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Note

High-performance liquid chromatographic assay of erythrocyte enzyme activity levels involved in vitamin B_6 metabolism

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Erythrocytes play an active role in human vitamin B_6 metabolism [1]. Three enzymes involved in vitamin B_6 metabolism are expressed in the erythrocyte [2–7]. Pyridoxal kinase (PLK; EC 2.7.1.35) catalyses phosphorylation of pyridoxine (PN), pyridoxamine (PM) and pyridoxal (PL), with ATP as the phosphate donor [2–4]. Pyridoxamine (pyridoxine-5′-phosphate) oxidase (PMP(PNP)Ox; EC 1.4.3.5) oxidizes pyridoxamine-5′-phosphate (PMP) and pyridoxine-5′-phosphate (PNP) to pyridoxal-5′-phosphate (PLP) [4,5], the co-enzyme form of vitamin B_6. Human erythrocytes also contain a PLP phosphatase activity [4,6,7], but cannot oxidize PL to 4-pyridoxic acid, the end-product of vitamin B_6 metabolism [7,8]. Therefore, erythrocytes release PL to the plasma [1,7,8], and thus contribute to plasma PL levels, which presumably serve as transport form of vitamin B_6 [8–10].

The erythrocyte is, therefore, a convenient cell system to study vitamin B_6 metabolism in general and in relation to disease. However, PLK and PMP(PNP)Ox activities in erythrocytes are much lower than those of liver tissue [8], for example, implying that analytical methods employed in the assay of PLK and PMP(PNP)Ox should have adequate sensitivity. Several individual methods for the determination of erythrocyte PLK [2,4,11,12], PMP(PNP)Ox [4,5,13] and PLP phosphatase [14] activities have been described, but quantification of all three enzyme activities in clinical studies remains a tedious task owing to different enzyme assay procedures used.

Recently, we described an isocratic high-performance liquid chromatography

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(HPLC) method for simultaneous quantification of plasma PLP and PL levels [15]. We have now optimized this method for the analysis of three reactions involved in vitamin B_6 metabolism, namely the assay of erythrocyte PLK, PMP(PNP)Ox and PLP phosphatase activities. Since both PLP and PL are determined by this HPLC method, PLK and PMP(PNP)Ox activities are monitored by PLP production from PL and PMP, respectively, and PLP phosphatase activity is determined by release of PL after PLP dephosphorylation. The HPLC assay for PLP and PL is very sensitive [15], and above-mentioned enzyme activities can be determined in less than 0.5 ml of whole blood.

EXPERIMENTAL

Reagents

PMP, PLP and PL were obtained from Merck (Darmstadt, F.R.G.). Triethanolamine hydrochloride was bought from Sigma (St. Louis, MO, U.S.A.) and adenosine-5′-triphosphate (ATP) was supplied by Boehringer Mannheim (Mannheim, F.R.G.). HPLC-grade acetonitrile was obtained from Saarchem (Muldersdrift, South Africa), and all other reagents were of analytical grade and supplied by Merck.

Haemolysate preparation and enzyme assays

Venous blood was collected with EDTA as anticoagulant; the plasma and white cells were removed after low speed centrifugation (1600 *g* for 10 min), and the red cells were washed twice with 0.154 *M* sodium chloride. A 100-μl volume of packed erythrocytes (taken from the bottom layer) was added to 0.5 ml of haemolysis buffer (10 m*M* triethanolamine containing 0.1% Triton X-100; pH 7.4) and the haemolysate haemoglobin content was measured by the spectrophotometric method of Drabkin and Austin [16]. For vitamin B_6 enzyme assays, the haemolysate was diluted as follows. For PLK assay, 60 μl of haemolysate were diluted to 0.4 ml using buffer A (10 m*M* triethanolamine, 90 m*M* dipotassium hydrogenphosphate, 2 m*M* magnesium chloride, 2 m*M* ATP; pH 7.4); for PLP phosphatase assay, 10 μl of haemolysate were diluted to 0.4 ml using buffer B (50 m*M* triethanolamine, 5 m*M* magnesium chloride; pH 7.4); the assay for PMP(PNP)Ox activity required dilution of 200 μl of haemolysate to 0.4 ml with buffer C (10 m*M* triethanolamine, 160 m*M* dipotassium hydrogenphosphate, 2 m*M* magnesium chloride; pH 7.4). The latter dilution was done in duplicate to determine basal erythrocyte PLP and PL levels. The diluted haemolysates were incubated for 10 min at 30°C. The reactions were started by adding 0.05 ml of 1.8 m*M* substrate (PL for PLK, PMP for PMP(PNP)Ox, and PLP for PLP phosphatase) solutions. Incubation times were 20 min for the PLP phosphatase assay, and 45 and 120 min for PLK and PMP(PNP)Ox assays, respectively. After the incubation period, the reactions were terminated by adding 0.2 ml of 10% trichloroacetic acid and 0.1 ml of 0.5 *M* semicarbazide. The mixture was mixed vigorously and then heated at 40°C for 30 min. The clear supernatant obtained after centrifugation was used for HPLC analysis of PLP and PL as described previously [15], except that the acetonitrile concentration of the mobile phase

