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Optimisation and validation of a sensitive high-performance liquid chromatography assay for routine measurement of pyridoxal 5-phosphate in human plasma and red cells using pre-column semicarbazide derivatisation

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Abstract

There are few studies in which direct measurement of vitamin B6 status in both plasma and red cells has been assessed. The aims of the present study were to evaluate the use of a simple, robust HPLC method of direct pyridoxal 5′-phosphate (PLP) measurement in plasma and red cells and to assess its use in establishing reference ranges in a healthy population. A reverse phase HPLC method with pre-column derivatisation using semicarbazide for the simultaneous measurement of PLP, its degradation product, 4-pyridoxic acid (PA) and pyridoxal (PL) in plasma and red cells was developed. Pre-column derivatisation, reverse phase chromatography and detection procedures were optimised. The recovery, precision, linearity and sensitivity of the assay for plasma and red cell PLP, PA and PL was established. The recovery of PLP was greater than 95% for both plasma and red cell samples. The Intra and Inter batch imprecision for PLP was less than 6% and 7%, respectively. The method for PLP was linear up to at least 1000 nmol/l and the detection limit was 2.1 nmol/l (limit of quantification; 5.8 nmol/l). Accuracy of PLP measurements in plasma were acceptable, showing a mean bias of 4.5% from the mean value of laboratories ($N=34$) participating in an external quality assurance scheme. Geometric mean (95% reference intervals) for plasma and red cell PLP in the healthy subjects ($N=126$) were 56 (21–138) nmol/l and 410 (250–680) pmol/g Hb, respectively. There was a strong positive correlation ($r^2=0.81$) between plasma and red cell PLP levels in the reference population. The HPLC method described was found to be suitable for the routine measurement of PLP in both plasma and red cells.

Keywords: Optimisation; Validation; Derivitisation, LC; Vitamins; Pyridoxal 5-phosphate

1. Introduction

Vitamin B6 (active 3 hydroxy-2-methyl pyridine derivatives) is an essential precursor of pyridoxal and pyridoxamine phosphate coenzymes, which are im-

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portant for a variety of enzymes of intermediary metabolism [1]. In plasma, pyridoxal 5′-phosphate (PLP) is the major form whereas PLP and pyridoxamine 5′-phosphate (PMP) predominate in the cell. For example, the average concentration of PLP in whole blood is approximately 65 nmol/l with about 60% present in red cells [2]. Recent clinical studies suggest that low plasma PLP concentrations are an independent risk factor for cardiovascular and other diseases [3–5].

A variety of methods have been proposed for the assessment of vitamin B6 nutritional status in humans [2,6]. The most widely used method to detect vitamin B6 deficiency is the indirect measurement of PLP in erythrocytes with the erythrocyte AST activation assay [2,6–8]. However, since this test is a functional rather than a direct measurement of PLP status, it may be affected by factors other than PLP deficiency [2,6]. Other factors that have reduced the usefulness of the activation test have been the difficulties with standardisation and defining the normal reference range with confidence [6]. Furthermore, the activation assay cannot be used to detect excessive vitamin B6 intake, which is increasingly being recognised as hazardous [9].

Direct measurement of PLP in blood by HPLC is more promising. Although vitamin concentration in blood cells tends to be a better marker of cellular stores [10], most HPLC assays described in the literature measure PLP concentration in plasma for assessing vitamin B6 nutritional status. In apparently healthy subjects, plasma PLP concentration is now considered one of the better indicators of vitamin B6 status [1,11–14] and has been shown to be well correlated with tissue PLP concentrations [12]. However, plasma PLP levels have been shown to fall transiently in patients with acute systemic inflammation, independent of nutritional intake [15].

In the present study we first describe the validation of a simple, robust reverse phase HPLC method with pre-column derivatisation using semicarbazide [16,17] that is suitable for the simultaneous measurement of PLP, its degradation product, 4-pyridoxic acid (PA) and pyridoxal (PL) in plasma and red cells. Second, its application in measuring plasma and intracellular PLP concentrations in healthy subjects in order to establish population reference ranges.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade. PLP, PA, PL, PMP, pyridoxamine (PN), sodium EDTA and semicarbazide were obtained from Sigma Chemical Company (Poole, UK). Glycine, orthophosphoric acid, perchloric acid and Analar grade methanol were purchased from BDH (Poole, UK) and HPLC analytical and guard columns were purchased from Phenomenex (Macclesfield, UK). Plasma based calibrator and quality control (QC) material containing PLP, PA and PL were obtained from Chromsystems (Munchen, Germany) and prepared according to manufacturers instructions.

