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STABILITY OF PYRIDOXAL-5-PHOSPHATE SEMICARBAZONE: APPLICATIONS IN PLASMA VITAMIN B_6 ANALYSIS AND POPULATION SURVEYS OF VITAMIN B_6 NUTRITIONAL STATUS

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SUMMARY

The determination of pyridoxal-5-phosphate (PLP) and pyridoxal (PL) in plasma requires the availability of dark room facilities, due to the photosensitivity of these vitamin B_6 vitamers. The fact that the semicarbazone forms of PL and PLP are more strongly fluorescent than the underivatized B_6 vitamers has been exploited in plasma analyses, but it was not previously realised that these semicarbazone forms are also very stable even under conditions that lead to rapid decomposition of free PL and PLP. The stabilisation of PLP and PL obtained in this manner is sufficient and fully adequate to meet the practical requirements of clinical field studies. We report a high-performance liquid chromatographic method for plasma PLP and PL determinations based on precolumn semicarbazone formation and fluorescence detection. The method is sensitive enough for quantitative plasma PLP determinations even in B_6-deficient patients.

INTRODUCTION

The analytical problems associated with the determination of vitamin B_6 (B_6) vitamers in biological material are many and varied and these have been studied for decades. The disadvantages of microbiological assays, which includes the lack of selectivity, have been summarised recently [1].

Since pyridoxal-5-phosphate (PLP) is the physiologically active form of vitamin B_6 and since plasma PLP measurements afford a good indication of B_6 nutritional status [2], several alternative methods have been developed for PLP estimations in plasma or whole blood. A complicating aspect of such analyses is the fact that the B_6 vitamers are water-soluble, which prohibits the use of extraction procedures as a means of purification. Direct fluorimetric

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determination of PLP and pyridoxal (PL) in plasma is either unreliable due to interfering fluorescent plasma compounds [3, 4], or tedious due to the requirement of sample clean-up procedures like column and/or thin-layer chromatography [5—7].

Several methods based on high-performance liquid chromatography (HPLC) for the separation of some or all B_6 vitamers have been described [8—12] but these are not suitable for routine measurement of blood levels, due to lack of sensitivity [13]. Direct HPLC analyses of plasma samples and fluorimetric quantification of all six B_6 vitamers [14, 15] have been reported, but these methods require sophisticated HPLC equipment and/or complicated column-switching procedures and are thus in general not suitable for clinical applications where a simple but highly sensitive method for the determination of PLP is required. Schrijver et al. [1] reported a rapid and sensitive HPLC method based on post-column semicarbazone formation of PLP and PL followed by fluorescence detection, but this method suffers from the disadvantage that no internal standard was used.

All of the above mentioned HPLC methods suffer from the further disadvantage that the analyses have to be carried out in a specially equipped dark room [14, 15], due to the photosensitivity of PLP and PL. This requirement has obvious disadvantages in the application of these methods in an ordinary laboratory. Moreover, the recent upsurge of interest in B_6 as a possible aetiological agent in the development of coronary artery disease [16, 17] requires the availability of a practical procedure to determine the B_6 status of various population groups. The instability and photosensitivity of the B_6 vitamers is thus a serious problem in conducting field studies and population screening surveys. In this study we show that PLP semicarbazone (PLPSC) is sufficiently stable under ordinary conditions of light and temperature to form the basis of a new isocratic HPLC method, based on previous work by Gregory [18], which fulfills all of the above-mentioned requirements.

An important and distinguishing feature of the method is the introduction of 6-methyl-2-pyridine carboxaldehyde semicarbazone (MPCSC) as an internal standard. This compound is a suitable internal standard which greatly improves the utility and precision of the method under practical conditions.

EXPERIMENTAL

Reagents

PL and PLP were obtained from Merck (Darmstadt, F.R.G.). The internal standard, 6-methyl-2-pyridine carboxaldehyde (MPC), was bought from Aldrich (Milwaukee, WI, U.S.A.). Chromatography-grade dichloromethane and acetonitrile were supplied by Merck. All other chemicals were analytical-reagent grade and obtained from Merck.

Purification of PL and PLP

The commercial PLP and PL preparations were purified by reversed-phase HPLC (Whatman Partisil 10 ODS-3 column; mobile phase: 10% methanol and 0.1% glacial acetic acid in water) and then lyophilized. Purified PLP and PL were checked for impurities by reversed-phase ion-pair chromatography [10],

UV detection (290 nm) and wavelength scanning (210—360 nm) at different stages of peak elution. UV absorption spectra of the purified PLP and PL were determined in 0.1 *M* hydrochloric acid, 0.1 *M* sodium hydroxide and 0.1 *M* sodium phosphate buffer, pH 7.0.