was reduced to 2.5%. Throughout the assay procedure, samples and substrate solutions were protected from light because vitamin B_6 compounds are light-sensitive.

Apparent K_M values and linearity of assays

Apparent Michaelis constants (K_M) for PLK, PMP(PNP)Ox and PLP phosphatase were determined according to the method of Lineweaver and Burk [17]. The linearity of PLP production from PL (PLK assay) and PMP (PMP(PNP)Ox assay) was tested up to 100 and 180 min, respectively, and the linearity of the PLP phosphatase assay was investigated up to 40 min.

Subjects studied

Enzymes of vitamin B_6 metabolism were assayed in erythrocyte haemolysates prepared from 28 healthy, female subjects, aged 18–39.

RESULTS

Employing HPLC analysis as reported previously [15], PMP(PNP)Ox and PLK activities were reported as nmol PLP formed/g Hb/h, and PLP phosphatase activity was expressed as μmol PL formed/g Hb/h.

The HPLC analysis is specific for vitamin B_6 aldehyde derivatives, which form highly fluorescent semicarbazone derivatives in the presence of semicarbazide [15]. PMP, which was used as substrate in the assay for PMP(PNP)Ox, therefore did not interfere in the quantification of PLP formed (Fig. 1E). However,

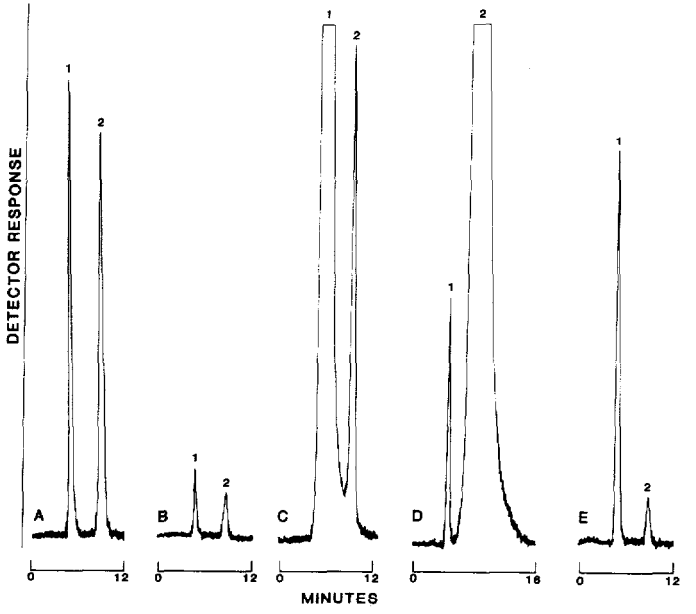

Fig. 1. HPLC assay of enzymes involved in vitamin B_6 metabolism. (A) Standard solution, containing 226 n*M* PLP and 147 n*M* PL; (B) erythrocyte haemolysate; (C–E) the same erythrocyte haemolysate to which PLP (C), PL (D) and PMP (E) were added to measure PLP phosphatase, PLK and PMP(PNP)Ox activities, respectively. Peaks: 1 = PLP; 2 = PL.

