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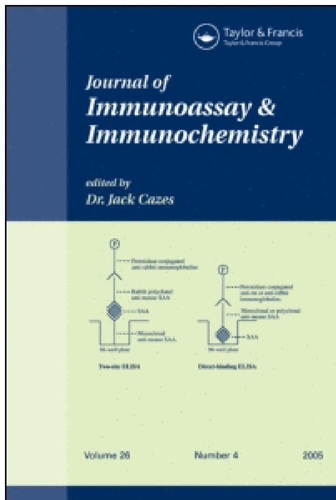

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Development of an ELISA for Pantothenic Acid (Vitamin B_5) for Application in the Nutrition and Biological Fields

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DEVELOPMENT OF AN ELISA FOR PANTOTHENIC ACID (VITAMIN B_5) FOR APPLICATION IN THE NUTRITION AND BIOLOGICAL FIELDS

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ABSTRACT

Immunological assays appear to be the only alternative to the microbiological method for analysis of pantothenic acid in foods and blood. In order to evaluate the influence of the linker on the immunogenicity of the hapten, we have tried to raise antisera against pantothenic acid in rabbits using different conjugates. The hapten was coupled to a carrier protein (BSA or thyroglobulin) using adipoyl dichloride (adipoyl conjugate) or bromoacetyl bromide (acetyl conjugate). Only the acetyl conjugate has induced the production of a specific antibody. With this antibody, an assay on microplate using the ELISA inhibition technique was developed to measure pantothenic acid. The use of pantothenic acid coupled to thyroglobulin with adipoyl dichloride as the capture antigen has improved the sensitivity of the ELISA. This assay was applied to food products and blood.

Key Words: pantothenic acid, ELISA, Food, Blood.

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INTRODUCTION

Pantothenic acid or vitamin B_5, a water-soluble vitamin of the B-group, is encountered in all types of animal and plant tissues and particularly in liver, eggs, yeasts and cereals. It occurs to a small extent as a free form, but mostly bound as coenzyme A or acyl carrier protein (ACP) that are the biologically active complexes of the vitamin B_5. Coenzyme A and ACP function as carriers of acyl group in enzymatic reactions involved in the synthesis of fatty acids, cholesterol or sterols, the oxidation of fatty acids, pyruvate or α-ketoglutarate, and in biological acetylations.

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Numerous methods have been developed to measure pantothenic acid. Bioassays using the growth of chickens as measurable factors (1) are not in use yet; chemical methods based on hydrolysis of the vitamin and quantification of the hydrolysis products (pantolactone or ß-alanine) (2) have poor sensitivity and specificity. Enzymatic assay based on the exchange of radioactive ß-alanine with ß-alanine preexisting in pantothenic acid by pantothenase (EC 3.5.1.22) is influenced by different inhibiting compounds (3). Chromatographic methods lack of a specific and sensitive method of detection. With UV detection, there is no absorption of the pantothenic acid at wavelength above 220 nm. The quantification of the vitamin in complex matrix requires extensive sample preparation and derivatization steps and these chromatographic methods have been found suitable only for the determination of the vitamin in pharmaceutical preparations either by gas (4) or liquid (5) chromatography. Mass spectrometry

coupled to gas chromatography have been used for the quantification of pantothenic acid in blood and food products (6). This method also needs long purification and derivatization steps. The most currently employed analytical assay for pantothenic acid in foods is the microbiological method based on measurement of the growth of yeasts and bacteria and mainly the assay procedure using *Lactobacillus plantarum* (7). That is still the reference method, although this procedure is complex and time-consuming and is performed only by few laboratories.

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The only alternative method for the routine quantification of pantothenic acid in complex products seems to be an immunological assay. The production of specific pantothenic acid antibodies was first been described by Wyse et al. (8) for the development of a radioimmunoassay for pantothenic acid in blood. An Enzyme Linked Immunosorbent Assay has been used for the determination of the vitamin in foods (9; 10) and in blood (11) with a similar antibody production procedure. As a matter of fact, all these methods use the same immunogen, pantothenic acid coupled to reduced and denatured BSA by bromoacetyl bromide (2 carbon atoms). In order to evaluate the influence of the length of the linker, we prepared other conjugates using adipoyl dichloride (6 carbon atoms). The molecular structure of the vitamin is not differently modified by the coupling using either bromoacetyl bromide or adipoyl dichloride but the close environment of the hapten is likely to be more affected, after coupling, using the former than the latter.

This paper presents our results on the preparation of antibodies to pantothenic acid using different immunogens, the development of an ELISA and the application of the method to measure pantothenic acid in foods and blood.

MATERIALS AND METHODS

All the chemicals and the complete and incomplete Freund adjuvants were obtained from Sigma Chemical Company, France. Peroxidase-conjugated AffiniPure Goat anti Rabbit IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories, USA and 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB) from Amresco, USA. Different enzymes were used for pantothenic acid extraction : Mylase ME from United States Biochemical, USA (ref. 19300) or alkaline phosphatase (EC 3.1.3.1) from Sigma (ref. P 3877) used alone or in association to pigeon liver acetone powder containing a pantetheinase (EC 3.5.1.-) from Sigma too.