Standards

Purified PLP and PL were used to prepare working standards containing 1—20 ng PLP and PL per ml. To prepare the internal standard, 6-methyl-2-pyridine carboxaldehyde was dissolved in 25 ml of 0.1 *M* sodium dihydrogen phosphate; 20 ml of 0.5 *M* semicarbazide solution was added and the mixture was heated for 20 min at 40—45°C. After cooling, 0.1 *M* disodium hydrogen phosphate was added to adjust the pH to 7.0; the volume was adjusted to 500 ml using Na_2HPO_4—NaH_2PO_4 buffer (0.1 *M*; pH = 7.0). The MPCSC solution was then divided into 2.0-ml aliquots, freeze-dried, sealed under nitrogen and stored at −20°C.

Sample preparation

To 1.0 ml plasma, 50 μl reconstituted MPCSC solution as internal standard were added. Plasma proteins were precipitated by the addition of 0.5 ml 10% trichloroacetic acid (TCA) while mixing vigorously. To the clear supernatant obtained after centrifugation, 50 μl of 0.5 *M* semicarbazide were added and the mixture was incubated at 40°C for 10 min. Prior to injection, the supernatant was extracted twice with 3.0 ml diethyl ether; the diethyl ether was aspirated and the water phase was extracted with 3.0 ml dichloromethane. A 100-μl aliquot of the supernatant was injected onto the column for analysis.

Instrumentation

A Perkin-Elmer Series 2 liquid chromatograph was slightly modified so that one pump was used for solvent delivery and the other pump for post-column reagent addition. An LS 4 Perkin-Elmer fluorescence spectrometer (excitation wavelength: 367 nm; emission wavelength: 478 nm) was coupled to a Perkin-Elmer Sigma 10 chromatography data station.

Columns

A Whatman (Clifton, NJ, U.S.A.) Solvecon pre-column (25 cm × 4.6 mm) installed on-stream and before the injector, was followed by a Brownlee Labs. (Santa Clara, CA, U.S.A.) RP-18 Spheri-5 guard column (4 cm × 4.6 mm) and a Whatman Partisil 10 ODS-3 analytical column (25 cm × 4.6 mm).

Chromatographic conditions

A solution of 0.05 *M* potassium dihydrogen phosphate (pH adjusted to 2.9 with concentrated orthophosphoric acid) containing 7% acetonitrile was used as mobile phase. Sodium hydroxide (4%, w/v) was introduced for post-column alkalinisation. The flow-rates of the solvent delivery pump and the post-column reagent pump were 1.1 and 0.1 ml/min, respectively.

Sample stability

The following preparations were placed (15 cm) under a 100-W electric light

bulb, and at certain time intervals (t = 0, 1, 2, 3, 5, 6 and 24 h) a 1.0-ml aliquot was withdrawn and analysed as described under sample preparation. (a) $1.2 \cdot 10^{-7}$ M PLP; (b) a $1.2 \cdot 10^{-7}$ M PLP solution containing $2.5 \cdot 10^{-2}$ M semicarbazide; (c) $1.2 \cdot 10^{-7}$ M PLP in 3% TCA; (d) $1.2 \cdot 10^{-7}$ M PLP in 3% TCA containing $2.5 \cdot 10^{-2}$ M semicarbazide; (e) human plasma; (f) supernatant after the same human plasma sample had been treated with TCA to precipitate plasma proteins (TCA-supernatant); (g) TCA-supernatant containing $2.5 \cdot 10^{-2}$ M semicarbazide; (h) $1.65 \cdot 10^{-4}$ M MPC; and (i) MPC solution containing $2.5 \cdot 10^{-2}$ M semicarbazide.

Samples containing semicarbazide were pre-heated at 40°C for 20 min before exposure to light.

Plasma and TCA-supernatant were also kept at both 4°C and 25°C in the dark and 1.0-ml aliquots were analysed for PLP at time intervals described above.

A solution of TCA-supernatant, reacted with semicarbazide, was kept at 4°C in the dark and analysed daily to test the long-term stability of PLP.

RESULTS

The commercial preparations of PLP and PL were not completely pure, but reversed-phase HPLC was very effective in separating PLP and PL from minor impurities. Purified PLP and PL eluted as single peaks during reversed-phase ion-pair chromatography [10], and peak homogeneity was confirmed by wavelength scanning at different stages of peak elution. UV absorbance spectra of purified PLP and PL were in close agreement with previously published results [19].

