B_2 vitamers in 9 different lots of plasma enriched with 30 nmol/L each of PN, PNP, PL, PM, riboflavin, and FMN and 150 nmol/L each of PLP, PMP, PA, and FAD after the

Table 1. Retention times, molecular weights, and optimized time-scheduled instrument settings.

Analyte	t_r,[a] min	M_r	Transitions, m/z: Parent ion	Transitions, m/z: Product ion	DT, ms	IS, V	DP, V	CE, V	CXP, V
Scan segment 1 (0–3.5 min)									
PMP	2.4	248.2	249.3	232.1	100	4500	41	19	6
PLP	2.6	247.2	248.1	149.9	100	4500	90	24	12
d_2-PLP	2.6	249.2	250.1	151.9	100	4500	90	24	12
PNP	2.9	249.2	250.2	134.1	100	4500	56	29	12
Scan segment 2 (3.5–5.1 min)									
PA	4.3	183.2	184.1	148.0	50	5500	61	31	12
FAD	4.3	785.5	786.3	348.0	50	5500	106	31	12
PL	4.5	167.1	168.1	150.1	100	4500	51	17	14
d_3-PL	4.5	170.1	171.1	153.1	100	4500	51	17	14
FMN	4.6	456.3	457.4	358.9/359.3[b]	100	5500	100	23	14
PN	4.7	169.2	170.1	134.1	50	5500	66	29	10
PM	4.9	168.2	169.3	134.1	100	4500	46	29	14
Scan segment 3 (5.1–8 min)									
RF	5.2	376.4	377.4	243.3	50	4500	90	30	6
d_8-RF	5.2	384.4	385.4	251.1	50	4500	90	30	6

[a] t_r, retention time; DT, dwell time; IS, ion spray; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; RF, riboflavin.
[b] For FMN, we used 2 product ions to increase the number of data points over the peak.

protein precipitation step. The increases in peak intensities of the vitamers were compared with the respective intensities measured in an aqueous calibrator solution to which the same concentrations of analytes had been added. The matrix effect was calculated as:

Matrix effect (%) =

$$\frac{\text{Peak area (added)} - \text{peak area (endogenous)}}{\text{Peak area (aqueous solution)}} \times 100$$

LINEARITY AND LIMITS OF DETECTION

It was impossible to obtain plasma devoid of all B_6 and B_2 vitamers by dialysis, as reported previously for PLP *(20)*. We therefore prepared solutions containing 40 g/L albumin in phosphate-buffered saline, to which all vitamers were added to obtain final concentrations in the range 0.1–400 nmol/L. The albumin–phosphate-buffered saline solutions were used to assess linear ranges and limits of detection (LOD).

The signal-to-noise (S/N) ratio for each ion pair was calculated as S/N = (peak height − baseline)/SD (baseline), using the S/N script supplied by Applied Biosystems (Analyst Ver. 1.3.1). LOD were determined from the linearity data as the lowest concentrations that gave peaks with S/N ratios of 5.

RECOVERY AND WITHIN- AND BETWEEN-DAY CVS

The plasma was divided into 3 portions, and to 2 of these portions we added medium (10 nmol/L each of PN, PNP, PL, PM, riboflavin, and FMN and 50 nmol/L each of PLP, PMP, PA, and FAD) and high (30 nmol/L each of PN, PNP, PL, PM, riboflavin, and FMN and 150 nmol/L each of PLP, PMP, PA, and FAD) concentrations of vitamers. We analyzed 24 replicates at each vitamin concentration (low, medium, and high) in 1 analytical run, and recovery was calculated according to the formula:

$$\text{Recovery (\%)} = \frac{\text{Measured concentration} - \text{endogenous concentration}}{\text{Added concentration}} \times 100$$

We calculated within-day precision of the assay from the data of the recovery experiments and determined the between-day precision by assaying the same samples on 22 different days over a period of 54 days.

PLASMA CONCENTRATIONS OF VITAMINS

EDTA-plasma was collected from 94 nonfasting healthy individuals (35% male) with a median (range) age of 57 (11–93) years. To study the effect of B_6 supplementation, we collected plasma from cardiovascular patients (78% male) with a median (range) age of 61 (41–76) years, who received PN (40 mg/day; n = 30) or placebo (n = 30) for 1 month.