2.2. Blood samples, calibrator, QC and stock standards

Blood samples for population references values were obtained from laboratory staff, from local health centers and from people attending a cardiovascular risk clinic. None of the subjects were taking any vitamin supplements or had any significant medical history or evidence of a systemic inflammatory response (serum C-reactive protein <10 mg/l). No formal diet histories were available for the healthy subjects.

Venous blood was collected into EDTA or heparin containing tubes, centrifuged (500 *g*, 4 °C, 10 min) and plasma removed into plastic tubes. Packed red cells were prepared by carefully removing all the remaining plasma and the buffy coat and stored at −70 °C until analysis. The type of anti-coagulant used was found to have no effect on the PLP, PA, and PL values obtained under the present study conditions.

The Chromsystem calibrator and QC levels 1 and 2 (lyophilised material based on human plasma) were reconstituted in distilled water according to manufacturers instructions, aliquoted and stored at −70 °C (stable for at least 12 months). The concentration of PLP, PA and PL in the calibrator was 59, 32 and 65 nmol/l, respectively. Commercial QC material (Chromsystem level 1 and 2) were used for internal QC. The performance of the method was monitored

by taking part in the external quality assurance scheme for B vitamins (Instand, Dusseldorf, Germany).

For the purpose of peak identification, stock aqueous solutions of PLP, PA and PL (10 mg/ml) were prepared individually. Stock solutions of PLP and PL were prepared in 0.02 mol/l HCl and stock PA prepared in 0.02 mol/l sodium hydroxide. These were diluted in distilled water to provide a final working concentration of 100 nmol/l (final pH 5.5).

2.3. Pre-column derivatisation

PLP and PL in blood samples were measured as their semicarbazone derivatives [16,17] whilst PA was measured using its natural fluorescence. Fluorescent semicarbazone derivatives of PLP (PLPSC) and PL (PLSC) were formed away from direct light by using semicarbazide plus glycine as the derivatisation agent (DA).

2.4. Plasma

To 500-μl plasma samples, calibrator or QC, 40 μl of DA (containing 250 mg/ml of both semicarbazide and glycine) was added in a microcentrifuge tube, vortex mixed and then incubated at room temperature in the dark for 30 min. To deproteinise the sample, 40 μl of 70% perchloric acid was added to each tube, thoroughly mixed for 1 min and centrifuged for 10 min (1000 *g*). Supernatant (300 μl) was then transferred to a fresh microcentrifuge tube, stabilised by the addition of 30 μl of 25% NaOH (final pH between 3.0 and 5.0) and 40 μl injected onto the HPLC column via an autosampler.

2.5. Red cells

To 300 μl of packed red cells, 700 μl of distilled water was added in microcentrifuge tube and vortex mixed for 30 s. A 500-μl aliquot of the diluted haemolysate was derivatised, stabilised and injected as described for plasma.

The remainder of the diluted haemolysate was used for haemoglobin estimation using drabkins reagent containing potassium ferricyanide and potassium cyanide (Sigma Diagnostics, Poole, UK). This treatment oxidised the haemoglobin and converted it to stable cyanmethaemoglobin. The absorbance of cyanmethaemoglobin was measured at 540 nm using the Cobas Mira analyser (Roche Products, Hertfordshire, UK).

2.6. Chromatographic conditions

The HPLC system consisted of a Waters solvent delivery system and a Waters fluorimeter, Model 474 (Waters, Watford, UK). The isocratic mobile phase for chromatography was 60 mmol/l disodium hydrogen phosphate containing 9.5% methanol (V/V) and 400 mg/l EDTA disodium salt, adjusted to pH 6.5 with concentrated phosphoric acid. The mobile phase was filtered through a 0.45-μm nylon filter and pumped to waste through a 5-μm reverse phase column (Luna C_{18}; 4.6×250 mm protected with a 3×4 mm guard column) at a flow-rate of 1.5 ml/min. The bonded phase of Luna C_{18} analytical column is stable between pH 2 and 10.

The mobile phase was optimised with respect to separation, signal response, reproducibility of results, column stability and analysis time by varying the pH and concentration of the methanol and phosphate buffer.

2.7. Detection

Following separation, PLPSC and PLSC were measured fluorimetrically using a programmable fluorescence detector: excitation (Ex) and emission (Em) wavelengths were 380 and 450 nm, respectively. At 16 min the detector was programmed to change the Ex and Em wavelengths after detecting PLPSC to measure PA using its natural fluorescence (Ex 320 nm; Em 420 nm) and then back to 380 nm (Ex) and 450 nm (Em) at 22.5 min to detect PLSC. Signals from the detector were collected by a data management system (Millennium 2010, Waters, Watford, UK).