Fig. 1 shows a typical separation of (a) a standard mixture of PLP and PL; (b) a normal human plasma sample pre-treated as described under Experimental; (c) the same plasma spiked with 4.0 ng PL and 6.0 ng PLP per ml plasma; and (d) plasma from a person taking an oral pyridoxine supplement of 20 mg per day.

The retention times of all three peaks shown were found to be very sensitive to the acetonitrile concentration in the solvent. The optimum acetonitrile concentration in the solvent differed when different columns were used and also changed with column ageing. Serum contains two background peaks eluting before the PLP peak, and the solvent acetonitrile concentration was chosen such that baseline resolution between the PLP peak and the background peaks was achieved.

The within-day precision of the method was determined by dividing a serum pool into fifteen 1-ml aliquots, and determining the PL and PLP concentrations in each aliquot. The coefficients of variation (C.V.) for PLP and PL were 5.9% and 8.1%, respectively. The day-to-day variation was determined by freeze-drying a serum pool in 1.0-ml aliquots. For twenty successive days a 1-ml aliquot was reconstituted for PLP and PL determination as previously described. The C.V. values of 11.8% and 12.5% which were found for PLP and PL, respectively, compares well with the within-day variation found.

Spiking of plasma with different amounts of PL and PLP indicated that the recovery of both vitamers was good. Table I shows that the recovery of PLP

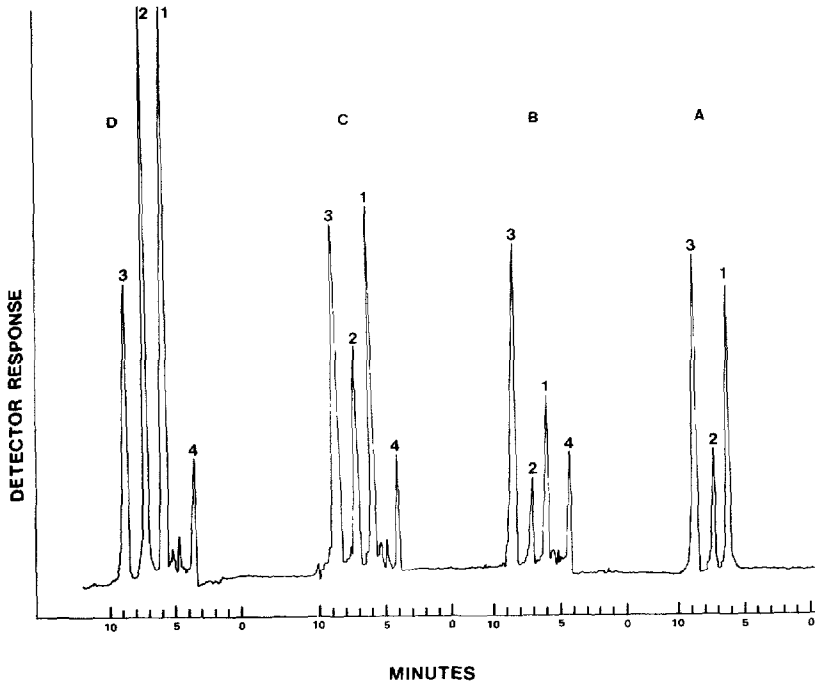

Fig. 1. The determination of PL and PLP in plasma using HPLC. (A) Standard, containing 10.0 ng PLP, 4.0 ng PL and 5.0 μg MPCSC per ml, (B) plasma sample from a normal, healthy person (PLP 5.7 ng/ml and PL 2.8 ng/ml); (C) the same plasma sample from B spiked with 6 ng PLP and 4 ng PL per ml plasma; (D) plasma sample from a person using oral pyridoxine supplements of 20 mg per day. Each sample was injected at t = 0 min. Peaks. 1 = PLPSC; 2 = PLSC; 3 = internal standard, MPCSC; 4 = plasma background.

TABLE I

RECOVERY OF PL AND PLP ADDED TO PLASMA

Concentration of vitamer added (ng/ml)	PLP found (ng/ml)	Percentage recovery	PL found (ng/ml)	Percentage recovery
0	6.8	—	0	—
4	10.5	92.5	3.6	90.0
5	11.7	98.0	5.1	102.0
8	14.9	101.3	6.5	81.3
10	16.9	101.0	8.8	88.0
20	26.0	96.0	20.0	100.0
Average (± S.D.)		97.8 ± 3.7		92.3 ± 8.6

was slightly better and more consistent than the recovery of PL, indicating that a small loss of PL may occur during the preparation procedure.