Results and Discussion

We have developed a high-throughput LC-MS/MS method for the measurement of 7 vitamin B_6 and 3 vitamin B_2 species in 60 μL of plasma. The sample processing and analyte extraction involved a simple protein precipitation step with an equal volume of TCA, which was carried out by a robotic workstation.

PROTEIN PRECIPITATION AND LIBERATION OF THE ANALYTES

The precipitating agent *(51, 52)* and incubation time *(53)* both affect the efficiency of protein precipitation and analyte extraction. TCA is efficient in precipitating human plasma proteins *(51, 52)*. Circulating PLP is almost entirely protein bound *(54)*, and incubation of the plasma/TCA mixture for 5 min at 50 °C has been recommended to liberate all PLP *(9)*.

Storage of the plasma/TCA mixture for 10 min at room temperature, or for 60 min at 0 °C, provided maximum yields of PLP, PL, and FAD but had no effect on the other vitamers. All analytes were stable during incubation. We used these experiments to establish the routine procedure for sample processing.

CHROMATOGRAPHY

The vitamers included in the assay span a wide range of chemical properties. The B_6 vitamers contain a pyridine ring, whereas all B_2 vitamers have a tricyclic isoalloxazine moiety (Fig. 1). These structural features confer a large range of polarities and variable chromatographic behaviors on the analytes. The highly polarized B_6 species containing phosphate groups were poorly retained by the column, whereas elution of the larger B_2 species required an acetonitrile gradient.

We used a high concentration of acetic acid (650 mmol/L) to lower the pH of the mobile phase without adding TFA or other strong acids, which may suppress ionization *(55)* and thus reduce sensitivity. The mobile phase also contained HFBA, which is a volatile perfluorinated carboxylic acid suitable for LC-MS/MS *(56)*. HFBA served as a counterion and was essential for the retention of PMP on the C_8 column.

The elution profiles of plasma to which all vitamers had been added are shown in Fig. 2, and the retention times are listed in Table 1. In plasma from healthy persons not taking B-vitamin supplements, only PLP, PA, PL, FAD, FMN, and riboflavin were detected (Fig. 2).

MS AND ACQUISITION SETTINGS

The mass spectrometer was used in MRM mode to optimize selectivity and sensitivity. All analytes gave a strong signal from the singly protonated molecule $[M+H]^+$, and we selected the most abundant product ions from the fragmentograms. The selected molecular transitions are listed in Table 1.

The parent–product ion pairs for the nonphosphorylated B_6 species suggest loss of $2 \cdot H_2O$ for PN and PA,

