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Determination of water-soluble vitamins in blood and plasma by coupled-column liquid chromatography

KURT JOHANSEN and PER OLOF EDLUND*
Bioanalytical Section, R & D, Kabi Pharma, Box 1828, S-17126 Solna (Sweden)

SUMMARY

A method is described for the simultaneous determination of riboflavin and the coenzymes related to pyridoxine and thiamine. Pyridoxal-5'-phosphate (PLP) bound to proteins as a Schiff base was liberated and stabilized by reaction with semicarbazide. Blood or plasma proteins were precipitated with acid and the supernatant formed was injected into a liquid chromatographic system consisting of one precolumn and two analytical reversed-phase columns. Riboflavin was detected by its native fluorescence with a separate detector while post-column reactions based on the fluorescence of PLP semicarbazone in alkaline solution and the oxidation of thiamine to thiochrome were combined in the same reaction detector. Chromatographic separation was achieved within 7 min and normal endogenous levels were quantified with a precision of 2–5% (relative standard deviation).

INTRODUCTION

In spite of the well known biochemical functions of the common vitamers, it is still difficult to find established methods for the assessment of vitamin status in health and disease. The vitamin concentrations in plasma varies with the daily intake of nutrients and does not reflect the degree of tissue saturation. Several approaches have been suggested for overcoming this problem, usually by some method of indirect measurement of the coenzyme related to the vitamin of interest. Several review books are available on the subject[1–3]. Vitamin B_6 status has been studied by xanthurenic acid excretion following a tryptophan load[4], by measurement of enzyme activity without[5] or with[6] *in vitro* stimulation with excess pyridoxal-5'-phosphate (PLP). Measurement of the urinary excretion of 4-pyridoxic acid, the principal B_6 metabolite, has also been used[7]. There has been some debate on the subject of which parameter gives the most relevant information about vitamin B_6 status[2]. Plasma PLP appears to be a good candidate[8] and several chemical methods based on liquid chromatography (LC) have been described[9–12].

Brin[13] demonstrated that erythrocyte transketolase activity is a reliable index of the availability of the coenzyme thiamine pyrophosphate (TPP) and thus correlated

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with the degree of thiamine deficiency in both humans and animals. This coenzyme can also be quantified by LC[14–16].

Plasma levels of riboflavin tend to reflect current intake and erythrocyte levels have been used more often to study riboflavin status[1]. An enzymatic method based on *in vitro* stimulation of erythrocyte glutathione reductase, an enzyme containing FAD, has been used as an alternative to riboflavin levels[1,17].

This paper describes how coupled-column LC combined with fluorescence detection can be used for the rapid and simultaneous quantification of PLP, TPP and riboflavin, three analytes useful for the assessment of vitamin status.

EXPERIMENTAL

Chemicals and reagents

Riboflavin (R), pyridoxal-5'-phosphate (PLP), thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP, cocarboxylase) were obtained from Sigma (St. Louis, MO, U.S.A.). Pyrophosphoric acid and triethylamine (TEA) were obtained from Fluka (Buchs, Switzerland) and acetonitrile and potassium hexacyanoferrate(III) from Merck (Darmstadt, F.R.G.). Sodium hexylsulphate ($HeSO_4$) was obtained from Kodak (Rochester, NY, U.S.A.). All other chemicals were of analytical-reagent grade. Water was purified in a Milli-Q water purification system (Millipore).

Apparatus

The liquid chromatograph consisted of three pumps (Model LC-6A), an autosampler (Model SIL-6A), two six-port valves (FCV-2AH) for chromatography, a fluorescence detector (Model RF-530) and a system controller (SCL-6A), all from Shimadzu (Kyoto, Japan). The system controller was programmed to control the column-switching valves and the detector was used for detection of TMP, TPP and PLP at excitation and emission wavelengths of 367 and 470 nm, respectively. An additional fluorescence detector (Model LS-4) from Perkin-Elmer (Norwalk, CT, U.S.A.) operated at excitation and emission wavelengths of 446 and 524 nm, respectively, was used for detection of riboflavin.