Routinely, 100-μl samples were used per injection, and reliable PLP detection at concentrations as low as 1 ng PLP per ml plasma was achieved. However, when using a 500-μl sample loop, the injection volume can be increased four-fold, thus setting the limit of sensitivity for PLP determinations at 0.25 ng PLP per ml plasma.

Plasma samples from 28 healthy, middle-aged (age ± 1 S.D.: 46.2 ± 9.6 years) men were analysed for PL and PLP and the plasma levels were 1.94 ± 1.12 and 11.44 ± 6.31 ng/ml, respectively, thus indicating that normal plasma PL and PLP levels are well within the sensitivity limit of this method.

The stability of PLP was studied under various conditions. In Figs. 2—4, the PLP concentration at $t = 0$ was assumed to be 100%. Fig. 2 indicates that PLP was stable in plasma for at least 6 h if the plasma was kept in the dark at either 4°C or room temperature. After 24 h, however, up to 10% loss of PLP from plasma was evident. However, direct light from a 100-W electric light bulb as described above results in a high loss of PLP from plasma due to PLP photodecomposition [15].

Fig. 3 shows that although a standard solution of PLP was prone to photodecomposition whether or not TCA was added to the solution, PLPSC was stable for a whole working day and after 24 h the loss was only 10% for a pure PLPSC solution. The same observation was made for the supernatant obtained after plasma protein precipitation (Fig. 4).

Fig. 4 indicates that supernatant treated with semicarbazide as described under Experimental, was stable under direct lighting conditions, while the PLP of an untreated supernatant was prone to photodecomposition. In fact, a supernatant treated with semicarbazide and stored at 4°C in the dark gave consistent results for PLP and PL determinations for at least 28 successive days.

As indicated in Fig. 5, the same principle applies in the case of internal standard: MPC concentration decreased when a solution containing MPC was placed directly under a 100-W electric light bulb, but the semicarbazide derivative concentration remained constant under these conditions.

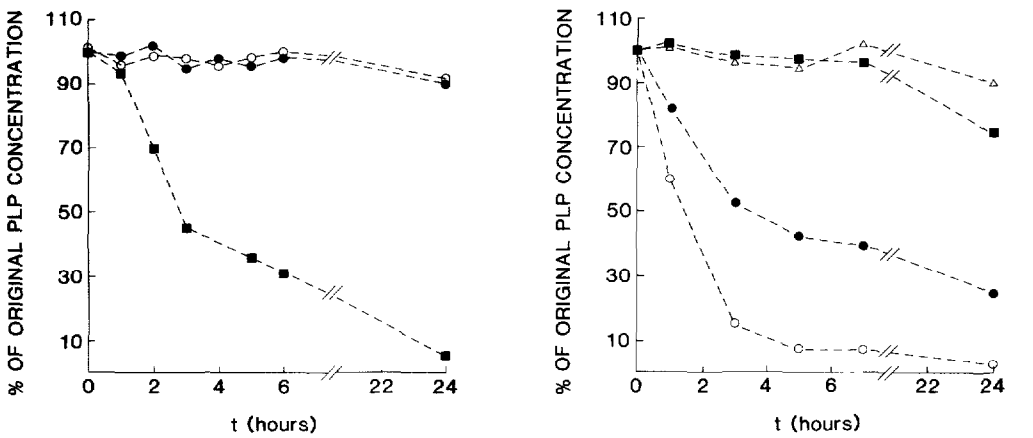

Fig. 2. The stability of PLP in heparin-plasma. The results presented are the average of three independent experiments. (•) Heparin-plasma stored in the dark at room temperature, (○) heparin-plasma stored at 4°C (dark); (■) heparin-plasma stored under a 100-W electric light bulb (15 cm).

Fig. 3. Stability of different PLP solutions under a 100-W electric light bulb (15 cm). (○) $1.2 \cdot 10^{-7}$ *M* PLP; (△) $1.2 \cdot 10^{-7}$ *M* PLP in $2.5 \cdot 10^{-2}$ *M* semicarbazide solution; (■) 3% TCA, containing $1.2 \cdot 10^{-7}$ *M* PLP and $2.5 \cdot 10^{-2}$ *M* semicarbazide; (•) 3% TCA, containing $1.2 \cdot 10^{-7}$ *M* PLP.

