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Assay Values for Thiamine or Thiamine Phosphate Esters in Whole Blood Do Not Depend on the Anticoagulant Used

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We compared the whole blood, plasma, and erythrocyte (red blood cell (RBC)) concentrations of thiamine and thiamine phosphate esters in the presence of heparin or EDTA as anticoagulants. Three blood specimens were collected from each of 24 healthy volunteers into evacuated collection tubes containing the following anticoagulants: heparin, Na2EDTA, or K2EDTA. The concentrations of nonphosphorylated free thiamine (T), thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate (TTP) were determined by the NH2-column HPLC method. The anticoagulant used had no effect on the concentrations obtained in whole blood and plasma of thiamine or any of the above thiamine compounds ($P > 0.05$). RBCs were isolated by centrifugation and washed with isotonic saline, and the cell counts of the washed cells were adjusted to their whole blood values. In the washed RBCs with any anticoagulant, the concentrations of T, TMP, and TDP expressed either as nmol/L of whole blood or a ratio to hemoglobin were significantly lower ($P < 0.05$) than those in whole blood. J. Clin. Lab. Anal. 19:205–208, 2005.

Key words: vitamin B1; thiamine diphosphate; erythrocyte; heparin; EDTA; blood collection; HPLC

INTRODUCTION

In vivo, vitamin B1 (thiamine) exists as the nonphosphorylated free thiamine (T) and its phosphate esters: thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate (TTP). TDP acts as a coenzyme in cells. T is the only precursor of TDP. With regard to circulating blood, T and TMP are present in plasma, while T, TMP, TDP, and TTP are found in erythrocytes and leukocytes (1,2). Because the concentration of TDP in erythrocytes (red blood cells (RBCs)) parallels its concentration in other tissues (3), whole-blood TDP is now measured simultaneously with T, TMP, and TTP to evaluate vitamin B1 levels by high-performance liquid chromatography (HPLC). TMP has been reported to be a more sensitive marker of deficiency in whole blood than TDP, since the only source of TMP is the dephosphorylation of TDP (4). In this study whole blood was collected with heparin or ethylenediamine tetraacetic acid (EDTA), based on an implicit but unsubstantiated assumption that anticoagulants would not affect the assay values of T and its phosphate esters in whole blood. Therefore, the aim of this study was to ascertain more systematically whether the assay values for T, TMP, TDP, and TTP in any specimen (i.e., whole blood, plasma, and erythrocytes) were comparable with the results obtained using different anticoagulants, heparin, and EDTA.

MATERIALS AND METHODS

Blood Collection

Three 5.0-mL volumes of whole blood were collected by venipuncture into evacuated collection tubes containing 4.0 mmol/L EDTA disodium salt, Na2EDTA in the final concentration (Venoject VP-NA050, Terumo, Tokyo, Japan); 3.0 mmol/L EDTA dipotassium salt, K2EDTA in the final concentration (Venoject VP-DK050); or 38 units of sodium heparin in the tube (Venoject VP-H050). The blood was collected from 24 healthy volunteers (12 men, 21–54 years old; and 12 women, 21–60 years old). The EDTA and

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heparin-anticoagulated blood tubes were centrifuged at 2,000*g* for 10 min. The plasma was removed, and the RBCs were washed twice (or eight times) with isotonic saline solution, with care taken to remove the buffy coat, and spun at 1,000*g* for 5 min after each washing. After the resuspended RBCs were counted (Cell-Dyn 3500R; Abbott Diagnostics Division, North Chicago, IL), the material was diluted with distilled water to the same counts as in whole blood to hemolyze the cells.

HPLC Method

The concentrations of thiamine and its phosphate esters in an aliquot of whole blood, plasma, and washed RBCs were determined by a precolumn derivatization HPLC method (5). To each 0.5 mL of specimen we added 0.5 mL of 0.2 mol/L sodium acetate, pH 4.5, and 0.5 mL of 0.61 mol/L trichloroacetic acid to deproteinize the specimens. Following vortex mixing and centrifugation, 0.2 mL of the supernatant was adjusted to pH 4.5 with 4.0 mol/L sodium acetate, and then oxidized with 0.02 mL of cyanogen bromide and 0.02 mL of 2 mol/L sodium hydroxide. Cyanogen bromide was freshly prepared each day by titrating bromine water (2–3%, w/v) with 10% potassium cyanide. After 5-min incubation at room temperature (25°C), the resulting thiochrome derivatives (0.05 mL) were injected into the HPLC column (Asahipack NH2-504E column, 4.6 × 250 mm; Shodex, Tokyo, Japan). The samples were isocratically eluted at a flow rate of 1.2 mL/min, with the following eluent heated at 40°C: 90 mmol/L phosphate buffer (pH 8.6)/acetonitrile, 40/60. The spectrofluorimeter was set with the excitation wavelength at 375 nm and the emission wavelength at 430 nm. A common practice is to express the values of thiamine analyte in whole blood or RBC as a ratio to hemoglobin (6). However, by adjusting the RBC counts to their whole-blood values in this study, the T, TMP, TDP, and TTP values in hemolyzed RBC could be directly compared with their whole-blood values. These values in plasma were expressed as nmol/L of plasma, and not as nmol/L of whole blood.