The precolumn, column 1 (15 × 3.2 mm I.D. Brownlee cartridge) contained 7-μm polymer particles (Applied Biosystems, Santa Clara, CA, U.S.A.). The analytical column for the separation of TPP, TMP and PLP (150 × 4.6 mm I.D.), column 2, contained 5-μm styrene–divinylbenzene copolymer particles from Polymer Labs. (Shropshire, U.K.) and the column for riboflavin (100 × 4 mm I.D.), column 3, contained 3-μm Spherisorb ODS-silica (Phase Separations, Queensferry, U.K.). The effluent from column 2 was connected to a low-dead-volume tee (Valco) for addition of post-column reagent [200 mg/l potassium hexacyanoferrate(III) in 2 *M* sodium hydroxide solution], followed by a stitched PTFE capillary (3.5 m × 0.38 mm I.D.) connected to the Shimadzu detector (see Fig. 1). The PTFE capillary was stitched into a metal net with a mesh size of 1 mm. The post-column reagent was pumped from a glass bottle with aid of a static nitrogen gas pressure of 28 p.s.i., which resulted in a flow-rate of 0.25 ml/min.

The mobile phase for the precolumn (C1) was pumped by pump 1 at a flow-rate of 1 ml/min and consisted of 0.1 *M* NaH_2PO_4 and 10 m*M* pyrophosphoric acid with

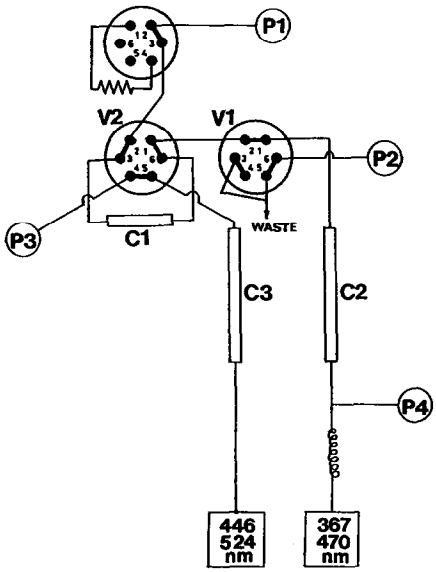

Fig. 1. Chromatographic system consisting of precolumn (C1) and polymeric column for separation of phosphates (C2) and ODS column for riboflavin (C3). V1 and V2 = six-port electric switching valves.

pH adjusted to 4 with 1 *M* sodium hydroxide solution. The mobile phase for the second column (C2) consisted of 10 m*M* triethylamine, 10 m*M* pyrophosphoric acid and 1 m*M* hexylsulphate in 0.1 *M* NaH_2PO_4 (pH 4), pumped by P2 at 1 ml/min. The mobile phase for the third column consisted of 13% acetonitrile in 0.1 *M* phosphate buffer (pH 4), pumped by P3 at 0.8 ml/min.

Sample preparation

Blood or plasma (0.5 ml) was diluted with 0.5 ml of mobile phase 2 from the chromatograph. The blood samples were mixed with 0.25 ml of 1 *M* semicarbazide, while 0.15 ml was used for plasma samples. The samples were derivatized in the dark at ambient temperature for 15 min, followed by protein precipitation with 0.4 ml of 25% (w/v) trichloroacetic acid and centrifugation for 5 min. The supernatants were filtered through cotton-wool wound around the tip of a Pasteur pipette and dispensed to autosampler vials. A volume of 80 μl was injected into the chromatograph for analysis. Calibration was performed by standard addition of TPP, PLP and riboflavin to normal blank plasma or blood to obtain a concentration of 200 ng/ml of the analytes. A duplicate of the blank standard and at least four fortified standards were extracted along with unknown samples as described above. The slope of the calibration graph of peak height *versus* concentration added was used for quantification of unknown samples.