2.8. Quantitation

Quantitation was carried out by the method of external standardisation using a single level calibration. PLP, PA and PL concentrations (nmol/l) in

plasma and red cells were calculated by multiplying the peak height ratio of the analyte in the sample and the plasma based calibrator chromatogram with the concentration of the analyte in the plasma based calibrator. Peak heights were measured in fluorescence units by the data management system. The concentration of PLP, PA and PL in red cells was related to haemoglobin (pmol/g Hb).

2.9. Stability studies

Since PLP is photo-labile, its stability in plasma and red cells at room temperature was monitored by analysing aliquots immediately and after 2, 6, 12, 24 and 48 h of (i) storage in the dark; and (ii) exposure to light. This study was also repeated after aliquots of the pooled plasma and red cell sample were derivatised.

The long term storage stability of plasma and packed cells was monitored by storing aliquots of the pooled plasma and red cell sample at −70 °C and analysing them over a period of 12 months.

2.10. Albumin and C-reactive protein (CRP)

Albumin and CRP concentrations in plasma were measured on an automated analyser (ADVIA 1650, Bayer, Munchen, Germany) using the bromocresol green method and enhanced immunoturbimetric assay, respectively.

2.11. Statistics

In the reference population, since it was clear from the plotted data that PLP, PA and PL concentrations were not normally distributed they were log transformed and the geometric mean and 95% confidence intervals calculated. Correlations were carried out using Spearman's rank correlation test. Analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Pre-column derivatisation of PLP and PL

Preliminary work with aqueous standards had shown that maximum fluorescence intensity of PLPSC and PLSC derivatives was achieved when the final concentration of semicarbazide in the derivatisation reaction mixture was >10 mg/ml, the pH of the reaction mixture between 2.0 and 4.0 and incubation time >20 min. Increasing the incubation temperature to 60 °C did not increase the rate or yield of PLP/PL derivatisation.

In the derivatisation procedure that we have described, the final concentration of semicarbazide used was 20 mg/ml. The optimal pH (3.2) was achieved by addition of glycine (final concentration 20 mg/ml) to the derivatisation reaction.

3.2. Stability studies

Fig. 1a shows that after only 2 h of exposure to natural light, there was a significant loss of PLP from the underivatised red cell (13%) and plasma sample (17%) due to its photochemical degradation. After 24 h of exposure to light, there was a high loss of PLP from red cells (60%) and plasma (67%). However, there was no significant loss of PLP from derivatised plasma or red cells for at least 48 h at room temperature allowing a batch of derivatised samples to be analysed overnight by HPLC with an autosampler. PLP in underivatised plasma and red cells was stable for at least 12 h at room temperature if the samples were kept in the dark (Fig. 1b). After 24 h, however, up to 7% loss of PLP from plasma and 9% from red cells was evident. PLP in plasma and red cells stored at −70 °C was stable for at least 12 months. The mean value of PLP from a pooled red cell sample at the start of a 12-month storage period was 378 pmol/g Hb (range 367–390; $n=4$) and at the end it was 381 pmol/g Hb (range 370–397; $n=4$). The corresponding mean values for plasma were 49.5 nmol/l at the start (range 47–52; $n=4$) and 48.8 nmol/l after 12 months (range 46–51; $n=4$).

The stability of PL in plasma and red cells when exposed to light was similar to that of PLP (data not shown). PA in blood samples was stable for at least 48 h when exposed to light.

