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Competitive Protein-binding Radioassay of Thiamine in Simple Solutions and in Multivitamin Pharmaceuticals

ANDRZEJ KOZIK, RADOSLAWA WRÓBEL AND IZABELA TURYNA

Jagiellonian University, Institute of Molecular Biology, Al. Mickiewicza 3, 31-120 Kraków, Poland

Abstract—A competitive inhibition radioassay of thiamine is described using a gel obtained by coupling a buckwheat-seed thiamine-binding protein to CNBr-activated Sepharose. The sample to be analysed is incubated with gel suspension and [^{14}C]thiamine and after centrifugation the radioactivity of the supernatant is measured. The method is simple and specific, and applicable over a thiamine concentration range 0·5–5 μM with a coefficient of variation typically below 5%. The gel is reusable and stable for several months. Applicability of the method for direct determination of thiamine in multivitamin pharmaceuticals is demonstrated.

A predominant chemical method of thiamine assay is the fluorometric determination of thiochrome formed upon oxidation of this vitamin (Ellefson 1985). The method is sensitive and applicable to a broad range of various materials. For samples of relatively high thiamine concentration, like pharmaceutical preparations, several other chemical procedures are of choice for thiamine assay, including colorimetry (Das Gupta & Cadwallader 1970), UV absorptiometry (Strohecker & Henning 1966), polarography (Vergara et al 1980), potentiometry (Hassan et al 1985) and gas chromatography (Echols et al 1985).

In this paper we introduce a new competitive inhibition radioassay of thiamine.

Materials and Methods

Chemicals

Bio-Rex 70 was obtained from Bio-Rad (USA), CNBr-activated Sepharose 4B and DEAE-Sephadex A-25 from Pharmacia (Sweden), thiamine HCl, riboflavin, and vitamin B_{12} from Sigma (USA), nicotinic acid from Koch-Light (England), other water-soluble vitamins and Carbowax 20M from Serva (West Germany). Spectrally pure isobutanol was purchased from Aldrich (USA). All standard chemicals were from POCh (Poland).

[Thiazole-2-^{14}C]thiamine of specific activity 2·85 MBq mg^{-1} (batch 35) was purchased from Amersham UK. A radio-chemical purity of the radiolabelled vitamin was at least 97% as promised by the manufacturer and as checked by thin layer chromatography in two recommended systems.

Multivitamin preparations.

Multivitaminum (I), Vitaminum B compositum (II), Falvit (III) and Visolvit (IV) were commercial products of Polish Pharmaceuticals (Polfa).

Purification of thiamine-binding protein (TBP)

TBP was isolated from buckwheat seeds by the method of Mitsunaga et al (1986). As only partial purification was necessary, the last step (Sephacryl gel filtration) was omitted. Activity of protein fractions (i.e. thiamine-binding capacity) was determined by equilibrium dialysis (Nishimune & Hayashi 1979) and protein was quantitated by the method of Lowry et al (1951). Purified protein preparations could bind 3·6–4·6 nmol thiamine per mg protein. TBP was stored frozen in 0·05 M phosphate buffer pH 7·5 containing 1% NaCl.

Correspondence: A. Kozik, Jagiellonian University, Institute of Molecular Biology, Al. Mickiewicza 3, 31-120 Kraków, Poland.

Immobilization of TBP in Sepharose

For coupling TBP to CNBr-activated Sepharose, the protein solutions were concentrated by dialysis against 20% Carbowax 20M and then dialysed against coupling buffer. An immobilization procedure followed in general the manufacturer's instruction. Final coupling suspensions (20 mL) contained 5 mL of CNBr-Sepharose and TBP (about 1·5 mg mL^{-1}) in 0·1 M bicarbonate buffer pH 9·0 containing 0·5 M NaCl. An excess of reactive groups was blocked with ethanolamine.

The gel was stored refrigerated in 0·05 M sodium phosphate buffer pH 8·0 saturated to 80% with ammonium sulphate. Under such storage conditions, immobilized TBP appeared stable for several months.