RESULTS AND DISCUSSION

Sample preparation

PLP is bound to ε-amino acid residues of albumin in plasma[18]. The Schiff base or

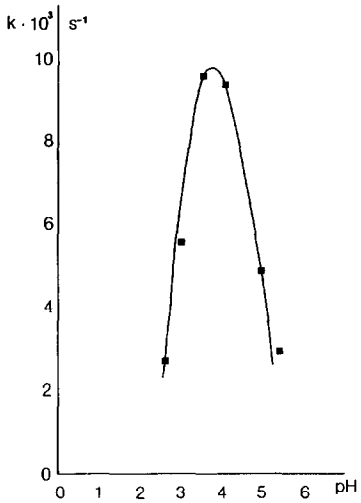

Fig. 2. Pseudo-first-order rate constants for derivatization of PLP with semicarbazide as a function of pH determined by LC at a reagent concentration of 3 m*M* at 26°C.

semicarbazone formation is dependent on pH with a maximum at *ca.* pH 3.5 (see Fig. 2). The most common methods for extraction of PLP from plasma involve protein precipitation with trichloroacetic[9–11] or perchloric acid[12], which acidifies the samples sufficiently to dissociate PLP from proteins[18]. Competitive reaction with excess of semicarbazide can also be used to liberate and stabilize PLP. Semicarbazon formation improves the fluorescence compared with free PLP and stabilizes the analyte towards photochemical decomposition[19]. Riboflavin is also sensitive to light and samples must be protected from strong daylight during handling and extraction. The extraction method with TCA described under Experimental gave recoveries close to 100% for all of the analytes studied except riboflavin in blood, where the recovery was 76% (see Table I). Riboflavin is less hydrophilic than TPP and PLP and it has not been possible

TABLE I

RECOVERY AND PRECISION

Compound	*Matrix*	*Recovery (%)*	*n*	*Precision*[a] *(%)*	*Detection limit (ng/ml)*
TPP	Blood	93 ± 7.8	13	1.2	2
	Plasma	85 ± 3.2	8	8.5	2
TMP	Blood	103 ± 8	13	14	1
	Plasma	104 ± 2	8	5.8	1
PLP	Blood	93 ± 2.8	13	3.6	2
	Plasma	100 ± 9.8	13	2.0	2
R	Blood	76 ± 5.6	13	9.1	1
	Plasma	94 ± 6.5	13	1.7	0.5

[a] Inter-assay precision (relative standard deviation) at endogeneous levels.

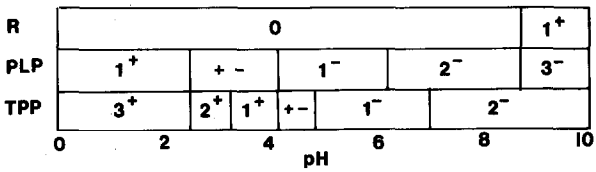

Fig. 3. Estimation of the state of ionization of the analytes as a function of pH. The pK_a values were estimated from pK_a values of the vitamins in ref. 1 combined with pK_a values of pyrophosphoric acid.

to obtain a quantitative recovery of this compound without excessive dilution of the sample. FAD was strongly (covalently) bound to enzymes and recoveries were too low for meaningful quantification. Extraction under more drastic conditions, *e.g.*, heating, or the use of a stronger acid, hydrolysed FAD to FMN and riboflavin. Therefore, only riboflavin concentrations were quantified.