TABLE I

ASSAY CONDITIONS FOR ENZYMES OF VITAMIN B_6 METABOLISM

Enzyme	Substrate	Apparent K_M (μM)	Haemolysate volume (μl)	Final substrate concentration	
				μM	$\times K_M$
PLP phosphatase	PLP	5.50	10	85.7	15.6
PLK	PL	3.36	60	85.7	25.5
PMP(PNP)Ox	PMP	0.95	200	85.7	90.2

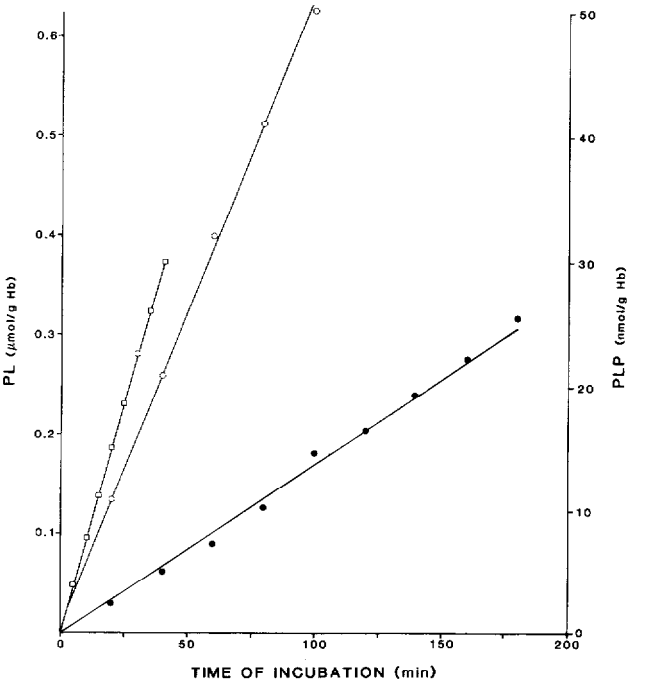

Fig. 2. Linearity of PLK (○), PMP(PNP)Ox (●) and PLP phosphatase (□) assays with time. PLK and PMP(PNP)Ox activities are expressed as PLP formed (nmol/g Hb) while PLP phosphatase activity is expressed as PL formed (μmol/g Hb).

assays for PLK and PLP phosphatase used, respectively, PL and PLP as substrates, and high substrate levels could interfere with product quantification. Therefore, the acetonitrile concentration in the HPLC mobile phase was considerably reduced when compared with normal plasma analysis [15]. This resulted in baseline separation of PLP from the substrate (PL) in the assay for PLK (Fig. 1D), and separation of PL from the substrate (PLP) in the PLP phosphatase assay (Fig. 1C) also allowed reliable determination of PL formation due to PLP hydrolysis. PLP formation resulting from PLK or PMP(PNP)Ox activity was corrected for basal erythrocyte levels (Fig. 1B), but the high dilution of haemolysate used in the PLP phosphatase assay made adjustment of PL levels unnec-

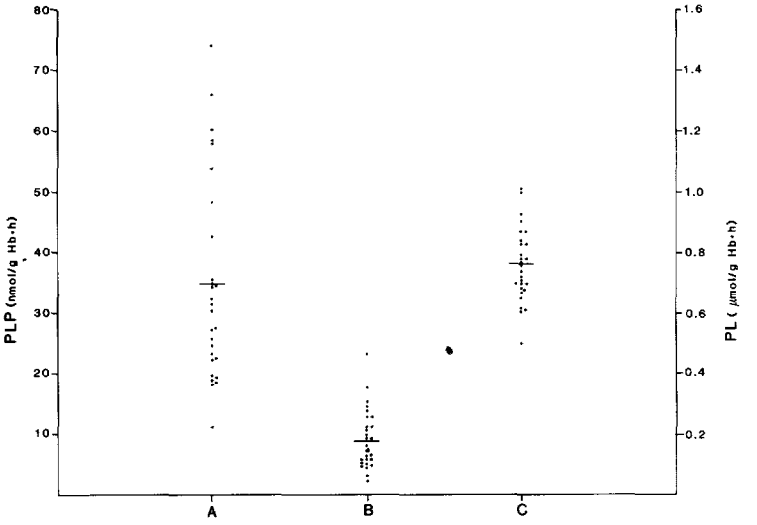

Fig. 3. Relative activities of (A) PL kinase, (B) PMP(PNP)Ox and (C) PLP phosphatase, in erythrocytes of Caucasian females ($n=28$). PLK and PMP(PNP)Ox activities are expressed as PLP (nmol/g Hb/h) formed from PL and PMP, respectively. PLP phosphatase activity is expressed as PL (μmol/g Hb/h) released from PLP.

essary. Substrate concentrations chosen for PLK, PMP(PNP)Ox and PLP phosphatase assays were 25.5 K_M; 90.2 K_M and 15.6 K_M, respectively (Table I). Under these conditions, product formation increased linearly for all three enzymes assayed (Fig. 2), indicating that these methods are valid for enzyme assays.