Competitive inhibition radioassay of thiamine using TBP-Sepharose

A diluted gel suspension in 0·05 M sodium phosphate buffer pH 8·0 was prepared in a glass round-bottomed flask. Under continuous shaking, just vigorous enough to keep the suspension uniform, 0·7 mL portions were transferred to Eppendorf-type plastic tubes. The gel was forced to settle by centrifugation. From a supernatant above the gel, 0·4 mL portions were taken and discarded. To a sample to be analysed (~1 mL), prepared in the same buffer, an amount of [^{14}C]thiamine was added, a portion (say 0·4 mL) was taken for precise determination of total activity added, and a further 0·4 mL was mixed with gel in the tube. Final incubation mixtures (0·7 mL) contained 0·4–0·45 μM [^{14}C]thiamine, 0·5–5 μM cold thiamine as standard or analyte, and TBP-Sepharose in amount sufficient to keep in the suspension a thiamine-binding capacity (B_{max}, see Results) of

1–1·5 μM. The suspensions were incubated for 2 h at room temperature (20°C) with occasional shaking, and then briefly centrifuged to settle the gel. Samples (0·4 mL) were carefully taken out from supernatant layers and their radioactivities were determined in 10 mL of Bray scintillation fluid using an LKB 1211 Rackbeta counter.

To each array of tubes, at least four standard samples were included for determination of binding parameters. The calibration was performed each day to assure a good precision of thiamine determinations. All results were analysed numerically. Data from standard samples were transformed on Scatchard plots, and the binding parameters, K_d and B_{max} (see Results) were found by linear regression. From values of $B/T = (T-F)/T$ (T,F,B being mean total, free and bound radioactivity, respectively, each in counts $min^{-1}/0{\cdot}4$ mL sample) determined for analysed samples, thiamine concentrations (x) were found by solving the equation:

$$x = B_{max}/(B/T) - K_d/(1-B/T)\text{-}H$$

where H is the concentration of radioligand.

TBP-Sepharose could be reused many times without any special regeneration procedure. The used gel suspensions were transferred to a small column and extensively washed at 4°C with 0·05 M sodium phosphate pH 8·0 (at least 1 L per 5 mL of TBP-Sepharose). A packed column, frequently washed with the buffer and kept cold, was suitable for storing TBP-Sepharose between assays.

Fluorimetric determination of thiamine

Thiamine was determined by a standard manual thiochrome method (Ellefson 1985), including purification on Bio-Rex 70. Fluorescence of thiochrome, extracted into isobutanol, was read in a Specol 11 fluorimeter (Carl Zeiss-Jena, Germany), with excitation wavelength set at 365 nm.

Determination of thiamine in multivitamin pharmaceuticals

Pills were ground thoroughly and extracted with water to obtain an expected concentration of vitamin B_1 in a range 10–100 μg mL^{-1}. Extracts were clarified by centrifugation and kept frozen until used. For fluorimetric determinations, a portion of extract containing 1–3 μg of thiamine was mixed with 10 mL of 0·1 M sodium acetate buffer pH 4·5 and applied to a Bio-Rex 70 column. For the radioassay the extract was diluted so as to contain 1–10 μM thiamine in 0·05 M sodium phosphate buffer pH 8·0.

Results and Discussion

After coupling to CNBr-Sepharose, the TBP from buckwheat seeds retains its high affinity to thiamine. The binding could be conveniently quantitated by measuring radioactivity of supernatant samples redrawn from the gel suspensions pre-incubated with [^{14}C]thiamine. Time-course of the [^{14}C]thiamine sorption is presented in Fig. 1A, as determined for a representative batch of immobilized TBP. The process seemed to reach a steady state within 2 h and appeared fully reversible. Scatchard plots were typically found to be linear (Fig. 1B), at least in the concentration range studied (0·2–5 μM [^{14}C]thiamine).

In 0·05 M sodium phosphate buffer pH 8·0, the dissociation constant K_d was found to be $0{\cdot}62 \pm 0{\cdot}13$ (mean ± s.d., 5 batches of TBP-Sepharose). The parameter was quite reproducible within the same preparation of immobilized TBP (5–7% between-day coefficient of variation). The maximal amount of thiamine bound (B_{max}), expressed here as concentration in the final incubation mixture, was in a range 0·3–5 μM and was a linear function of gel volume in the suspension (results not shown). The coupling of TBP to CNBr-Sepharose resulted in gels of thiamine-binding capacity as high as 25 nmol thiamine per mL of packed gel. However, gels of moderate binding capacity (10–12 nmol thiamine mL^{-1}) were used in most experiments presented here.