Statistics

The data were analyzed by means of the Wilcoxon matched-pairs test for nonparametric variables. Statistical significance was defined as $P<0.05$.

RESULTS AND DISCUSSION

The HPLC method was linear from 1 to 250 nmol/L for T, TMP, TDP, and TTP. The coefficient of variations (CVs) of within-day precision were 2%, 6%, 2%, and 12% for T, TMP, TDP and TTP, respectively. The between-day precision CV was 2%, 11%, 2%, and 21% for T, TMP, TDP and TTP, respectively. The recovery of T, TMP, TDP, and TTP added to whole blood was 100–101% in heparin, 100–103% in Na2EDTA, and 93–104% in K2EDTA.

Confirming the findings of a previous report (2), TDP was predominantly located in RBC but scant in plasma (see Table 1). Plasma also contained T and TMP. We considered that the TDP observed in plasma may originate from RBC during blood collection. Of the 24 volunteers examined, only four revealed the presence of TTP (1–6 nmol/L) in whole blood anticoagulated with heparin, Na2EDTA, and K2EDTA. The apparent absence of TTP in washed RBC can be explained by the loss of erythrocyte TTP during washing to a level below the detection limit of the HPLC method. Although Egi et al. (7) hypothesized that erythrocyte TTP is formed from TDP in the RBC by adenylate kinase (EC, 2.7.4.3) in the presence of ADP and Mg^{2+}, significantly elevated levels of TDP were not observed in subjects who had TTP in whole blood.

Whole-blood concentrations of T, TMP, and TDP were the same for specimens collected with heparin or EDTA ($P>0.05$). Plasma concentrations of T, TMP, and TDP were also the same for specimens collected with heparin or EDTA ($P>0.05$), whereas the values of T, TMP, and TDP in washed RBCs collected with three anticoagulants were significantly lower than their respective concentrations in whole blood ($P<0.05$; see Table 1). These decreases would not be due to removal of the buffy coat, although leukocytes are a rich source of TDP. In another experiment, only 20–40% of leukocytes were removed from twice-washed RBCs, and TDP concentrations in the twice-washed RBCs with the buffy coat removed were 1–3 nmol/L lower compared to those with the buffy coat retained. The TDP levels in erythrocytes and leukocytes were ca. 10^{-11} nmol/cell and 10^{-9} nmol/cell, respectively. Considering that erythrocytes had higher cell counts than leukocytes, the effect of leukocyte TDP on the concentration of TDP was negligible in washed RBCs.

Based on the above findings, we consider that intracellular T, TMP, and TDP were released from the RBCs during washing with isotonic saline, thus decreasing their values in the washed RBCs. The decreases of TDP in the washed RBCs that paralleled the increased washing times were also found even if their values were expressed as a ratio to hemoglobin (Hb) (Fig. 1). These decreases were significantly larger ($P<0.05$) than those that spontaneously occurred during the post-collection degradation of whole blood TDP that was stored 25°C in the dark for 2 hr. The decreases of the TDP/Hb ratio ($\times 10^{-3}$) in twice-washed RBCs were, on average, 3.8 for heparin, 3.8 for Na2EDTA, and 3.2 for K2EDTA

TABLE 1. Effect of anticoagulant on the values of thiamine (T) and thiamine phosphate esters (TMP, TDP and TTP) in an aliquot of whole blood, plasma, and washed RBC*