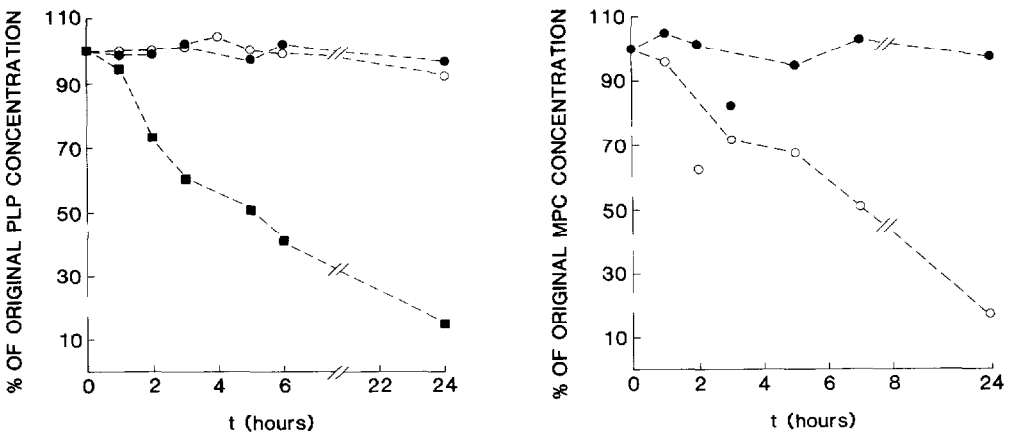

Fig. 4. Stability of PLP in the supernatant after precipitation of plasma proteins with 10% TCA. Supernatant stored at 4° C in the dark (○), at room temperature under a 100-W electric light bulb (15 cm) (■); and containing $2.5 \cdot 10^{-2}$ *M* semicarbazide, stored under a 100-W electric light bulb (15 cm) at room temperature (●).

Fig. 5. Stability of 6-methyl-2-pyridine carboxaldehyde (MPC) solutions under a 100-W electric light bulb. (○) $1.65 \cdot 10^{-4}$ *M* MPC; (●) $1.65 \cdot 10^{-4}$ *M* MPC in $2.5 \cdot 10^{-2}$ *M* semicarbazide solution.

DISCUSSION

The reaction of PL and PLP with semicarbazide as originally described by Cordes and Jencks [20] has frequently been utilized in the quantitative determination of PL and PLP in biological samples, because the semicarbazone derivatives show better fluorescence characteristics than the underivatized vitamers. Gregory [18] used pre-column derivatisation in determining tissue PLP levels as PLPSC. Schrijver et al. [1] in order to fully automate their HPLC system, used post-column derivatisation, while Vanderslice et al. [14] used on-column derivatisation.

Since a recent study indicated that regular laboratory light is extremely destructive to vitamin B_6 [21], our findings on the stability of PLPSC under ordinary laboratory light conditions stress the desirability of pre-column derivatisation. With pre-column derivatisation under the above mentioned conditions, HPLC analysis can be carried out in an ordinary laboratory, thus eliminating the need of a specially equipped dark room as used by others [14, 15]. Moreover, since PLPSC is stable under our conditions, TCA-supernatants reacted with semicarbazone can be kept in a fridge and analysed at a convenient time. It is therefore now possible to screen remote, rural populations with respect to their B_6 status, because only the most basic laboratory facilities are needed to prepare and store plasma PLP in the stable semicarbazone form.

The sensitivity of this method is very good. Since PLPSC is only moderately fluorescent in acidic to neutral solutions, Gregory [18] was unable to determine low plasma PLP levels. Schrijver et al. [1] using alkaline detection conditions, reported a sensitivity of up to 1.2 ng/ml. The sensitivity of our method is therefore as good as that described by Schrijver et al. [1] and considerably better than others. Although HPLC methods have been published

for the determination of all six B_6 vitamers and pyridoxic acid in one run [14, 15], these methods are generally less sensitive, because the fluorescence characteristics of the different B_6 vitamers differ widely [22], and the detection conditions adopted are usually a compromise between the optimum detection conditions of each vitamer. Thus Vanderslice et al. [14] reported the lower limit of sensitivity for PLP as 3.4 ng/ml, while Coburn and Mahuren [15] using a cation-exchange procedure not based on semicarbazone derivatisation, report that integration errors are significant at concentrations below 5 ng per injection. In our case the sensitivity of semicarbazone measurement in alkaline medium was such that reliable results were still obtainable at a level of 0.1 ng per injection.

The use of MPC as internal standard deserves comment. The semicarbazone derivative of MPC is less fluorescent than PLPSC and PLSC at the wavelengths selected and for this reason the MPC concentration chosen was much higher than that of PLP or PL concentrations in standard solutions. However, this concentration (0.5 μg per injection) is still well within the analytical capabilities of the column used and MPCSC elutes as a sharp, constant peak from the column under these conditions.

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