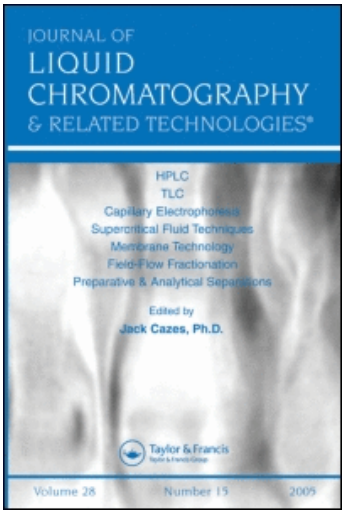

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Simple Internally-Standardised Isocratic HPLC Assay for Vitamin B_6 in Human Serum

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To cite this Article Reynolds, T. M. and Brain, A.(1992) 'A Simple Internally-Standardised Isocratic HPLC Assay for Vitamin B_6 in Human Serum', Journal of Liquid Chromatography & Related Technologies, 15: 5, 897 — 914
To link to this Article: DOI: 10.1080/10826079208018843
URL: http://dx.doi.org/10.1080/10826079208018843

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(5), 897-914 (1992)

A SIMPLE INTERNALLY-STANDARDISED ISOCRATIC HPLC ASSAY FOR VITAMIN B_6 IN HUMAN SERUM

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ABSTRACT

A simple method which is practical for use in a routine clinical chemistry laboratory,for assay of vitamin B_6 vitamers in serum is presented. The procedure uses a C_{18} (ODS) analytical column and fluorimetric detection (325 nm excitation, 400 nm emission) after post column derivatisation with pH 7.5 phosphate buffer containing 1g/l sodium sulphite. The mobile phase is pH 2.5, 0.067 mol/l KH_2PO_4 adjusted with concentrated orthophosphoric acid and containing 125 umol/l sodium hexanesulphonate as an ion pairing agent. Pyridoxamine 5'-phosphate is suggested for use as an internal standard when assaying human serum.

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INTRODUCTION

A multitude of methods have been developed for the assay of vitamin B_6 in biological fluids. Indirect assessment giving an index of vitamin status without assaying absolute vitamin concentration is possible by measurement of the enhancement effect due to addition of pyridoxal-5'-phosphate (PLP) on the activity of aspartate aminotransferase (AST) (1,2). Other methods measure vitamin B_6 concentration and include microbiological (3), radioenzymatic (4), non-isotopic enzymatic (5) and chromatographic methods. Only the latter have found widespread clinical application as the other techniques are too time consuming.

Chromatographic methods fall into two main groups: open column methods (Amberlite resin or phosphocellulose with fluorimetric detection (6,7,8)) and high-performance liquid chromatography (HPLC). There is a wide variety of HPLC methods but many are also too time consuming or complex for routine clinical use and include; eg. ternary gradient chromatography requiring 5 different buffer compositions (9), twin column chromatography (10) (this method is no longer possible as the packing material is no longer available (11)), and paired ion binary gradient chromatography (12). Newer methods using C_{18} octadecylsilane (ODS) columns have been developed but either use hazardous reagents such as potassium cyanide for derivatisation (13) or acetonitrile in the mobile phase (14), or rely on complex gradient elution (11) requiring sophisticated pump systems.

We describe a simple internally standardised isocratic assay for PLP in serum which uses no organic solvents and has a total run time of 30 minutes per sample.

MATERIALS

Reagents

Pyridoxamine-5'-phosphate (PMP), Pyridoxamine (PM), PLP, Pyridoxal (PL), Pyridoxine (PN), hexane sulphonic acid (sodium salt) and sodium sulphite were obtained from Sigma Chemical Co, (Poole, Dorset, UK). Potassium dihydrogen phosphate, sodium monohydrogen phosphate, orthophosphoric acid, trichloroacetic acid and perchloric acid were obtained from Merck Ltd., (Poole, Dorset, UK).