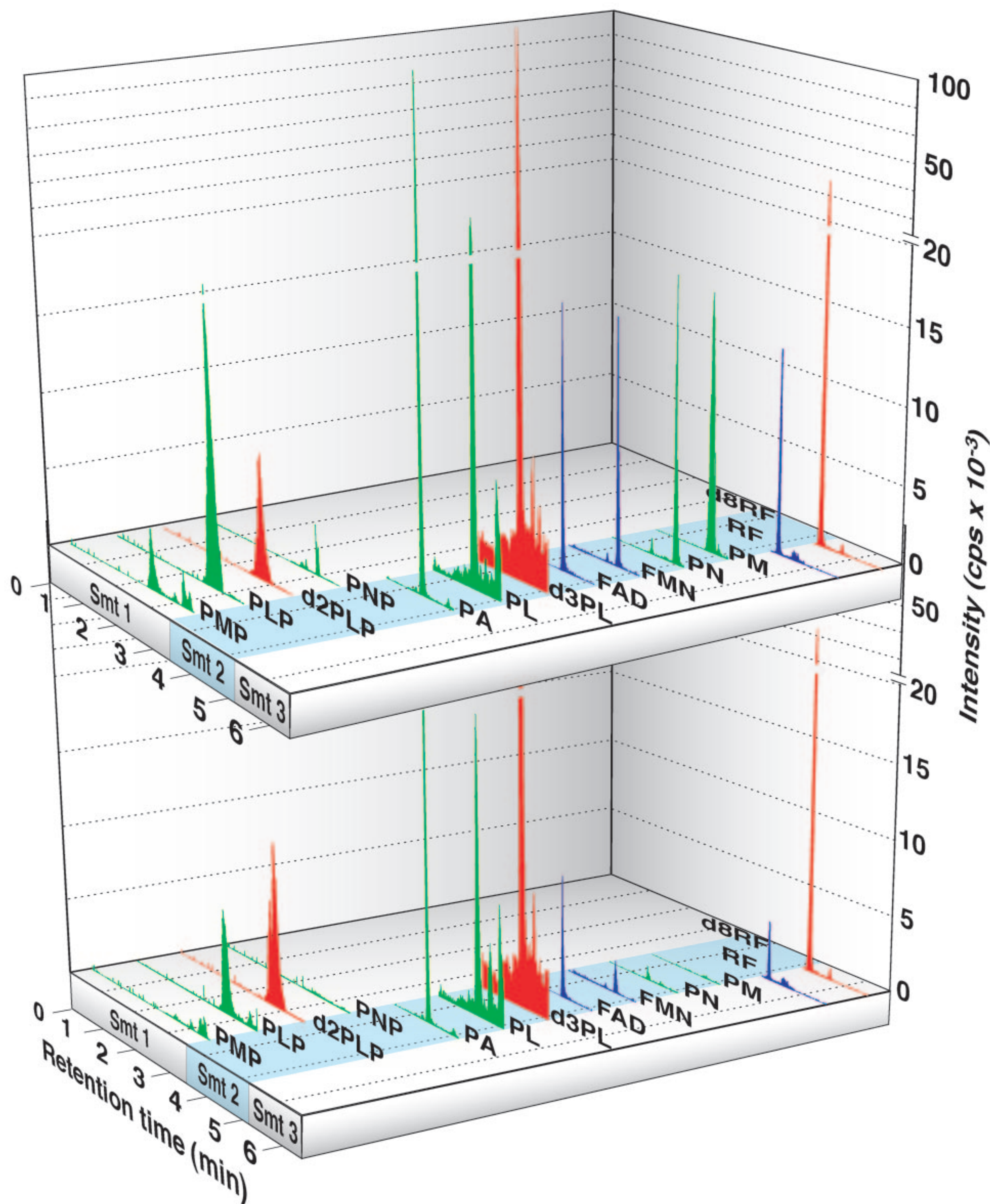

Fig. 2. LC-MS/MS MRM chromatograms of human plasma containing endogenous vitamin B_6 and B_2 (*bottom*) and plasma with added B_6 and B_2 vitamers (*top*).

The traces for the molecular transitions of various forms of vitamin B_6 (PMP, PLP, PNP, PA, PL, PN, and PM) are shown in *green*, vitamin B_2 species (FAD, FMN, and riboflavin) in *blue*, and the deuterated internal standards in *red*. (*Bottom*), native plasma; (*top*), the same plasma with added vitamers: 50 nmol/L PMP, 100 nmol/L PLP, 20 nmol/L PNP, 50 nmol/L PA, 20 nmol/L PL, 20 nmol/L PN, 20 nmol/L PM, 50 nmol/L FAD, 50 nmol/L FMN, and 20 nmol/L riboflavin. *Smt*, scan segment.

loss of H_2O for PL, and NH_3 plus H_2O for PM *(57)*. For the remaining B_6 vitamers, the fragmentograms suggest additional loss of HPO_3 for PNP and PLP and loss of H_2O for PMP compared with the nonphosphorylated B_6 forms. The ion transitions for the B_2 vitamers can be explained by elimination of the ribityl side chain for riboflavin and elimination of H_3PO_4 for FMN *(58)*. The product ion for FAD has the molecular mass of protonated adenylic acid *(58)*.

There was no cross-talk between ion pairs originating from different analytes or between analytes and the corresponding internal standards.

The tandem mass spectrometer allows for optimization of acquisition conditions for each analyte, and we used specific settings for each MRM transition (Table 1). The mass spectrometer also permits skipping between the various MRM channels in time steps of milliseconds. To improve sensitivity, we restricted the number of parallel MRM traces to 8 by dividing each chromatographic cycle into 3 scan segments. The optimized time-scheduled MRM conditions for analyzing 10 vitamers and 3 deuterated internal standards are summarized in Table 1.