Enzyme activities in normal subjects differed considerably (Fig. 3). PLK activity levels varied between 10.9 and 74.0 nmol PLP/g Hb/h, and PMP(PNP)Ox activity varied between 2.2 and 23.4 nmol PLP/g Hb/h. Erythrocyte PLP phosphatase levels varied between 0.49 and 1.10 μmol PL/g Hb/h, which was a much narrower range than observed for PLK and PMP(PNP)Ox. Erythrocyte PLP phosphatase activities were considerably higher than PLK or PMP(PNP)Ox activities. The mean PLP phosphatase activity of 0.76 μmol PL/g Hb/h was 21.8-fold higher than the mean PLK activity of 34.8 nmol PLP/g Hb/h, and the mean PMP(PNP)Ox activity of 8.7 nmol PLP/g Hb/h differed 87.4-fold from the mean PLP phosphatase activity.

DISCUSSION

HPLC is a very useful analytical tool for the monitoring of substrate depletion or product accumulation in enzyme-catalysed reactions. In the application reported here, a single HPLC method was used to measure activities of erythrocyte enzymes involved in vitamin B_6 metabolism. Owing to the high sensitivity of the HPLC method, only a small volume (0.5 ml) of whole blood is required to assay for PLK, PMP(PNP)Ox and PLP phosphatase activities in red blood cell haemolysates. This could therefore be the method of choice in studies where only small amounts of blood are available, i.e. from small laboratory animals of from

infants. It is interesting to note that newborns have much higher plasma PLP levels than older children or adults [18], however, enzymes of vitamin B_6 metabolism have not yet been determined in infants, presumably owing to a lack of suitable methods.

The high sensitivity of the HPLC assay for PLP and PL allows quantification of PLK activity at physiological pH. Previously reported methods were performed at the pH optimum (pH 8.0) of PLK [2,4]. However, assay of PLK at physiological pH could be advantageous when possible mechanisms of PLK inhibition are studied. In agreement with previous reports [4], we found no evidence of substrate inhibition of PLK in erythrocyte haemolysates.

Assays from PMP(PNP)Ox and PLP phosphatase were close to their reported pH optima of 7.1 and 7.4, respectively [4]. In the assay for PMP(PNP)Ox, PMP instead of PNP was preferred as substrate, because PMP is readily available commercially and is also more stable than PNP. Moreover, PMP(PNP)Ox is subject to substrate inhibition when PNP is used as substrate [4,19]. We found no evidence of substrate inhibition of PMP(PNP)Ox when PMP was used as substrate.

It is essential to perform the PLK activity and PMP(PNP)Ox activity assays in a phosphate buffer, since phosphate is an effective inhibitor of erythrocyte PLP phosphatase [4,5,7]. This eliminates the possibility that the PLP formed as product in the PLK and PMP(PNP)Ox reactions is again used as the substrate for PLP phosphatase.

In a study of synthesis and degradation of PLP in rat pineal glands, Ebadi [6] showed that PLK activity was 200-fold lower than PLP phosphatase activity. In our study of human red blood cells, we also found that PLP phosphatase activity was much higher (22-fold) than PLK activity, indicating that PLP dephosphorylation was favoured above PLP synthesis from PL. However, PLP has a high affinity for haemoglobin [20,21] and protein-bound PLP is protected against hydrolysis [10,21].

Depressed plasma PLP levels have been reported in a number of apparently unrelated diseases, such as myocardial infarction, diabetes mellitus, asthma and cancer (for a review, see ref. 22). It seems plausible that if depressed plasma PLP levels are not nutritionally induced, it should be explained in terms of altered vitamin B_6 metabolism. In fact, it has been shown that vitamin B_6 deficiency in alcoholism could be explained by accelerated degradation of PLP [23]; liver disease is characterized by enhanced clearance of PLP due to enhanced degradation [24], and increased PLK activity is implicated in anaemia [25]. The ability to study enzymes involved in vitamin B_6 metabolism from a small blood sample, as reported here, could provide valuable information that would help to elucidate further the relationship between vitamin B_6 metabolism and disease.

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