3.3. Chromatography

The chromatographic profiles corresponding to a

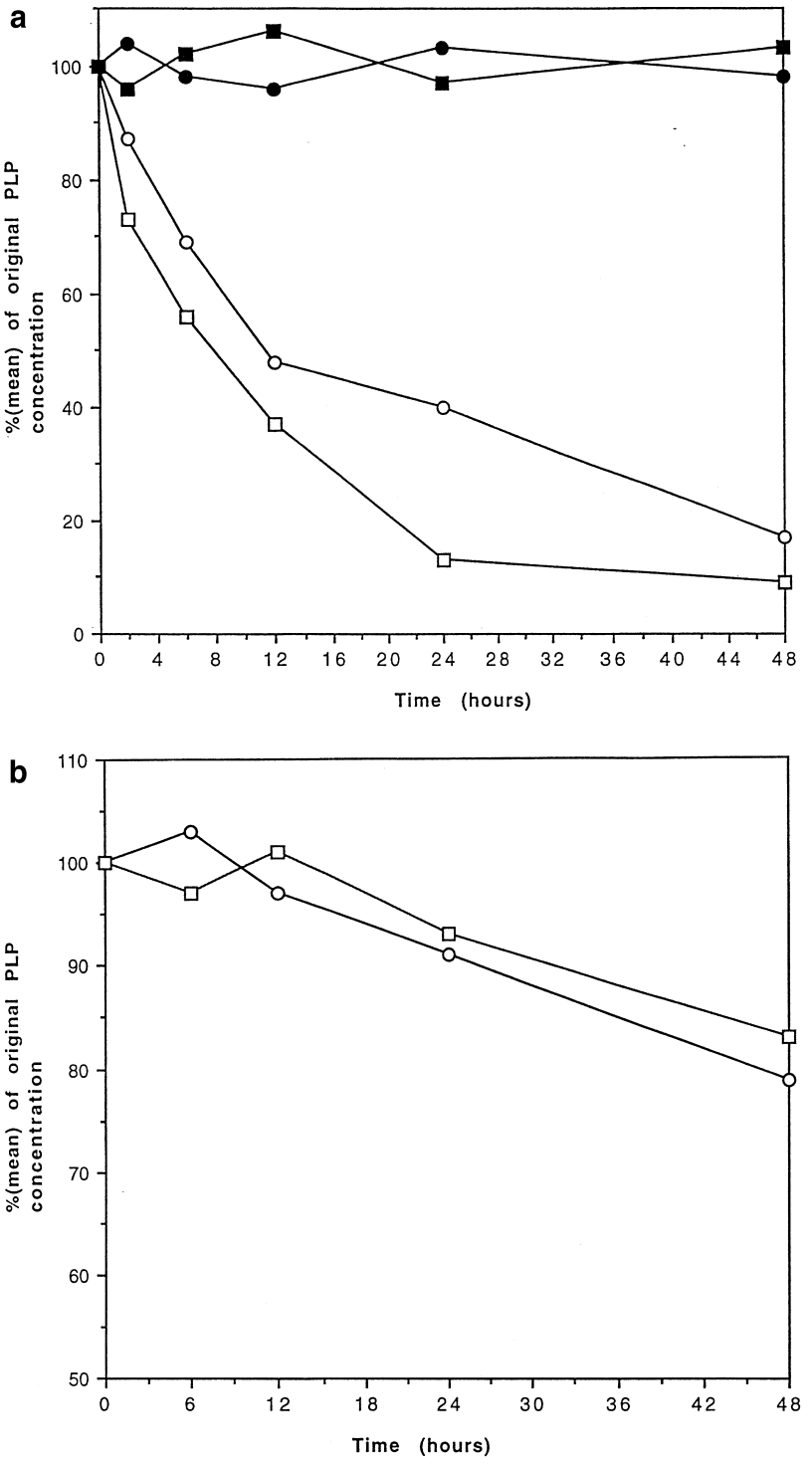

Fig. 1. Stability of underivatised and derivatised plasma and red cells. (a) When exposed to light and (b) when stored at room temperature. At each point, three aliquots of each sample were analysed to obtain mean PLP values. □, underivatised plasma; ○, underivatised red cells; ■, derivatised plasma; ●, derivatised red cells.

derivatised plasma and a red cell haemolysate extract are shown in Figs. 2a,b. The PLPSC, PA and PLSC peaks were well resolved with K' of 4.7, 11.6 and 19.8, respectively. The peaks were identified by comparing peak retention times with that of PLP, PA and PL aqueous standards after derivatisation. The peak corresponding to the retention time of PLP in plasma and red cell haemolysate extracts disappeared after pretreatment of the samples with acid phosphatase verifying that the peak was derived from a phosphorylated compound. Under the derivatisation and chromatographic conditions described, PMP and PN (100 nmol/l) did not produce a fluorescence response. PMP, which is present in red cells at concentrations similar to that of PLP, was therefore not detected using the method described.

The shape and efficiency of the PLPSC peak was found to be sensitive to the presence of EDTA in mobile phase especially with column aging. Addition of EDTA to the mobile phase at concentrations >200 mg/l yielded narrow symmetrical PLPSC peaks that were reproducible, whilst its absence resulted in broad tailing PLPSC peaks. This is possibly due to metal contamination of the HPLC column with use, which may interact with the PLPSC derivative during separation resulting in broad tailing peaks. Addition of EDTA to the mobile phase would chelate these metal contaminants from the column thus removing the interference. Neither PA nor PLSC peaks were affected by EDTA in mobile phase. As long as there was EDTA in the mobile phase, the type of anti-coagulant used in the blood collection tubes did not affect the shape or efficiency of PLPSC peak.