MATRIX EFFECTS

The mean (SD) matrix effect for 9 different plasma lots was 32 (2)% for PMP, 114 (10)% for PLP, 16 (2)% for PNP, 106 (9)% for PA, 87 (8) for PL, 48 (9)% for PN, 81 (18)% for PM, 527 (45)% for FAD, 100 (14)% for FMN, and 121 (4)% for riboflavin. Thus, ion suppression was most pronounced for PMP and PNP, whereas substantial ion enhancement was observed for FAD. Notably, the variability, also referred to as "relative matrix effect" *(49)*, was low (<10%) for most analytes.

Both ion suppression and ion enhancement occur in LC-MS/MS *(49, 59–61)*. A substantial matrix effect may contribute to low assay precision and low reproducibility. However, adequate assay performance and analytical recovery may be obtained if the relative matrix effect is low, if the external calibrators are composed in the same matrix as the samples, and if the internal standards correct for the matrix effect. Stable-isotope internal standards should afford optimal correction because the matrix effect is expected to be the same for an analyte and its labeled analog.

INTERNAL STANDARDS

A method based on mass spectrometry allows quantification by isotope dilution with inclusion of deuterated internal standards. This is in principle the ideal strategy to correct for variability in most analytical steps from analyte extraction to detection *(62)*. We obtained 3 deuterated internal standards, d_2-PLP, d_3-PL, and d_8-riboflavin, for the present assay. PMP, PLP, and PNP were corrected against d_2-PLP, the nonphosphorylated B_6 forms against d_3-PL, and vitamin B_2 species against d_8-riboflavin. However, we did not observe better recovery or precision for the vitamers (PLP, PL, and riboflavin) measured with authentic deuterated standards compared with those measured in the absence of such standards (Table S-1 in the online Data Supplement and Table 3).

PERFORMANCE OF THE METHOD

We assessed assay performance in terms of linearity, sensitivity, analytical recovery, and precision.

For estimation of the linear dynamic range, we constructed calibration curves in the range 0.1–400 nmol/L (Fig. 3). Correlation coefficients (r^2) >0.991 were obtained by least-square regression analysis for all compounds (Table 2).

LOD (defined as S/N ratio = 5) were calculated for all vitamers from the linearity experiments, and the results are listed in Table 2. LOD varied from 0.1 nmol/L for PN to 4 nmol/L for PMP and FAD. The LOD values obtained were comparable to those of other B_6 *(20, 25, 27, 29)* and B_2 *(39)* assays.

We investigated the recovery and precision of the assay by adding all vitamers at 2 (medium and high) concentrations to plasma containing endogenous vitamer concentrations.

Recoveries were in the range 78%–163% (Table S-1 of the online Data Supplement). Recoveries in the range 61%–110% have been reported for other vitamin B_6 *(9, 18, 29, 63)* and B_2 assays *(39, 40)*. Thus, recoveries that differ from 100% are a common finding. One possible explanation for this may be uneven distribution of the analytes between the protein pellet and the supernatant. This may affect the accuracy of the assay but has minor effects on precision as long as the variability is low.

The mean within-day CVs were <10% for PLP, PL, PA, and riboflavin; 10%–15% for PN, PM, FAD, and FMN; and 15%–20% for PMP and PNP (Table 3). The between-day CVs were in general somewhat higher than within-day CVs (Table 3).

CVs were highest for PNP and PMP (Table 3). These were the compounds with the highest ion suppression. Thus, the low precision could be related to the relative matrix effect, which was not totally corrected by the internal standard, d_2-PLP.

For published B_6 assays, there are often no data on assay imprecision, or the imprecision reported varies widely, from 0.6%–5.6% *(29)*, 2%–6% *(9, 20)*, and 4%–12% *(27)*, to 7%–67% *(18)*.

The precision of the vitamin B_2 measurements seems to be somewhat lower than that of our vitamin B_2 capillary electrophoresis assay *(40)*, but better than the precision reported for an HPLC assay *(47)*.

STABILITY

All B_6 vitamers except PNP have been reported to be photosensitive and unstable in aqueous solutions *(64–67)*. In plasma, PLP is unstable at room temperature *(68)* but is stable at low temperatures (−30 °C) *(69)*. FAD degrades at high TCA concentrations *(40)*.

