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Note

Analysis of vitamin B_6 vitamers in human tissue by cation-exchange high-performance liquid chromatography

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Vitamin B_6 is present in biological fluids and tissues as a number of related vitameric forms, viz. pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), as well as their corresponding 5′-phosphorylated analogues and their biologically inactive metabolic end-product, pyridoxic acid (PA). The recent application of high-performance liquid chromatography (HPLC) to the analysis of vitamin B_6 vitamers in plasma [1–4] has enhanced the feasibility of vitamer analysis in a range of physiological samples which would lead to a more complete understanding and definition of its nutriture.

As an easily sampled tissue, as well as a tissue involved in vitamin B_6 metabolism, erythrocytes have received relatively little attention. The analysis of vitamin B_6 in blood has generally only involved the determination of the coenzyme form, pyridoxal 5′-phosphate (PLP), and occasionally of PL in plasma or in whole blood [5–8], with little work reported on erythrocytes alone. PLP levels have been analysed in erythrocytes [9–11], leucocytes [11,12], lymphocytes, polymorphs and platelets [13], and the PL kinase activity in erythrocytes and leucocytes has been investigated [14]. As regards the vitamer distribution in blood cells, open-column phosphocellulose ion exchange with subsequent chemical analysis has been used to determine this distribution in whole blood [15], while thin-layer chromatography has been used to separate radiolabelled B_6 vitamers in leucocyte hydrolysates [16].

This publication reports the modification of existing methodology to analyse

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vitamin B_6 vitamers in human erythrocytes, mononuclear cells (MNCs) and human liver biopsy tissue. It further reports for the first time a complete comparison of plasma and erythrocyte vitamer levels in ten laboratory volunteers.

EXPERIMENTAL

The cation-exchange HPLC assay system, with post-column derivatisation, based on the method of Coburn and Mahuren [2], as well as the storage and preparation of vitamer standard solutions and the analysis of plasma samples have been previously described [1,17].

Blood samples were drawn by venipuncture from ten normal, healthy laboratory volunteers [three males, seven females, age 36 $\pm$ 10 years (mean $\pm$ S.D.)] none of whom were taking vitamin supplements. In order to analyse both erythrocyte and plasma vitamer levels, blood samples were collected in silicone-coated 'no additive' tubes (VAC-U-TEST®, red stopper, Cat. No. RE 0505, Radem Laboratory Equipment, Johannesburg, South Africa) to which 5 mg of solid disodium EDTA had been added as an anticoagulant [1].

The blood was centrifuged at 1200 *g* at 4°C for 15 min, plasma removed, and the packed erythrocytes were washed twice with equal volumes of saline (154 mmol/l). If not analysed immediately, erythrocytes were stored at −20°C, the vitamers being stable under these conditions for at least a month.

The vitamin B_6 vitamers were extracted by adding packed erythrocytes (0.25 ml) to distilled water (0.5 ml). After standing at room temperature for 5 min, proteins were precipitated by addition, while vortexing, of 40% trichloroacetic acid (125 μl). Internal standard solution [135 μl of a 0.01 *M* hydrochloric acid solution of 4′-deoxypyridoxine-5′-phosphate (DPNP) equivalent to 125 ng of DPNP] was added and the precipitated proteins were immediately spun down at 8700 *g* for 2 min at room temperature in a Microfuge centrifuge (Beckman, Palo Alto, CA, U.S.A.). The supernatant was withdrawn, extracted twice with diethyl ether (8 ml), purged with nitrogen to remove excess diethyl ether and injected (300–500 μl) onto the HPLC column.

MNCs were prepared from blood samples drawn by venipuncture and collected in heparin tubes. The heparinised blood was diluted 1:1 with phosphate-buffered saline (PBS; 0.85% saline buffered to pH 7.2, supplied by Difco Labs., Detroit, MI, U.S.A.) and loaded onto Lymphoprep (Nycomed, Oslo, Norway). After centrifuging at 2100 *g* for 20 min at room temperature, the MNC layer was collected and washed three times with PBS, once at 450 *g* for 15 min and twice at 750 *g* for 10 min. The final pellet was resuspended in a known quantity of PBS, and 20 μl of this cell suspension were diluted to 200 μl with Turk's solution for counting on an improved Neubauer counting chamber under a microscope at 40× magnification.