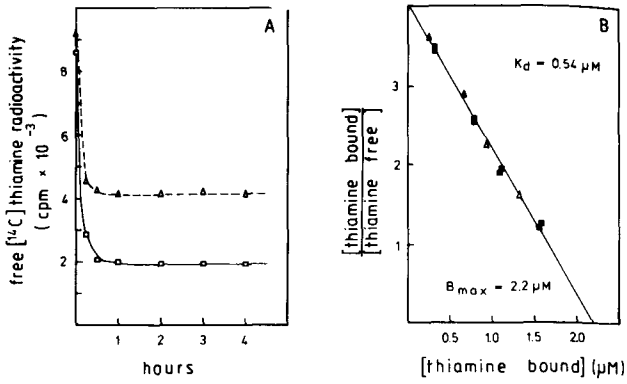

FIG. 1. Time-course (A) and Scatchard plot (B) for [^{14}C]thiamine binding to Sepharose-immobilized TBP. Results are presented from an experiment performed on a representative batch of TBP-Sepharose, in 0·05 M sodium phosphate buffer pH 8·0 at room temperature. Time-course was determined for 0·41 μM [^{14}C]thiamine (□) or for a mixture of 0·45 μM [^{14}C]thiamine with 2·4 μM cold thiamine (△). A Scatchard plot was first constructed for a range of [^{14}C]thiamine concentrations from 0·5 to 3 μM. After incubation for 2 h, 0·4 mL samples were removed for radioactivity counting (■) and then buffer (0·4 mL) was added for a further 2 h (△). From a regression line fitted to all points the binding parameters: dissociation constant (K_d) and maximal binding (B_{max}, in the incubation mixture) were calculated.

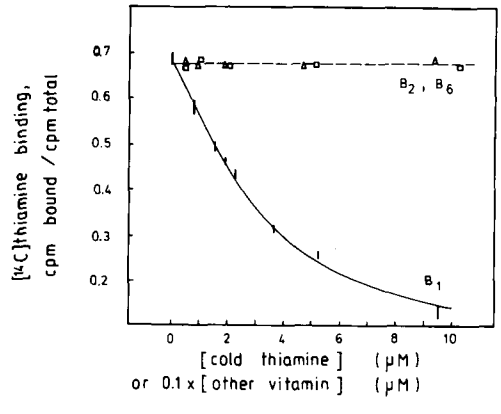

FIG. 2. Competition of cold thiamine and other water-soluble vitamins with [^{14}C]thiamine for binding sites of TBP-Sepharose. A displacement plot for thiamine was determined for three subsequent days using the same gel suspension in 0·05 M phosphate buffer pH 8·0 (one determination per day for each thiamine concentration). Points are presented as bars of 2 times s.d.; the displacement curve is drawn assuming $K_d = 0{\cdot}59$ μM and $B_{max} = 1{\cdot}57$ μM. Effects of pyridoxine (□, up to 100 μM) and riboflavin (△, up to 100 μM) on [^{14}C]thiamine binding are also presented. [^{14}C]Thiamine concentration was 0·45 μM.

Table 1. Competitive protein-binding radioassays of thiamine content in some multivitamin preparations.

Sample	Competitive radioassay of thiamine: Concentration in assay mixture (μM)	Total content (mg)	CV (%)	Reference thiamine content: Fluorimetry (mg)	Expected (mg)
I	0·40	4·74	5·1 (3)		
	1·12	5·09	2·9 (3)		
	2·62	5·00	1·1 (3)		
	4·23	4·61	0·6 (3)	4·70 ± 0·22 (3)	5
II	0·68	3·01	5·7 (3)		
	1·43	3·21	5·5 (3)		
	2·36	3·63	3·2 (3)		
	3·63	3·28	1·1 (3)	3·33 ± 0·09 (3)	3
III	1·29	3·81	6·0 (4)	3·78 ± 0·35 (3)	3
III+	1·76	+1·39	5·2 (4)		+1·5
	2·38	+3·21	4·3 (4)		+3·1
	2·98	+4·98	4·3 (3)		+4·6
IV	1·05	0·88	5·9 (6)	0·81 ± 0·06 (3)	1
IV+	1·48	+0·40	3·2 (3)		+0·34
	1·74	+0·62	3·0 (2)		+0·65
	2·14	+0·96	2·1 (3)		+0·98