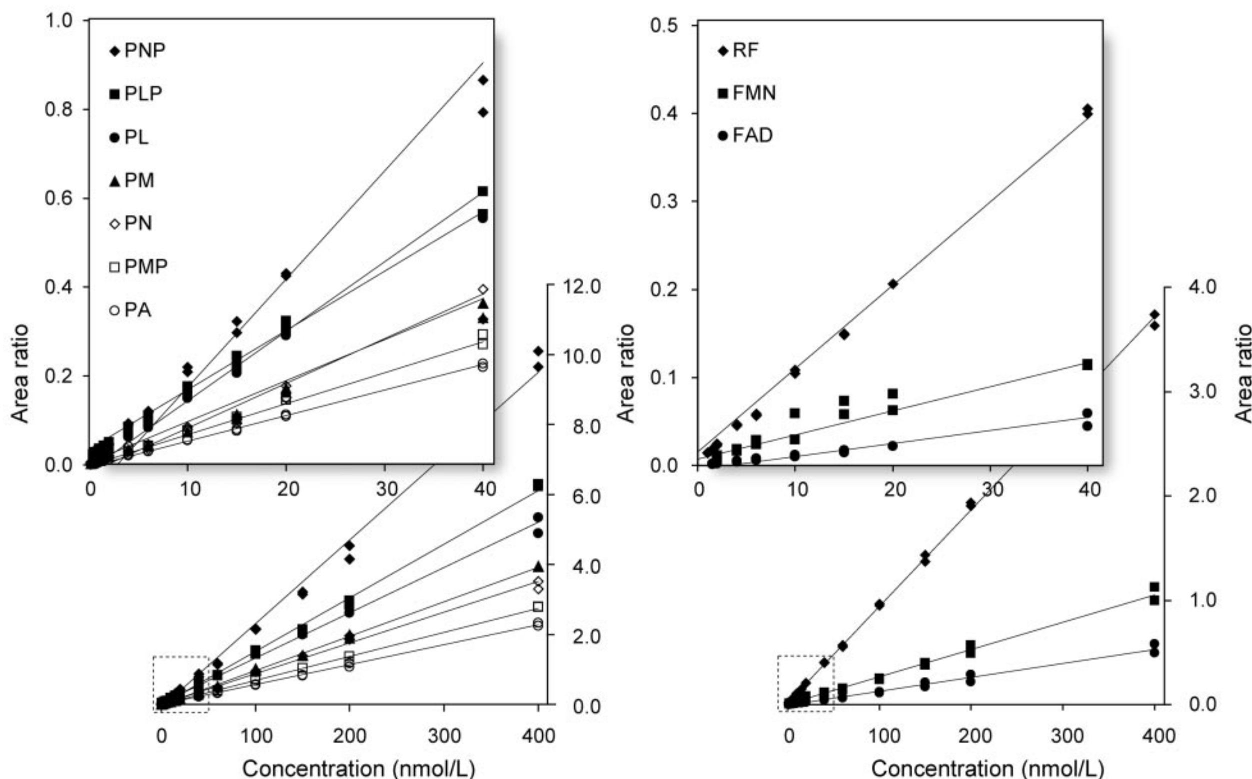

Fig. 3. Linear dynamic ranges for the B_6 and B_2 vitamers.

The curves for the B_6 vitamers are shown to the *left*, and those for the B_2 vitamers to the *right*. The low-concentration regions are shown enlarged in the *insets*. *RF*, riboflavin.

The aqueous stock solutions of vitamers; aqueous solutions of d_2-PLP, d_3-PL, and d_8-riboflavin, and the EDTA-plasma used as calibrators were aliquoted and stored at −80 °C. Under these conditions, we observed no degradation of the vitamers over a period of 2 years.

For protein precipitation, we chose a final TCA concentration of 25 g/L because at higher concentrations, we observed loss of FAD and a concurrent increase in FMN. After protein precipitation, the pH of the sample was ~1. We observed no instability for any B_6 or B_2 species in this acidic supernatant kept in subdued light at 4 °C for up to 96 h (data not shown). Thus, the B_6 and B_2 vitamers are stable under the storage and sample handling conditions used for this assay.

PLASMA CONCENTRATIONS OF VITAMIN B_6 AND B_2 SPECIES

We analyzed vitamins B_6 and B_2 in 94 healthy individuals (35% male) with a mean (range) age of 57 (11–93) years. Median vitamer concentrations (nmol/L) were 35.4 for PLP, 22.4 for PA, 16.9 for PL, 63.1 for FAD, 7.5 for FMN,

Table 2. LOD and linearity.