Vitamin B_6 vitamers were extracted from MNCs by cell lysis. Distilled water (500 μl) was added to the cell pellet (containing approximately $10 \cdot 10^6$ cells)

followed by a rapid freeze–thaw step using liquid nitrogen. After standing 5 min, the lysate was diluted with 250 μl of 0.01 *M* hydrochloric acid, proteins were precipitated with 250 μl of 40% trichloroacetic acid and internal standard was added (20 μl of a hydrochloric acid solution of DPNP equivalent to 125 ng DPNP). The extract stood for 10 min before being centrifuged, was extracted with diethyl ether and injected (500 μl) onto the column in the same manner as for the erythrocyte extract.

Human liver samples (obtained post-mortem or by needle biopsy) were prepared for HPLC analysis as follows. A weighed liver sample (50 mg) was homogenised in 2 ml of 0.01 *M* hydrochloric acid containing 10% trichloroacetic acid and 40 μl of a hydrochloric acid solution of DPNP (equivalent to 250 ng DPNP). The homogenate was centrifuged at 1200 *g* for 15 min at 4°C. The supernatant was extracted with diethyl ether and injected (150 μl) onto the column in the same manner as for the erythrocyte extract.

Analytical recoveries were performed on samples by addition of vitamer standards to the sample aliquot while vortexing with the trichloroacetic acid protein precipitant.

RESULTS AND DISCUSSION

This HPLC analysis of vitamin B_6 vitamers in erythrocytes and MNCs is based on that previously reported for plasma [1]. The same internal standard (DPNP) has been used as chromatograms of cell extracts have shown no interference in the region of the chromatogram where DPNP elutes. A typical chromatogram of a normal erythrocyte sample is shown in Fig. 1. The main vitamin B_6 vitamers detected were PLP, pyridoxamine 5′-phosphate (PMP) and PL, while PN, PM and PA were below detectable limits (<1 ng/ml of packed erythrocytes). Table I shows the precision for the replicate analysis of a single erythrocyte sample, as well as recovery data on vitamer standards added to the deproteinised hemolysate.

Table II compares the vitamin B_6 vitamer levels found in erythrocytes and plasma of ten normal laboratory volunteers. Plasma vitamer levels are similar to those previously published by various workers [1–3,18]. The erythrocyte PLP values fall within a similar range to the plasma PLP levels. Previous workers [8], analysing both plasma and whole blood, have inferred that PLP is approximately equally distributed between erythrocytes and plasma in non-B_6-supplemented subjects.

Whereas plasma acts as the transport medium for PA, negligible levels of PA were found in erythrocytes indicating that erythrocytes do not contribute significantly to the formation of this biologically inactive metabolite of vitamin B_6. The contrast in PMP levels between the two blood compartments is indicative of the differing activity of aminotransferases in plasma and erythrocytes.

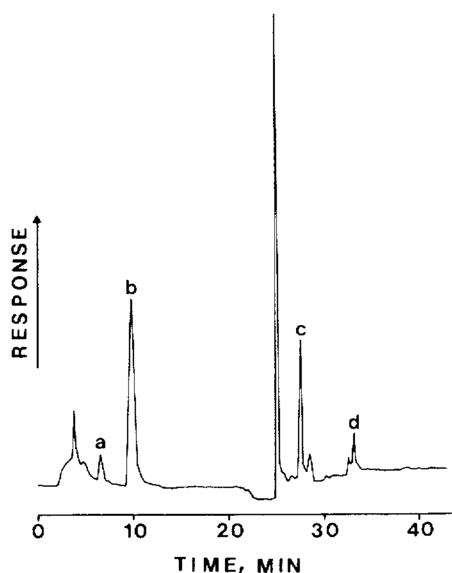

Fig. 1. Chromatogram of erythrocyte sample (300-μl injection). Peaks: a=PLP; b=DPNP; c=PMP; d=PL.

TABLE I

PRECISION AND RECOVERY DATA FOR THE ASSAY OF ERYTHROCYTE B_6 VITAMERS

Vitamer	Coefficient of variation[a] (%)	Recovery[b] (%)
PLP	4.1	84.4 ± 9.6
PMP	1.7	110.0 ± 15.9
PL	3.5	114.6 ± 11.9

[a]Six replicate analyses of a single sample.
[b]Recovery (mean ± S.D.) for five normal erythrocyte samples spiked with 7 ng PMP and PL and 16 ng PLP per ml packed erythrocytes.

The high level of these enzymes in erythrocytes leads to relatively high levels of PMP.