The concentration of the phosphate buffer in the mobile phase affected column stability and performance. At concentrations <10 m*M*, the peaks were broader and retention times longer and at concentrations >200 m*M*, the column was unstable. A phosphate concentration of 20–100 m*M* avoided both problems. The optimal concentration of the organic modifier, methanol, in the mobile phase was determined to be 9.5%, which resulted in PLPSC, PA and PLSC peaks that were well resolved with reasonable retention times. The K' of PLPSC and PLSC peaks decreased and that of PA increased when the pH of the mobile phase was varied between 4.0 and 10.0. A pH of 6.5 produced optimal resolution and retention times.

3.4. Detection of PLP and PL semicarbazone derivatives

Excitation (Ex) and Emission (Em) spectral studies had established that the optimal Ex and Em wavelength for derecting PLP and PL semicarbazone derivatives in mobile phase was 380 and 450 nm, respectively. At these wavelengths, the fluorescence intensity was independent of pH between 6.0 and 10.0. In the present study, PLP and PL semicarbazone derivatives were detected at pH 6.5.

3.5. Analytical validation

3.5.1. Linearity and detection limits

The method was linear up to at least 1000 nmol/l for PLP, PA and PL. The limits of detection, defined as three times the chromatographic base line noise, were 2.1 nmol/l for PLP, 1.0 nmol/l for PA and 2.8 nmol/l for PL. The limits of quantification, defined as 10 times the signal-to-noise ratio, were 5.8 nmol/l for PLP, 2.5 nmol/l for PA and 6.5 nmol/l for PL.

3.5.2. Recovery studies

For recovery studies, aliquots of a pooled plasma and red cell sample were analysed both before and after spiking with 40 and 80 nmol/l of PLP, PA and PL. With 40 nmol/l spike, the recoveries (mean±SD; $n=5$) of PLP, PA and PL from plasma were 97±3.9%, 91±4.4% and 94±3.0%, respectively. The corresponding recoveries from packed red cells were 97±3.1%, 89±3.7% and 91±4.1%, respectively. With 80 nmol/l spike, the recoveries (mean±SD; $n=5$) of PLP, PA and PL from plasma were 98±2.9%, 93±3.3% and 93±4.9%, respectively. The corresponding recoveries from red cells were 96±2.5%, 89±4.0% and 93±3.7%, respectively.

Quantitative extraction of PLP from red cells was only obtained when the sample was derivatised with semicarbazide before the acid precipitation step. PLP levels were much lower in red cell samples that were deproteinised prior to derivatisation (Table 1). Extraction of PLP from red cells was also incomplete if