HPLC Instrumentation

The HPLC system comprised a dual reciprocating Constametric Model III pump (LDC, Stone, Staffs, UK) with a 200ul Rheodyne 7125 injection loop and a 250 x 4.6mm Spherisorb ODS2 column (12%C end capped, 5um particle size). Within 50mm of the outlet from the column a T piece (Jones chromatography, Hengoed, South Wales) was attached to allow injection of post column derivatisation agent, pumped by a dual

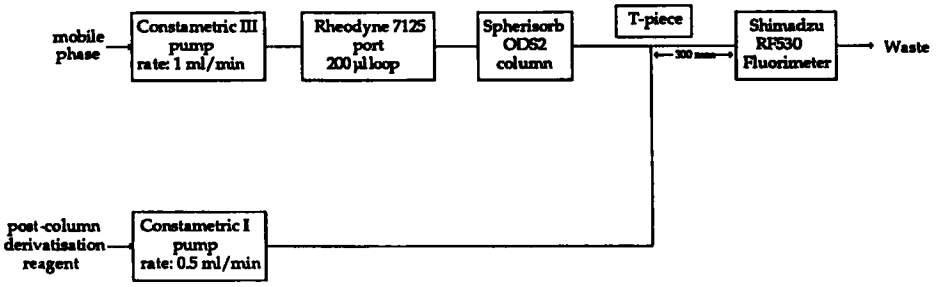

Fig 1. A schematic diagram showing the HPLC equipment used.

reciprocating Constametric Model I pump (LDC). There was a further 300mm of tube before the detector which was a Shimadzu RF530 fluorimeter (Dyson instrunents, Houghton-Le-Spring, UK) with a 12ul flow cell, wavelengths for maximum emission from derivatised PLP: 325nm excitation; 400nm emission (Fig 1).

METHODS

Buffers

The mobile phase buffer was 0.067 mol/l potassium dihydrogen phosphate soultion with 125 umol/l sodium hexane sulphonate adjusted to pH 2.5 with concentrated orthophosphoric acid. The pump rate was 1ml/min. The post column reagent was 0.067 mol/l phosphate buffer (potassium dihydrogen phosphate solution mixed with Disodium

hydrogen phosphate solution) at pH 7.5 with 1g/l sodium sulphite pumped at 0.5ml/min and was similar to a post-column derivatisation reagent previously described (9). As the column aged and consequently lost efficiency, it was necessary to increase the concentration of ion pair (up to a maximum of 250 umol/l) to maintain separation.

Standards

Stock standards (PMP (2.5 mmol/l); PM (5.0 mmol/l); PLP, PL, PN (10 mmol/l)) in mobile phase buffer were prepared and frozen at -20°C in 250ul aliquots. For method development concentrated mixed standard was prepared from the stock solutions by diluting 10ul aliquots of each to 10ml in mobile phase buffer. This concentrated mixed standard was used to supplement serum samples during method development. The working mixed standard (PMP (25 nmol/l); PM (50 nmol/l; PLP, PL, PN (100 nmol/l)) was prepared by diluting 100ul of concentrated mixed standard to 10ml with mobile phase buffer. Since the only vitamer found in serum was PLP, the working standard for assay standardization was simplified to PLP, 100 nmol/l which allowed other vitamers to be tested for use as an internal standard.

It has been reported that mixed B_6 standards are not stable even when frozen (15). To clarify this we froze freshly prepared concentrated and working mixed standards for 18 h at -20°C before analysis against freshly prepared standard. The stability of mixed standard at room

temperature was evaluated by running 28 consecutive working mixed standard samples at 30 min intervals using a Marathon autosampler (LDC) with a Waters 740 peak integrator (Millipore, Watford, UK) to measure peak area.

Samples

Sample preparation requires perchloric acid deproteinization (2ml serum plus 100ul of 4 mol/l perchloric acid) followed by centrifugation (1500g, 5 min) and filtration of the supernatant through a disposable syringe filter (0.45um pore size, 25mm diameter, BDH).