Single doses were ground if necessary (I–III), extracted with water, supplemented with thiamine as indicated by (+) and made to contain a buffer suitable for radioassay (0·0 5 M phosphate pH 8) or for the thiochrome method. CV is the within-day coefficient of variation. Number of independent determinations are given in parentheses. Thiamine contents found by the radioassay are compared with the results of the standard thiochrome method (mean ± s.d.) or with expected values as claimed by the manufacturer (Polfa).

A competition of [^{14}C]thiamine with cold thiamine for binding sites of TBP-Sepharose is demonstrated in Fig. 2. The detailed displacement plot presented was prepared in a range of thiamine concentrations 0·2–10 μM and should be regarded as a calibration plot for determination of this vitamin in 0·05 M phosphate buffer pH 8·0. Values of B/T could be determined with a very good precision: coefficients of variation from at least 3 determinations performed on the same samples were typically below 3% between days (Fig. 2) and well below 1% within a day. Though a limit of thiamine detection was as low as 0·2 μM, a comfortable analytical range for thiamine determinations appeared to be from 0·5 to about 5 μM. Within this range an overall precision might be kept at the 5% level with a sensitivity of about 0·2 μM.

Fig. 2 emphasizes a high specificity of competitive protein-binding radioassay of thiamine. A possible capability of binding to TBP-Sepharose was tested for a range of water soluble vitamins, including riboflavin and pyridoxine (Fig. 2), other B_6 vitamins, biotin, nicotinic acid, ascorbic acid, pantothenic acid and vitamin B_{12} (results not shown). In no case was any competition with [^{14}C]thiamine for TBP-Sepharose detected, even at ligand concentrations some hundred times higher than that of the radioisotope.

TBP-Sepharose used in an assay is recoverable for further thiamine determinations. An extensive washing (see Methods) completely clears the gel from any residual radioactivity. Moreover, immobilized TBP appears quite stable if properly handled and stored. After two months a gel frequently used for thiamine assays lost only 20% of starting thiamine-binding capacity but K_d remained almost unchanged.

Preliminary tests were performed on the applicability of the competitive inhibition radioassay for thiamine in commercial multivitamin pharmaceuticals. Results are summarized in Table 1. The method seems quite satisfactory in both precision and accuracy. A good consistency of thiamine contents from the competitive radioassay and from standard thiochrome fluorimetry was found, with a full recovery of thiamine added to analysed samples (102 ± 4%, 20 determinations, 2 multivitamin preparations).

A general principle of competitive protein-binding assay has been frequently exploited in analytical biochemistry of vitamins: biotin (Hood 1979), riboflavin (Lotter et al 1982), vitamin B_{12} (Lee & Griffiths 1985) and folates (Herbert & Colman 1985). Thiamine radioassay has not yet been suggested, probably because specific soluble thiamine-binding proteins are rare and poorly characterized. We focused our attention on TBP from buckwheat seeds because of its highest specificity toward unphosphorylated vitamin B_1 (Mitsunaga et al 1986). Thus, high selectivity is one of the advantages of the thiamine assay outlined here.

As a rule, proteins utilized in various competitive inhibition assays form very tight complexes with ligands to be determined. The analyte and its radiolabelled form compete for a limited number of binding sites on the specific protein. Separation of free and bound ligand does not provide any serious problems; usually the bound radioligand is quantitated in a solid phase, extensively washed of free (unbound)

radioactivity. Thiamine binding to TBP, however, is relatively weak (K_d in micromolar range). For separating free thiamine from its protein-bound form, a procedure is necessary which does not disturb the binding equilibrium. With Sepharose-immobilized TBP, this is simply performed by settling the thiamine-binding gel and counting free radioligand activity in a phase from above the gel. Such procedure may offer an advantage as TBP-Sepharose may be reused many times. Moreover, immobilized TBP is reasonably stable. Thus, our thiamine radioassay seems economic in comparison with other competitive inhibition assays.

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