Chromatographic separation

The analytes studied differed widely in acid–base properties (see Fig. 3) and polarity, with TPP being the most polar with little or no retention on conventional reversed-phase columns whereas riboflavin was strongly retained. Trace enrichment procedures have been used for this compound[20,21]. Thus gradient or coupled-column procedures must be used to elute these compounds in the same chromatographic

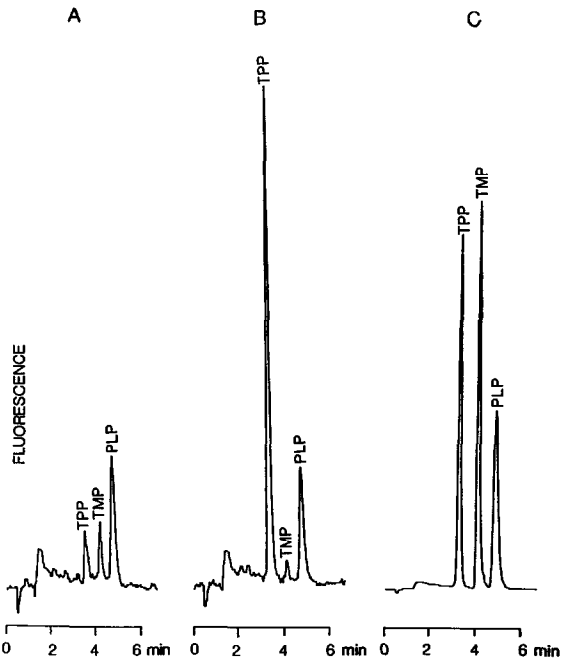

Fig. 4. Chromatograms obtained with (A) a plasma sample, (B) a blood sample and (C) a fortified blood sample. The phosphates were separated on a polymer column as described under Experimental during the experiment. The plasma concentrations were TPP 4 ng/ml, TMP 5 ng/ml and PLP 25 ng/ml. The blood concentrations were TPP 60 ng/ml, TMP 2 ng/ml and PLP 25 ng/ml.

system. We preferred the latter approach because faster separations with very short re-equilibration times can be achieved and better baseline stability is obtained for trace analysis. A small precolumn packed with polymeric reversed-phase particles was used for the separation of riboflavin from TPP, TMP and PLP. The more polar analytes were eluted from the precolumn with buffer for further separation by ion-pair chromatography with a mixture of triethylamine and hexylsulphate on a polymeric column (see Fig. 4). Riboflavin was eluted from the precolumn with a mobile phase containing 13% acetonitrile for separation on a separate column (Fig. 5). The column-switching events are summarized in Table II.

At pH 4 riboflavin was neutral whereas TPP was mostly present as a singly charged cation and PLP was present as an anion or zwitterion (see Fig. 3). Thus TPP and TMP were retained as ion pairs with hexylsulphate and PLP as an ion pair with triethylamine on polymeric reversed-phase columns and the selectivity for separation of thiamine phosphates from PLP can be controlled by the TEA–$HeSO_4$ ratio (Fig. 6). The hexylsulphate concentration required to obtain suitable selectivity varied between different columns. A new column required 3 m*M* hexylsulphate (Fig. 6) whereas a column that had been used for 1 year required a concentration of 1 m*M* hexylsulphate (Fig. 4). The column packed with polymeric particles gave a much stronger retention than silica-based ODS columns and symmetrical peaks were obtained (see Fig. 4). Semicarbazide is an oxidizable compound and had to be at least partially separated from TPP to avoid quenching of the post-column reaction with

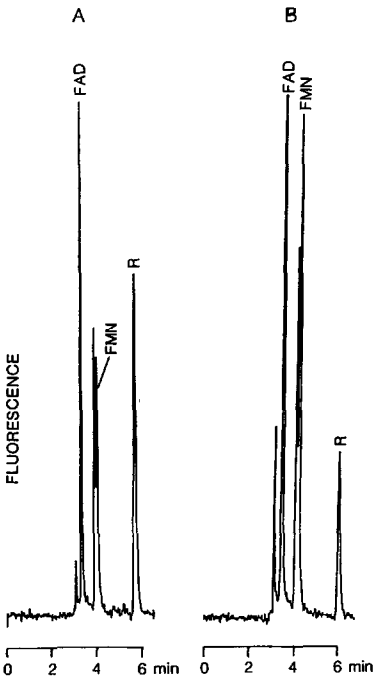

Fig. 5. Chromatograms obtained with (A) a plasma sample and (B) a blood sample with riboflavin concentrations of 50 and 20 ng/ml, respectively.