	Anticoagulant		
	Heparin	Na_2EDTA	K_2EDTA
Whole blood			
T, nmol/L	6 (2–10)	6 (3–11)	8 (3–17)
TMP, nmol/L	9 (1–16)	8 (1–17)	8 (1–32)
TDP, nmol/L	107 (53–131)	102 (44–126)	101 (45–128)
TTP, nmol/L	0 (0–2)	0 (0–6)	0 (0–4)
Plasma			
T, nmol/L	4 (1–15)	6 (1–14)	6 (2–15)
TMP, nmol/L	6 (1–16)	7 (4–16)	8 (3–14)
TDP, nmol/L	0 (0–2)	0 (0–3)	0 (0–3)
TTP, nmol/L	0 (0–0)	0 (0–0)	0 (0–0)
Washed RBC[a]			
T, nmol/L	4 (1–6)[b]	4 (2–17)[b]	5 (2–12)[b]
TMP, nmol/L	4 (1–16)[b]	4 (1–15)[b]	4 (1–8)[b]
TDP, nmol/L	82 (42–123)[b]	82 (40–125)[b]	81 (34–138)[b]
TTP, nmol/L	0 (0–0)	0 (0–0)	0 (0–0)

*Values represent the median (range) of 24 volunteers. The concentrations of T, TMP, TDP, and TTP in an aliquot of whole blood and washed RBC were expressed as nmol/L of whole blood, and these concentrations in plasma were expressed as nmol/L of plasma.
[a]RBC was washed twice with isotonic saline solution.
[b]Significantly ($P < 0.05$) lower than those in whole blood.

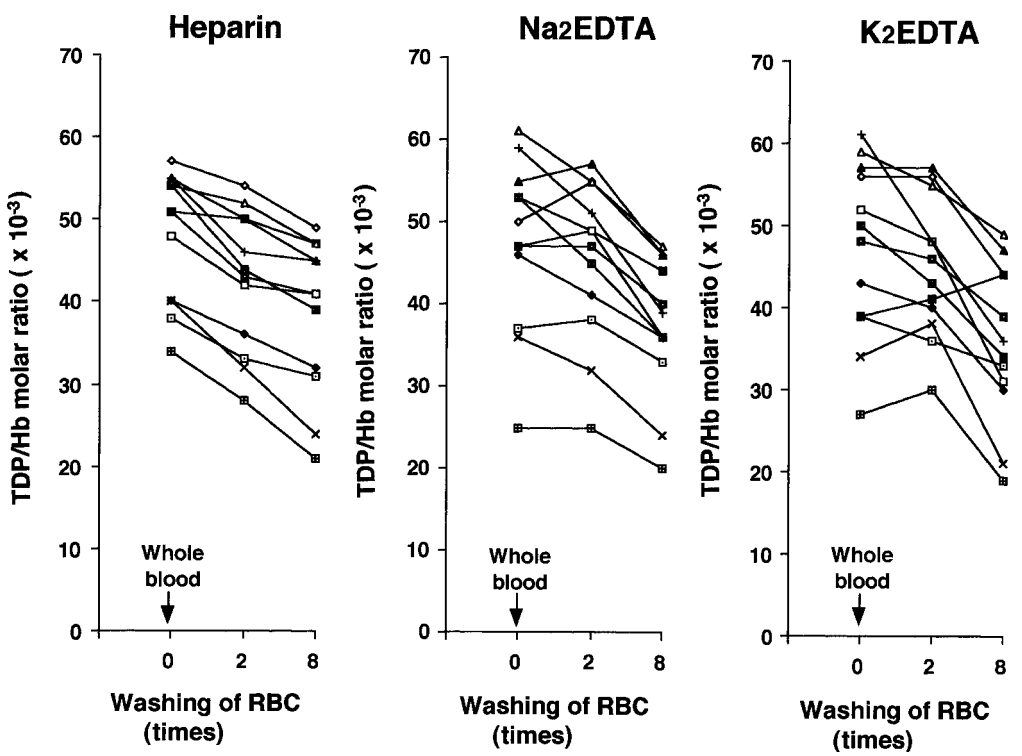

Fig. 1. Effect of washing RBCs on the values of TDP expressed as a molar ratio to hemoglobin (TDP/Hb) The RBCs were washed two to eight times with isotonic saline solution. Each symbol represents one subject.

anticoagulants; the spontaneous decreases after 2 hr, in the same sequence, were 1.0, 1.8, and 1.2, respectively. However, the values of T, TMP, and TDP in washed RBCs expressed either as nmol/L of whole blood or a ratio to hemoglobin were the same among the anticoagulants used.

In conclusion, the results obtained for T, TMP, and TDP in whole blood were not affected by the anticoagulants heparin, Na2EDTA, or K2EDTA. Since washing RBCs with isotonic saline reduces the T, TMP, and TDP in the RBCs, washed RBCs are unsuitable as specimens for the assay of T, TMP, and TDP. Based on our findings, we recommend that whole blood containing any of the above anticoagulants be used for the assay of thiamine and its phosphate esters.

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