Method development was performed using human serum and whole blood. The stability of vitamers in serum and heparinised whole blood at room temperature was examined by supplementation with mixed concentrated standard (final concentrations = Blank plus: PMP (25 nmol/l); PM, PLP, PN (50 nmol/l); PL (100 nmol/l)). A blank and zero time supplemented serum and separated whole blood samples were frozen at -20°C (16). At regular intervals a serum and separated whole blood sample were frozen for later batch analysis.

To examine the rate of disappearance of vitamers other than PLP, a blood sample from a volunteer was assayed within 5 min of venepuncture.

Analytical Evaluation

Linearity and within batch precision were assessed using doubling dilutions of mixed standard and of serum supplemented with concentrated mixed standard. Mean recoveries were estimated using supplemented serum (n=6) and calculated thus:

$$\text{Recovery} = \frac{(\text{amount in supplemented serum} - \text{amount in blank})}{(\text{amount of compound added})} \times 100\%$$

Internal Standardisation

DPN was unsatisfactory as an internal standard because the retention time was significantly longer than that for PLP. Pyridoxamine-5'-phosphate (100ul of 1000nmol/l PMP to 2ml serum or working standard before addition of 200 ul perchloric acid), proved to be ideal as it is not present in normal human serum and eluted before PLP (retention times shown in table 1.

Reference Range

A reference range was derived from 62 samples taken from randomly selected hospital out-patients (age range 23-82 years), having blood tests for other reasons. Forty-five of these samples were also assayed for AST, ALT, albumin, total protein and creatinine. The serum from these patients was frozen at -80^{o}C for up to 3 days before analysis.

Table 1. Analytical parameters calculated on results from a run of mixed standard performed using a new column and 125 umol/l sodium hexane sulphonate in the mobile phase.
k' = capacity factor
α = selectivity
R = resolution

	Retention time (min)	k'	α	R
PMP	4.6	0.26		
			6.9	2.0
PM	5.5	0.50		
			2.4	4.3
PLP	8.1	1.2		
			2.2	5.6
PL	13.4	2.7		
			1.5	3.7
PN	18.1	4.0		

RESULTS

Stability of Standards

Freezing of mixed standard resulted in significant loss of vitamers. After one freeze-thaw cycle the decreases in peak heights recorded were: PMP, 100%; PM, 60%; PLP, 30%; PL, PN, 12%. Mixed standard at room temperature under normal lighting conditions showed no significant loss of PLP ($p>0.05$) but significant changes in concentrations of other vitamers over 14 h. The changes measured in percentage change in peak area /hr were: PMP, +0.82%; PM, -1.23%; PL, +0.54%; PN, +0.65%.

Table 2. Linearity and detection limits assessed using serial dilutions of working mixed standard. Regression parameters relate vitamer concentration in nmol/l (x) to peak height in mm (y). Maximum limit of linearity is also given for extracted serum.

	PMP	PM	PLP	PL	PN
A	-1.0	1.1	2.0	3.2	2.8
B	2.1	1.3	0.45	0.53	0.34
r	0.993	0.996	0.992	0.999	0.993
Detection Limit (nmol/l)	1.5	1.5	5.0	12.5	12.5
Amount injected (pmol)	0.3	0.3	1.0	2.5	2.5
Limit of Linearity (nmol/l)	50	100	200	200	100

Analytical Parameters

When the ion pair concentration is optimised for column efficiency the method gives resolution to baseline for all vitamers studied. Resolutions, capacity factors and selectivities calculated for a run using a new column with 125 umol/l sodium hexanesulphonate in the mobile phase are shown in Table 1, linearity and detection limits assessed using doubling dilutions of mixed standard in Table 2. and within batch imprecision assessed at two levels for unextracted standard and serum supplemented with concentrated mixed standard in Table 3.