Whereas PLP is tightly bound to protein in both erythrocytes and plasma (predominantly hemoglobin and albumin, respectively), the binding of PL is much weaker. Thus, PL can readily cross the cellular membrane, and differences in PL distribution between erythrocytes and plasma result from differences in protein binding [10]. Previous studies on the uptake of PL by erythrocytes have shown there to be an approximate ratio of 2:1 in concentration of PL in erythrocytes and plasma [10]. The results in Table II show the higher

TABLE II

COMPARISON OF ERYTHROCYTE AND PLASMA VITAMIN B_6 VITAMER CONCENTRATIONS IN TEN NORMAL VOLUNTEERS

Vitamer	Concentration			
	Erythrocytes (ng/ml of packed cells)		Plasma (ng/ml)	
	Mean ± S.D.	Range	Mean ± S.D.	Range
PLP	9.6±3.9	15.4–4.4	11.0±4.3	20.6–5.9
PA	Not detected		6.9±5.8	22.0–2.5
PMP	10.8±2.7	15.0–6.6	Not detected	
PL	2.0±0.6	3.0–1.1	1.0±0.5	2.1–0.4
PM	Not detected		0.1±0.3	0.9–0.0
PN	Not detected		Not detected	

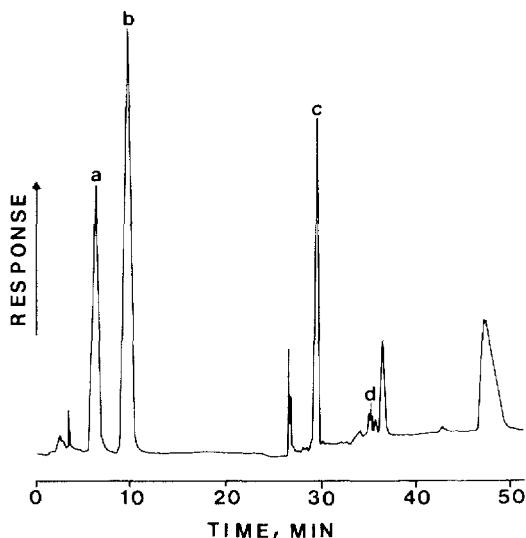

Fig. 2. Chromatogram of mononuclear cell sample (500-μl injection). Peaks: a = PLP; b = DPNP; c = PMP; d = PL.

levels of PL in erythrocytes as compared to plasma. Both PM and PN levels in the two blood compartments generally remain below detectable levels.

A chromatogram of an MNC extract is shown in Fig. 2. The main vitamers observed in MNCs were PLP and PMP, with only trace amounts of PL. Levels of PL in MNCs drawn from normal volunteers were generally below detectable limits (<0.01 ng per 10^6 cells). In addition, the PL peak was poorly resolved from another closely eluting unknown peak or appeared as a shoulder on this peak making identification and quantitation difficult. Changes in buffer gra-

dient failed to improve this separation. No detectable amounts of PA, PN or PM were found in any of the cells drawn from normal volunteers.

Precision and recovery data for the analysis of MNCs are shown in Table III. For optimum PLP recovery, it was found that the cell lysate in water must stand 5 min before and 10 min after protein precipitation. These periods had no effect on PMP levels. Table IV shows the PLP and PMP values found in MNCs drawn from eight normal volunteers and compares these with leucocyte PLP values reported previously, using non-HPLC methods. Leucocyte PMP values have not previously been reported. The significant levels found here are indicative of transaminase activity which, like PL kinase activity [14], has been shown, based on cell counts, to be considerably greater in leucocytes than erythrocytes [11]. The PLP values are similar in magnitude to those measured in leucocytes by previous workers, but are larger than values reported for lymphocytes [13]. This difference may reflect the different extraction methods used, as the latter workers used boiling hydroxide hydrolysis to extract the PLP vitamer.

TABLE III

PRECISION AND RECOVERY DATA FOR THE ASSAY OF VITAMIN B_6 VITAMERS IN MONONUCLEAR CELLS

Vitamer	Coefficient of variation[a] (%)	Recovery[b] (%)
PLP	4.1	101.1±8.2
PMP	5.5	96.1±3.3

[a]Six replicate analyses of a single sample.
[b]Recovery (mean ± S.D.) for five normal mononuclear cell samples spiked with 0.2 ng vitamer per 10^6 cells.