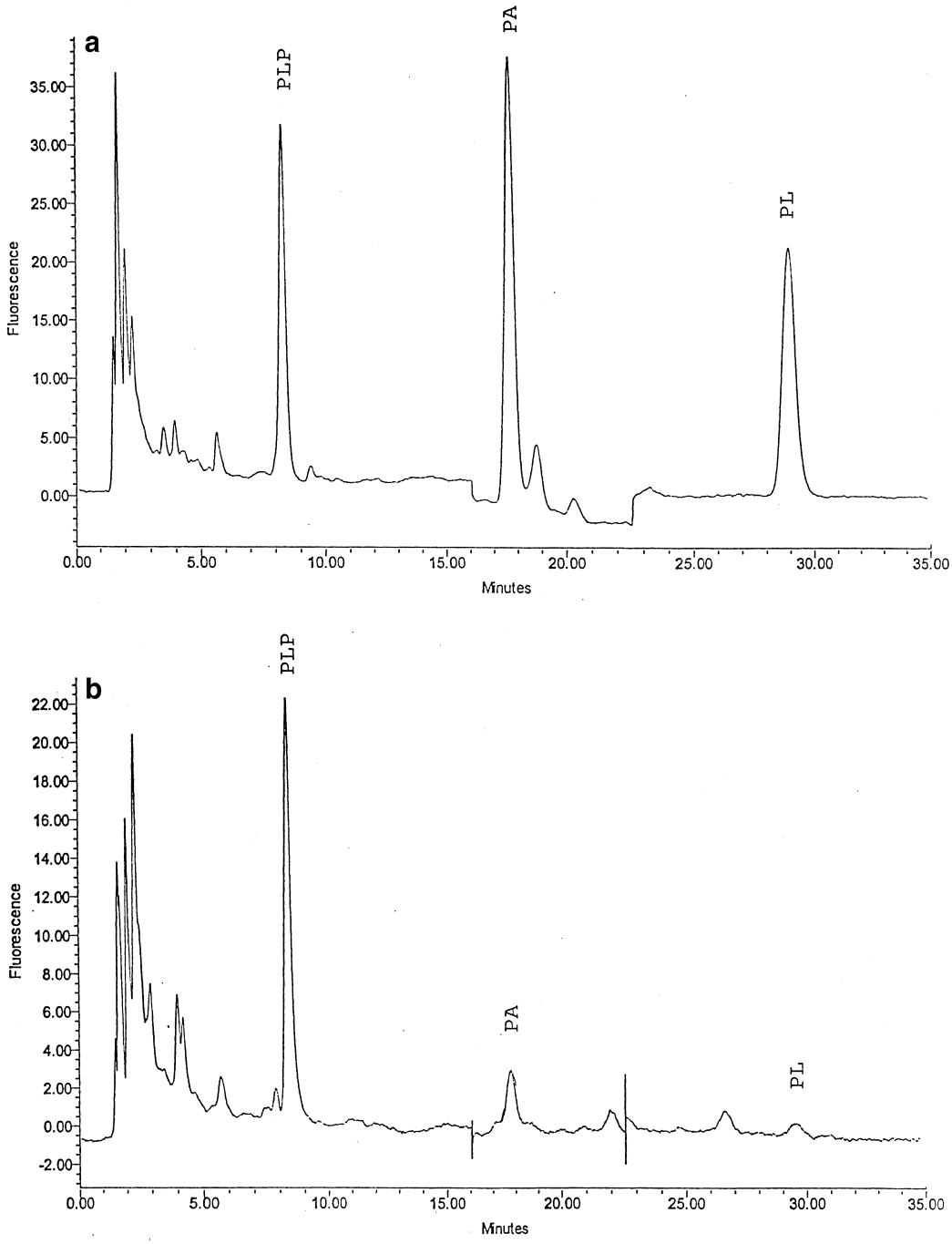

Fig. 2. Chromatographic profiles of derivatised plasma and red cells extracts. (a) Plasma based calibrator containing 59 nmol/l PLP, 32 nmol/l PA and 65 nmol/l PL; (b) a red cell haemolysate containing 425 pmol PLP/g Hb, 23 pmol PA/g Hb and 26 pmol, PL/g Hb.

Table 1
Effect of three different derivatisation methods on the efficiency of PLP extraction from pooled plasma (PP) and pooled red cells (RC)

Sample	PLP		
	Method 1[a]	Method 2[b]	Method 3[c]
PP sample1 (nmol/l)	45 (4)	47 (4)	47 (3)
PP sample 2 (nmol/l)	97 (6)	93 (5)	94 (4)
PP sample 3 (nmol/l)	53 (4)	52 (3)	50 (4)
RC sample 1 (pmol/g Hb)	369 (10)	321 (11)	188 (8)
RC sample 2 (pmol/g Hb)	481 (21)	408 (21)	280 (14)
RC sample 3 (pmol/g Hb)	318 (13)	277 (9)	181 (9)
RC sample 4 (pmol/g Hb)	581 (15)	484 (24)	367 (19)

Mean (SD) were obtained from analysing three aliquots of each sample (Intra batch).

[a] Method 1, aliquots of each sample were derivatised with semicarbazide plus glycine before protein precipitation.

[b] Method 2, aliquots of each samples were derivatised with semicarbazide without glycine before protein precipitation.

[c] Method 3, aliquots of each sample were deproteinised before derivatisation with semicarbazide plus glycine.

the sample was derivatised in the absence of glycine (Table 1).

3.5.3. Precision studies

Precision data are shown in Table 2. The Intra batch imprecision for the measurement of PLP, PL and PL in plasma was calculated by analysing commercial QC, levels 1 and 2, 10 times on the same day. The Inter batch imprecision data were obtained from level 1 and 2 analysed 19 times over a period of 5 months.

The Intra batch imprecision data for the measurement of PLP, PA and PL in red cells was obtained from a pooled red cell sample analysed 10 times on the same day. Inter batch imprecision was obtained by analysing the pooled red cell sample aliquoted and stored at −70 °C over a period of 3 months (n=17).