Table 3. Within batch precision for standard and supplemented serum

Concentration (nmol/l)	PMP	PM	PLP	PL	PN
Unextracted standard					
12.5	4.0%				
25		8.4%	7.8%		9.1%
50	6.4%			4.1%	
100		3.4%	6.1%		3.9%
200				2.0%	
Supplemented serum					
12.5	7.9%				
25		5.0%	2.3%		
50	1.9%			11.6%	6.7%
75					5.4%
100		2.5%	6.9%		
160				14.0%	

Recovery

Recoveries assessed by supplementing serum to 50% of working mixed standard concentrations were: PMP, 101±4.2%; PM, 100±5%; PLP, 103±6.7%; PL, 100±11%; PN, 110±5.4%. Values quoted are mean and standard deviation (n=6).

Vitamers in Serum and Effect of Immediate Analysis

In a serum sample taken from a healthy volunteer and analysed immediately after venepuncture, the only vitamer peak observed was PLP.

Stability of Vitamers in Supplemented Serum and Blood at Room Temperature

The concentrations of vitamers expressed as percentage of vitamer concentration at t=0 for heparinised blood and serum are shown in Table 4.

Internal Standardization

PMP was found to be an ideal internal standard as it ran close to the PLP peak without interfering. Also it did not cause an increase in run time and recovery of PMP was approximately 100%. A typical chromatogram for serum with internal standard is shown in figure 2.

Co-eluting Substances

The column effluent was passed through a UV detector after the fluorimetric detector to determine whether there were any co-eluting

Table 4. Loss of vitamers in serum and whole blood at room temperature. Values in table are quantity of vitamer remaining as percentage of vitamer measured at time = zero (serum) or time = 1 h (plasma).

		Time (h) 0	1	2	4	6	9	12	24
PMP	serum	100	31	31	0	0			
	plasma		100	96	78				69
PM	serum	100	100	102	97	100	95	97	95
	plasma		100	69	45	30			10
PLP	serum	100	100	100	100	100		89	93
	plasma		100	101	96	98			91
PL	serum	100	107	93	93	100	93	110	100
	plasma		100	92	81		97		49
PN	serum	100	100	80	80	100		90	
	plasma		100	83	0	0			0

peaks. For working mixed standard there were no detectable absorbance changes at 292 nm or 254 nm. When serum was injected, there was 1 peak at 292 nm and 4 at 254 nm which eluted at a similar time to PLP. However, none of these peaks were detectable by fluorescence. It was therefore concluded that although there were substances which eluted with similar retention times to PLP, the fluorimetric method of detection was sufficiently specific to render these insignificant.

Relationship with Other Analytes

No significant correlations between PLP and other analytes were demonstrated.

Reference Range

PLP was determined on 62 samples. To test whether there was any significant age or sex differences the results were separated into 4 approximately equally sized groups: (Male/Female, Age<60/Age>60). Since the distribution of results was non-Gaussian, the null hypothesis that all results came from the same distribution was tested non-parametrically using the Smirnoff test. The test statistic for each pairing was (critical value for rejection of null hypothesis at 95% level in brackets): male<60/male>60, 0.375 (0.519); female<60/female>60, 0.157 (0.474); male <60/female<60, 0.188 (0.454); male>60/female>60, 0.202 (0.535). Since no significant differences were shown all results were used to generate a single reference range by probability plotting of LOG transformed data. The range determined using cutoffs at the 2.5 and 97.5 percentiles was 13 - 106 nmol/l.

DISCUSSION

Primary dietary deficiency of vitamin B_6 is rarely seen in developed countries because the vitamin is found in a large variety of foods (17) but