		Regression parameters		
Analyte	**LOD, nmol/L**	**Slope**	**Intercept, nmol/L**	r^2
PMP	4.0	0.0069	0.00	0.999
PLP	0.4	0.015	−0.01	0.999
PNP	0.2	0.024	−0.08	0.997
PA	0.4	0.0058	0.02	0.999
PL	0.4	0.016	0.04	0.999
PN	0.1	0.012	−0.04	0.995
PM	0.4	0.011	−0.02	0.999
FAD	4.0	0.0025	−0.01	0.999
FMN	0.4	0.0039	0.04	0.991
RF[a]	0.2	0.0092	0.00	0.999

[a] RF, riboflavin.

Table 3. Precision of the assay.[a]

	Within-day CV, % (n = 24)			Between-day CV, % (n = 22)		
Analyte	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
PMP		20	18		18	19
PLP	5.7	6.1	5.1	7.4	5.5	6.4
PNP		19	17		22	19
PA	5.1	4.5	3.1	9.9	11	9.4
PL	9.2	3.4	3.5	8.5	7.6	6.4
PN		10	10		19	21
PM		11	11		19	17
FAD	15	10	8.3	11	11	11
FMN	18	12	13	22	13	15
RF	5.4	5.4	3.1	7.8	5.9	7.3

[a] The within-day experiments are identical to the recovery experiments given in Table S-1 of the online Data Supplement.

Table 4. Vitamer concentrations in healthy persons and cardiovascular patients.[a]

Analyte	Healthy persons (n = 94)	Cardiovascular patients	
		Placebo (n = 30)	PN supplementation (n = 30)
PMP	ND[b]	ND	ND
PLP	34.4 (17.0–102.3)	30.8 (18.3–53.9)	356 (234–585)
PNP	ND	2.6 (2.0–3.5)	2.5 (1.5–3.4)
PA	22.4 (11.2–88.0)	19.2 (12.6–26.6)	832 (262–2222)
PL	9.9 (5.7–28.2)	15.0 (9.6–20.1)	812 (170–2073)
PN	ND	ND (ND–0.1)	0.4 (ND–164)
PM	ND	0.1 (ND–0.1)	0.3 (0.1–1.3)
FAD	63.1 (49.9–84.8)	75.0 (60.4–98.6)	75.2 (59.8–103)
FMN	7.5 (3.3–13.4)	7.0 (4.8–13.9)	6.9 (4.7–12.7)
RF	10.3 (4.9–38.4)	9.0 (5.6–21.8)	6.7 (3.2–24.4)

[a] Concentrations are given as the median (10th–90th percentiles). All concentrations are in nmol/L.
[b] ND, not detectable; RF, riboflavin.

and 10.3 for riboflavin (Table 4). These concentrations are similar to those reported by others *(9, 22, 27, 40)*.

We also analyzed samples from patients with cardiovascular disease who received vitamin B_6 (40 mg/day PN; n = 30) or placebo (n = 30) for 1 month and observed that PLP, PL, and PA concentrations were 10- to 100-fold higher in vitamin B_6-supplemented patients than in the placebo group (Table 4). This difference was of a magnitude similar to that observed in previous studies of vitamin B_6 supplementation *(36, 63)*.

No patients in the placebo group, but some patients in the B_6 group, had detectable PN (n = 18) and PM (n = 5). PN and PM were detected only in 3 and 5 of the healthy individuals, respectively.

PNP was detected in most patients in the B_6 and placebo groups, but not in healthy persons, whereas PMP was not detected in any of the samples. Two other studies *(28, 63)* have reported the presence of PNP in plasma. PMP has been found in plasma in some *(19, 25, 26, 28, 70)*, but not all studies *(18, 21, 22)*. It is uncertain whether the presence of PMP and PNP in plasma is the result of limited hemolysis.

The B_2 concentrations were similar in the B_6-supplemented and nonsupplemented groups.

In conclusion, the present method exploits the sensitivity and selectivity of the latest generation of tandem mass spectrometers for the measurement of all known forms of vitamins B_6 and B_2 in human plasma. The assay includes vitamers with a wide range of polarities, and the method has the advantage of high sample throughput and low sample volume requirement. The present method thus may be useful for large-scale studies of vitamins B_6 and B_2 in health and disease.

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