3.5.4. Accuracy of PLP measurements in plasma

The method showed a mean deviation of 4.8% from the all laboratory mean value (N=34) participating in the external assurance scheme (Instand e.V., Institut fur Standardisierung und Dockumentation im medizinischen Laboratorium, 40223/40093 Dusseldorf) for the measurement of PLP in plasma by HPLC for at least 12 months. There was no external assurance scheme for the equivalent red cell measurements.

3.6. Recalibration of assay

Within the same HPLC batch, the assay was recalibrated after every 10 samples to correct for any drift and to monitor the stability of the chromatographic system. No significant drift was observed in

Table 2
Precision (C.V.) of PLP, PA and PL measurements in plasma (P) and in pooled red cells (RC)

	PLP		PA		PL	
	Mean	C.V.	Mean	C.V.	Mean	C.V.
Intra batch (n=10)						
P1	59 (2.9)	4.9	12 (1.0)	8.2	36 (1.7)	4.6
P2	119 (4.4)	3.7	26 (1.9)	7.6	144 (4.3)	3.0
RC	367 (19)	5.2	24 (2.8)	11.6	36 (1.7)	4.6
Inter batch[a] (n=10)						
P1	57 (3.3)	5.8	14 (1.6)	11.5	37 (2.1)	5.7
P2	121 (5.5)	4.6	23 (1.9)	8.4	146 (6.2)	4.3
RC	370 (26)	6.9	27 (3.7)	13.7	43 (3.4)	7.9

P1 and P2, plasma QC levels 1 and 2. Mean (SD) are expressed as nmol/l for plasma samples and pmol/g Hb for pooled red cell samples.

[a] Inter batch imprecision data were obtained by analysing P1 and P2 over a period of 3 months (n=19) and a pooled red cell sample (n=17) over a period of 5 months.

calibrator at start and end of a batch containing 30 samples.

3.7. Population reference values

PLP, PA and PL concentrations in plasma and red cells were measured in 126 subjects, 67 males and 59 females with an average age of 53 years (range 31–73 years). Plasma albumin concentrations were all within the laboratory derived reference range (36–52 g/l).

Plasma and red cell PLP, PA and PL values had a non-Gaussian distribution. Geometric means and 95% reference intervals for plasma and red cell levels obtained are shown in Table 3. There was no relationship between age or gender and PLP, PA or PL concentrations in plasma or red cells.

In plasma PLP concentrations correlated with both that of PA (r^2=0.31; P<0.0001) and PL (r^2=0.49; P<0.0001) whilst in red cells PLP concentrations correlated with PL (r^2=0.55; P<0.0001) but not with PA. Red cell PLP concentrations showed a strong positive correlation with PLP concentrations in plasma (r^2=0.810, P<0.0001; Fig. 3).

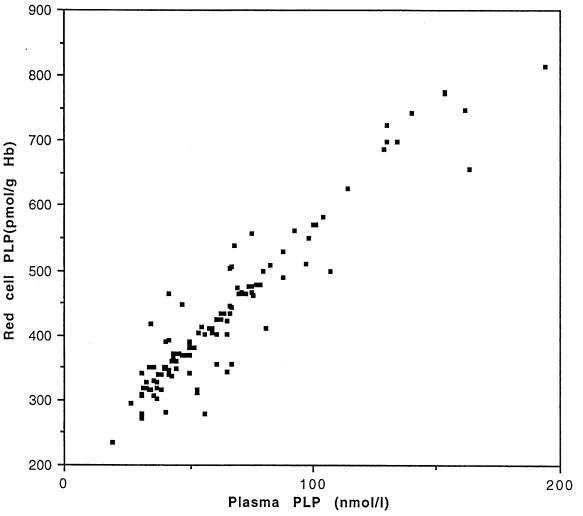

Fig. 3. The relationship between PLP concentration in plasma and red cells in a reference population (n=126).

4. Discussion

In this study we evaluated a reverse phase HPLC method for the simultaneous measurement of PLP, its degradation product PA and PL in plasma and red cells. In blood, the measurement of PLP by HPLC requires its conversion to a fluorescent derivative for adequate sensitivity. Methods have described pre- or post-column derivatisation of PLP in plasma to its fluorescent derivative using semicarbazide, cyanide or bisulfite as derivatisation agents [2,18–24]. However, post-column derivatisation requires additional equipment, a post-column pump and a reaction column, which can lead to peak broadening. Moreover, unlike pre-column derivatisation methods, the major disadvantage of post-column derivatisation procedures is that due to the photosensitivity of PLP, significant losses of PLP may occur during sample processing and analysis unless they are carried out away from UV light [18,24].