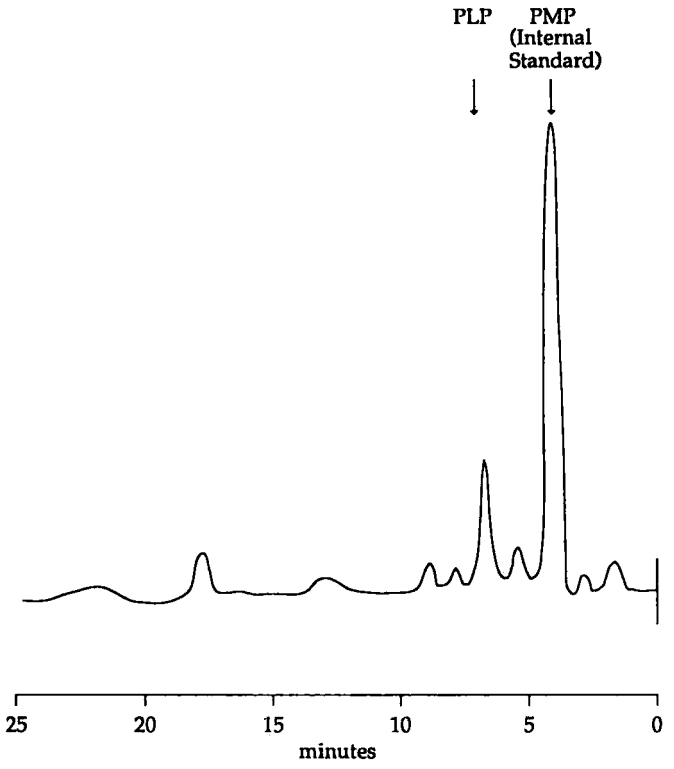

Fig 2. A typical chromatogram from a patient sample with PMP as internal standard.

it is seen in malnourised populations and in cystic fibrosis (18), asthma (19), renal failure (20) and some other clinical conditions. Pharmacological supplementation of the diet with vitamin B_6 must however, be performed with caution because overdosage has been associated with sensory neuropathy (21) and possible teratogenesis (22). This is particularly important as the vitamin is popularly associated with prevention of premenstrual syndrome (22) and is frequently self prescribed in large

doses. A simple assay for the vitamin would therefore be extremely useful.

The only vitamer which we found to be present in human serum in significant quantities was PLP. This is important for two reasons: Firstly because it allows the use of one of the other four vitamers as an internal standard. Pyridoxal and PN were not good candidates due to wide peaks and the presence of interfering substances while PM was unsuitable because it elutes very near to PLP. Furthermore, PN is the vitamer widely employed in pharmaceutical preparations (23). Therefore, PMP is the only vitamer which can reasonably be used as the internal standard. Our evaluation of PMP found it to be entirely adequate for this purpose. Secondly, there is relatively poor efficiency for assay of PL and PN but since these vitamers are not present in human serum in significant concentrations, this is not important.

The stability of mixed standard appears to cause a problem but since individual stock standards are stable when frozen this does not in practice create any difficulties. Providing new working standard is prepared each day, the method gives very consistent results. Furthermore PLP is the only vitamer which needs to be present in the standard if the internal standardization method is employed which simplifies working standard preparation.

Previous reports have shown that PLP is rapidly lost when exposed to high intensity light (100W light bulb at 15 cm) and that there is a 10%

Table 5. Reference values for PLP reported by other workers. Mean and sd is not quoted for our reference data because we found a non-Gaussian distribution. The range presented as 'this paper' refers to the 2.5-97.5 inter-percentile range.

Reference	n	Mean[PLP] (±sd) (nmol/l)	Range (nmol/l)
4	29	68.7 ± 40.4	20 - 202
9	38	57.0 ± 26.0	
11	4	75.0 ± 24.0	
13	30	58.5 ± 19.4	24 - 93
14	21	69.0 ± 28.0	20 - 134
24	27	50.5 ± 25.0	
25	9	29.5 ± 17.0	
This paper	62		13 - 106

loss of PLP after 24 h in the dark (26). We found that loss of PLP was not significant during the first 6 h after specimen collection with the sample stored at room temperature under normal lighting conditions. Thus, providing the specimen reaches the laboratory on the day it is collected special blood collection protocols are unneccessary.