TABLE II

COLUMN-SWITCHING TIMETABLE

Time (min)	*Valve operated*	*Event*
0	Injector	The sample is injected on C1
0.1	1	TPP, TMP and PLP are eluted from C1 with buffer for separation on C2
0.9	1	C1 is washed with buffer
2.0	2	Riboflavin is eluted from C1 with 13% acetonitrile for separation on C2
5.0	2	C1 is conditioned with buffer
8.0	Injector	Next sample is injected

Fe^{3+} ions. Semicarbazide was eluted with the interstitial column volume and the polymeric column gave sufficient retention of TPP, but silica-based ODS columns were ineffective and quenching frequently occurred. Initially a simplified system was investigated where the sample was injected on the precolumn with the mobile phase for separation of the phosphates. The TEA–$HeSO_4$ ion pair interfered with the subsequent separation of riboflavin, shown as peak splitting or excessive band broadening; a buffer without ion-pairing reagents had to be used for sample injection.

Attempts were made to include ascorbic acid in the same assay but the retention on the polymer column was not sufficient to separate ascorbic acid from uric acid.

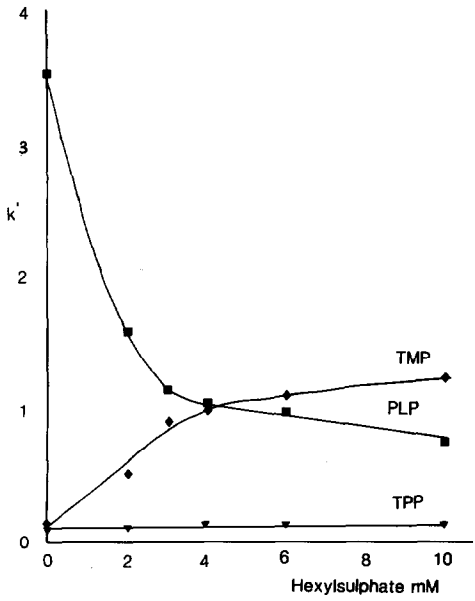

Fig. 6. Capacity factors of TPP, TMP and PLP on a polymer column as a function of hexylsulphate concentration. The mobile phase contained 10 m*M* triethylamine and 10 m*M* pyrophosphoric acid in 0.1 *M* phosphate buffer (pH 4) and various concentrations of hexylsulphate.

These acids have similar UV spectra and oxidation potentials and must be separated. However, precolumn separation is a very flexible technique and separations can often be tailored according to the analytical demand. The determination of ascorbic acid can, for instance, be combined with the determination of riboflavin in the system above if the polymeric column is replaced with an anion exchange-column. The same extraction method can be used if dithiothreitol is added to the TCA solution as an antioxidant.

Detection

Riboflavin was detected with high sensitivity and selectivity by its native fluorescence. The fluorescence of PLP semicarbazone gave an optimum intensity at *ca.* pH 12 (data not shown) and was not influenced by the addition of a low concentration of hexacyanoferrate(III). Hence the post-column alkalinization of PLP and the alkaline oxidation of thiamine phosphates can be combined in the same reaction detector. The oxidation of TPP occurred quickly and a 3.5 m × 0.38 mm I.D. PTFE reactor was sufficient to obtain a quantitative reaction. The reactor was stitched to improve radial mixing and to reduce band broadening. The optimum wavelength for excitation was 367 and 369 nm for PLP and TPP, respectively. The optimum wavelengths for emission were 478 and 435 nm for PLP and TPP, respectively. The emission bands are wide and TPP can be detected at the emission wavelength of PLP with about a 50% decrease in sensitivity.

Accuracy and precision

The identity of the phosphates was confirmed by the disappearance of the chromatographic peaks after enzymatic hydrolysis with acid phosphatase. Further, no peaks corresponding to TPP and PLP were detected when the post- and precolumn reagents were excluded from the analysis (data not shown).