The HPLC method that we have described using pre-column semicarbazide derivatisation is simple to perform, reliable and has adequate sensitivity and precision for the routine measurement of PLP in plasma and red cells. The sensitivity of this method is comparable to that reported by Vuilleumier et al. [2] and Millart et al. [19]. Although the method described has a relatively long separation time (30 min) for the simultaneous measurement of PLP, PL and PL in blood, this is of little consequence since the routine analysis of derivatised samples were performed as a batch overnight. The method does not detect PMP, which is present in significant concentration in red cells. However, the usefulness of red cell PMP measurements for assessing vitamin B6 status has not been established.

Several pre-column derivatisation procedures using potassium cyanide or semicarbazide as de-

Table 3
Plasma and red cell levels of PLP, PA and PL in a reference group (n=126)

	Geometric mean	95% reference interval
Plasma		
PLP (nmol/l)	56	21–138
PA (nmol/l)	23	9–60
PL (nmol/l)	11	5–26
Red cell		
PLP (pmol/g Hb)	410	250–680
PA (pmol/g Hb)	22	9–54
PL (pmol/g Hb)	68	25–195

Geometric mean, i.e. antilog10 of log10−mean.

rivatising agents have been described for measurement of PLP in plasma [2,18–21]. Although the amounts of potassium cyanide used for derivatisation are small, the use of this reagent has safety implications. Pre-column semicarbazide derivatisation procedures that have been reported [2,18] require post-column alkalinisation to detect PLP with adequate sensitivity. In addition, the method described by Ubbink et al. [18] used trichoroacetic acid, rather than perchloric acid used in the present study, for protein precipitation, which had to be removed with diethyl ether prior to injection. Therefore, compared with the present method, these methods are less suitable for routine application.

Using perchloric acid to precipitate the proteins, derivatisation with semicarbazide was not a limiting factor for the extraction of PLP from plasma as we found that PLP concentrations were not significantly different in plasma samples that were derivatised before or after protein precipitation. However, in erythrocytes, PLP has to be derivatised before protein precipitation in order to dissociate PLP quantitatively from red cell proteins. This is probably because the binding of PLP to plasma proteins, predominately albumin, is not as strong as its binding to haemoglobin in red cells (PLP binds to haemoglobin about twice as strongly as to serum albumin) [25].

These observations are consistent with those reported by Srivastava et al. [17] who demonstrated that an excess of semicarbazide can also be used to liberate PLP from red cell proteins, mainly haemoglobin, by breaking the schiff base of PLP and proteins to form semicarbazone. The PLP schiff base with red cell proteins is very stable and in the absence of semicarbazide may not be quantitatively broken by protein precipitating agents like perchloric acid and is therefore removed in the precipitated protein resulting in low recoveries [17]. Glycine was also found to influence extraction of PLP from red cells. The mechanism of its action is unclear, but it may act as a nucleophillic catalyst for PLP semicarbazone formation [26].

In the present study we also compared the HPLC measurement of PLP in plasma with that of red cells and established population reference ranges for PLP, PA and PL. The reference values for PLP, PA and PL in plasma are in good agreement with data reported by other workers [19–22]. We related PLP concentrations in red cells to Hb rather than to volume of packed red cells because, due to their viscosity, accurate pippeting of packed red cells is difficult and therefore, adversely affects the precision of the HPLC assay. There was a strong positive correlation ($r^2=0.81$) between plasma and red cell PLP levels in the reference population. Given that plasma PLP concentrations are valid indicators of vitamin B6 status, this would suggest that in the reference population at least, plasma PLP concentration reflects that in the red cell and therefore either plasma or red cell PLP measurements can be used as markers of vitamin B6 status. It is therefore of interest that we have recently demonstrated a lack of concordance between plasma and red cell PLP levels for assessing vitamin B6 status in patients with an acute systemic inflammatory response (median CRP, 130 mg/l) and receiving vitamin B6 supplementation [27]. In this group of patients, PLP concentrations, compared with the reference population, PLP concentrations were significantly lower in plasma (approx. 30% of control) but significantly higher in red cells (approx. 150% of control). Therefore, red cell PLP levels, in contrast to plasma PLP levels, may be useful in differentiating true from apparent vitamin B6 deficiency in patients with a systemic inflammatory response.

In summary, we have analytically validated a HPLC method coupled with pre-column derivatisation and showed that it is suitable for the routine measurement of PLP in both plasma and red cells. Since PA (metabolite of PLP) and PL can be simultaneously measured with PLP, the method is also suitable for research studies on PLP metabolism in humans.

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