Our reference values were slightly lower than others previously reported (Table 5). This is possibly because we used an out-patient population while other studies used healthy volunteers. We are unaware

of any previous report of a non-gaussian distribution for PLP although the range (20-202 nmol/l) with mean of 69 nmol/l quoted by Camp et al (4) suggests a similarly skewed distribution. In common with other workers we found no significant sex or age differences for PLP (14).

Reports of other vitamer concentrations vary. PL would be detectable by our assay if it were present in the concentrations reported by Coburn and Mahuren (23±10 nmol/l) (9), but not at the concentration reported by Shephard et al (7.4±6 nmol/l) (24). Other vitamer concentrations are below our detection limits (11,24).

The HPLC method we present has several advantages over others previously described: we have eliminated organic solvents; there is no requirement for gradient elution and consequently no time-consuming column re-equilibration step; the total run time is only 30 min allowing a good throughput even without an autosampler; a practical internal standard (PMP) has been identified; perchloric acid does not need to be removed so sample preparation is simple. Consequently this method represents a practical assay for use in routine clinical chemistry laboratories.

REFERENCES

1. R. Bayoumi, R. Rosalki, Clin. Chem., 22: 327-335 (1976)

2. J. Mount, E. Heduan, C. Herd, R. Jupp, E. Kearney, A. Marsh, Ann. Clin. Biochem., 24: 41-46 (1987)

3. H. Sauberlich, Ann. Rev. Nutr., 4: 377-407 (1984)

4. V. Camp, J. Chipponi, B. Faraj, Clin. Chem., 29: 642-644 (1983)

5. P. Edwards, P. Liu, G. Rose, Ann. Clin. Biochem., 26 158-163 (1989)

6. M. Chauhan, K. Dakshinamurti, Anal. Biochem., 96: 426-432 (1979)

7. M. Chauhan, K. Dakshinamurti, Clin. Chim. Acta., 109: 159-167 (1981)

8. S. Contractor, B. Shane, Clin. Chim. Acta., 21: 71-77 (1968)

9. S. Coburn, D. Mahuren, Anal. Biochem., 129: 310-317 (1983)

10. J. Vanderslice, C. Maire, J. Chromatogr., 196: 176-179 (1980)

11. D. Sampson, K. O'Conner, Nutr. Res., 9: 259-272 (1989)

12. G. Tryfiates, S. Sattsangi, J. Chromatogr., 227: 181-186 (1982)

13. H. Millart, D. Lamiable, Analyst, 114: 1225-1228 (1989)

14. P. Edwards, P. Liu, G. Rose, Clin. Chem., 35: 241-245 (1989)

15. G. Shephard, D. Labadarios, Clin. Chim. Acta., 160: 307-312 (1986)

16. M. Andon, R. Reynolds, Am. J. Clin. Nutr., 45: 1461-1465 (1987)

17. A. Truswell, Br. Med. J., 291: 1103-1106 (1985)

18. B. Faraj, D. Caplan, V. Camp, E. Pilzer, M. Kutner, Pediatrics, 78: 278-282 (1986)

19. R. Reynolds, C. Natta, Am. J. Clin. Nutr., 41: 684-688 (1985)

20. B. Lacour, C. Parry, T. Druecke, M. Touam, H. Kreis, M. Bailly, D. Durand, Clin. Chim. Acta., 127: 205-215 (1983)

21. H. Schaumberg, J. Kaplan, A. Windebank, N. Vick, S. Rasmus, D. Pleasure, M. Brown, New Engl. J. Med., 309: 445-448 (1983)

22. L. Gardner, J. Welsh-Sloan, R. Cady, Lancet 1: 636 (1985)

23. British National Formulary, 20: 313 (1990)

24. G. Shephard, M. Louw, D. Labadarios, J. Chromatogr., 416: 138-143 (1987)

25. A. Lui, L. Lumeng, T Li, Am. J. Clin. Nutr., 41: 1236-1243 (1985)

26. J. Ubbink, W. Serfontein, L. DeVilliers, J. Chromatogr., 342: 277-284 (1985)