TABLE IV

VITAMIN B_6 VITAMER LEVELS IN LEUCOCYTES

Study	Sample	Concentration (ng per 10^6 cells)			
		PLP		PMP	
		Mean ± S.D.	Range	Mean ± S.D.	Range
Present	Mononuclear cells	0.36 ±0.25	0.91–0.19	0.13±0.03	0.19–0.09
Hamfelt [11]	Leucocytes	0.27 ±0.19	–	–	–
Wachstein et al. [12]	Leucocytes	0.23 ±0.05	0.36–0.14	–	–
Mahuren and Coburn [13]	Mixed leucocytes	0.243±0.073	–	–	–
	Lymphocytes	0.076±0.023	–	–	–
	Polymorphnuclear cells	0.237±0.069	–	–	–
Donald and Ferguson [19]	Leucocytes	0.30 ±0.05	0.38–0.22	–	–

Although not readily sampled, liver is an important tissue with regard to vitamin B_6 metabolism. The feasibility of HPLC analysis of small liver samples such as obtained by needle biopsy is shown by the chromatogram of a liver extract (Fig. 3) in which the injection volume represents approximately 2 mg of liver tissue. DPNP was again found to be a suitable internal standard for this analysis. The main vitamers observed were PLP, PMP and PM, although smaller amounts of PL, PN and PA were also observed. Precision and recovery data for the analysis are shown in Table V.

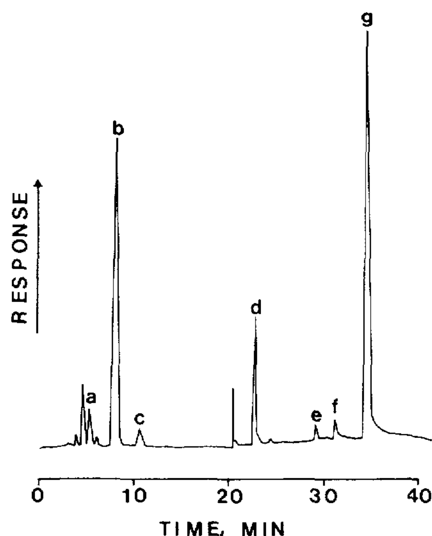

Fig. 3. Chromatogram of human cirrhotic liver sample obtained at post-mortem (50-μl injection). Peaks: a=PLP; b=DPNP; c=PA; d=PMP; e=PL; f=PN; g=PM.

TABLE V

PRECISION AND RECOVERY DATA FOR THE ASSAY OF VITAMIN B_6 VITAMERS IN A LIVER HOMOGENATE

Vitamer	Coefficient of variation[a] (%)	Recovery[b] (%)
PLP	3.3	106.9 ± 13.5
PA	1.0	110.2 ± 3.3
PMP	1.3	104.4 ± 3.2
PL	3.1	98.5 ± 1.4
PN	1.4	102.6 ± 4.7
PM	1.2	96.2 ± 12.7

[a]Five replicate analyses of single homogenate.
[b]Recovery (mean ± S.D.) of B_6 vitamers from five replicate analyses of liver homogenate spiked with 25 ng of each vitamer.

In the analysis of cellular samples, it should be remembered that cell lysates may contain vitamin B_6 metabolising enzymes or other enzymes, such as phosphatase, for which B_6 vitamers can act as substrates. It has previously been shown that such enzymes, when present and active in the extraction system, can influence the distribution and values of B_6 vitamers in rat liver and other tissue samples [20]. Interference by phosphatase enzymes was observed during this study when DPNP was added to the hemolysate prior to deproteinisation. The resulting dephosphorylation reaction was readily observed on chromatographic analysis as a reduced internal standard peak and by the appearance of a new peak corresponding in retention time to the dephosphorylated product, deoxypyridoxine. Hence in this study the internal standard and exogenous vitamers used in recovery determinations were added to the hemolysate immediately after trichloroacetic acid, and while vortexing which caused immediate protein denaturation and at the same time released bound vitamers. Our data indicate that this procedure together with its short duration and the fact that endogenous vitamers are protein-bound and hence protected from enzymes such as phosphatases ensured that any such potential interconversions were kept to a minimum. Thus, in the case of liver, this was achieved by homogenising the cells in a trichloroacetic acid solution. Furthermore we have found that in the case of blood cell lysis, adequate time must be allowed for complete and effective lysis to occur prior to deproteinisation. Analysis of the B_6 vitamers in erythrocytes, after allowing the lysate to stand for varying periods, indicated that for optimum PMP extraction, the hemolysate must stand 5–10 min prior to deproteinisation, the analysis of the other vitamers being unaffected by this procedure. The contrast between the reactivity of added DPNP and that of the endogenous phosphorylated vitamers emphasizes the importance of the protein binding of these vitamers in protecting them from the action of phosphatases [21].

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