The inter-assay precision (relative standard deviation) varied from 1.2% for TPP in blood to 9.1% for riboflavin (Table II). Both plasma and blood TMP levels were close to the method detection limit of 1 ng/ml for this analyte. The sensitivity of the method was sufficient to determine TPP, PLP and riboflavin in plasma and blood at reduced levels.

CONCLUSIONS

Precolumn derivatization with semicarbazide not only dissociated PLP bound to proteins but also stabilized the analyte to avoid photochemical decomposition.

Ion-pair chromatography with TEA–$HeSO_4$ on a polymer column was useful to control the selectivity for separation of TPP, TMP and PLP. These phosphates were detected with the same reaction detector after a minor compromise in emission wavelength. The use of ion-pair reagents interfered with the subsequent separation of riboflavin and therefore a separate buffer had to be used for sample injection.

Fast separations were achieved with precolumn fractionation and TPP, PLP and riboflavin were determined in plasma and blood with favourable precision within 7 min.

REFERENCES

1 L. J. Machlin (Editor), *Handbook of Vitamins*, Marcel Dekker, New York, 1984.
2 J. E. Lekelm and R. D. Reynolds (Editors), *Methods in Vitamin B-6 Nutrition, Proceedings of Workshop on Analytical Methodology and Criteria for Assesing Nutritional Status, Mt. Hood, Oregon, June 29–July 2, 1980*, Plenum Press, New York, 1981.
3 A. P. De Leenheer, W. E. Lambert and M. G. M. De Ruyter (Editors), *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, 1985.
4 L. D. Greenberg, D. F. Bohr, H. McGrath and J. F. Reinhart, *Arch. Biochem.*, 21 (1949) 237.
5 M. J. Woodring and C. A. Storwick, *Am. J. Clin. Nutr.*, 23 (1970) 1385.
6 B. Chabner and D. Livingstone, *Anal. Biochem.*, 34 (1970) 413.
7 J. F. Gregory and J. R. Kirk, *Am. J. Clin. Nutr.*, 32 (1979) 879.
8 J. Leiner, I. Simon and D. Hötzel, *Int. J. Vitam. Nutr. Res.*, 53 (1983) 166.
9 J. Schrijver, A. J. Speek and M. P. Schreurs, *Int. J. Vitam. Nutr. Res.*, 51 (1984) 216.
10 S. P. Coburn and J. D. Mahuren, *Anal. Biochem.*, 129 (1983) 310.
11 T. E. Heffaran, B. M. Chrisley and J. A. Driskell, *J. Chromatogr.*, 374 (1986) 155.
12 P. Edwards, P. K. S. Liu and G. A. Rose, *Clin. Chem.*, 35 (1989) 241.
13 M. Brin, *Ann. N.Y. Acad. Sci.*, 98 (1962) 528.
14 H. Sanemori, H. Ueki and T. Kawasaki, *Anal. Biochem.*, 107 (1980) 451.
15 J. Bontemps, L. Bettendorff, J. Lombet, G. Danrifosse, E. Schoffeniels, F. Nevejans, Y. Yang and M. Verzele, *Chromatographia*, 18 (1984) 424.
16 M. Baines, *Clin. Chim. Acta*, 153 (1985) 43.
17 A. M. Prentice and J. Bates, *Br. J. Nutr.*, 45 (1981) 37.
18 J. B. Ubbink, W. J. Serfontein and L. S. DeVilliers, *J. Chromatogr.*, 375 (1986) 399.
19 J. B. Ubbink, W. J. Serfontein and L. S. DeVilliers, *J. Chromatogr.*, 342 (1985) 277.
20 H. Y. Mohammed and H. Vening, *J. Chromatogr.*, 226 (1981) 471.
21 G. Jaumann and H. Engelhardt, *Chromatographia*, 20 (1985) 615.