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Blood and serum were obtained from healthy subjects, the first on EDTA and the second on dry tubes and immediately centrifuged at +4°C. Food were purchased at a local supermarket

Synthesis of Immunogens

As pantothenic acid (MW 219 Da) is not directly immunogenic, it must be coupled to a carrier protein to induce the production of antibodies. Different conjugates were synthesized with two carrier protein : bovine serum albumin

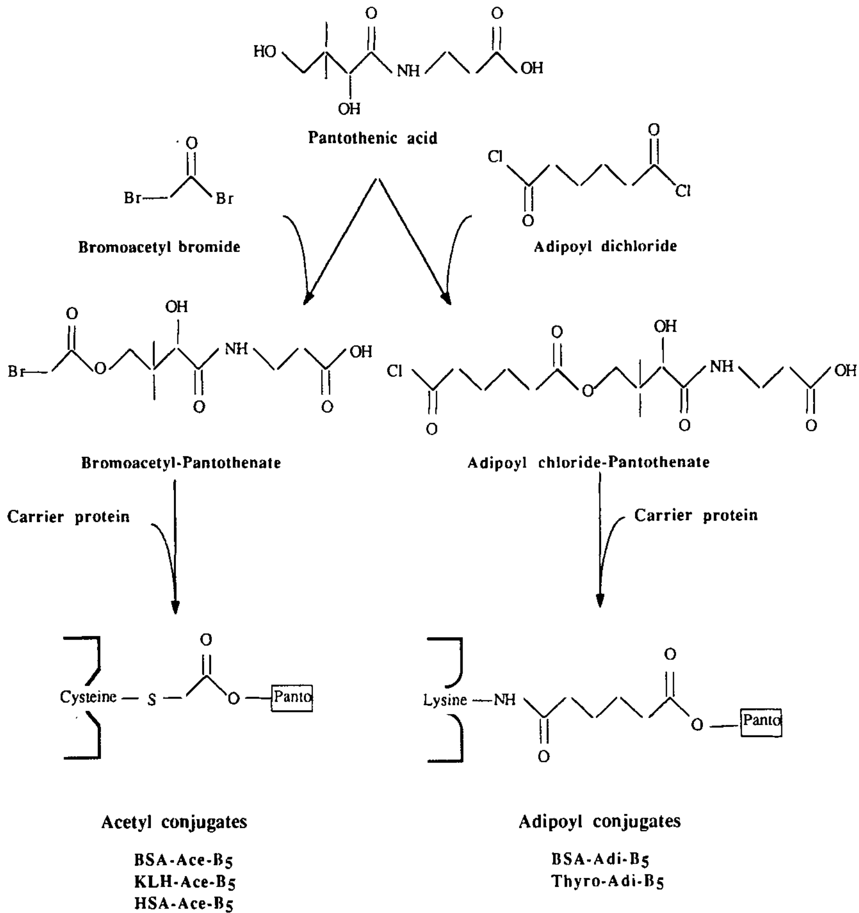

FIGURE 1: schematic synthesis of the different conjugates

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(BSA) or swine thyroglobulin (Thyro), and two linkers : adipoyl dichloride (Adi) or bromoacetyl bromide (Ace) (figure 1). Two other conjugates using Keyhole Limpet Hemocyanin (KLH) and human serum albumin (HSA) as carrier protein and bromoacetyl bromide as linker were also synthesized. They were used only as capture antigens.

Adipoyl Conjugates

Oxalic acid (2 mmol) was added to a 0.2 mol/L aqueous solution of D(+) calcium pantothenate (953 mg in 10 mL corresponding to 4 mmol of pantothenic acid). The calcium oxalate precipitate was removed by centrifugation (2000g for 10 min). The supernatant was then concentrated by rotary evaporation. The resulting pantothenic acid (which is soluble in organic solvent, in contrast with the calcium salt) was dissolved in 15 mL of ethyl acetate and the medium dried overnight at +4°C with anhydrous sodium sulfate. Adipoyl dichloride (380 µL corresponding to 2.6 mmol) was added to the solution. After 1 h at + 4°C, the ethyl acetate was evaporated and the conjugate dissolved with 5 mL of N,N dimethylformamide (DMF) previously cooled at +4°C. In parallel, 60 mg of the carrier protein (BSA or thyroglobulin) were dissolved in 5 mL of 0.05mol/L phosphate buffer pH 7 and cooled also at +4°C. This solution was mixed with the Adipoyl chloride-Pantothenate conjugate in DMF and stirred gently for 2 h at +4°C.

Both conjugates (BSA-Adi-B_5 or Thyro-Adi-B_5) were dialyzed against 4 L of 0.5 mol/L NaCl (4 times) and against 4 L of distilled water (4 times) (Dialysis membrane Millipore ND, retention size 10000 Da), freeze-dried and stored at -18°C.

Since adipoyl dichloride is not routinely used as a linker, the Adipoyl-Pantothenate conjugate was carefully characterized. After reaction with an excess of dry methanol, it was transformed to its terminal, unreactive methyl ester

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counterpart. The latter was analyzed by HPLC (phenyl-grafted silica gel-5μm, eluent: 1:4 methanol-water adjusted to pH 2.2 with HCl, evaporative light scattering detection), ^{1}H and ^{13}C nmr. The conjugate was shown to contain 15-20% of free vitamin B_5 that can be removed by column chromatography (silica gel, eluent: 4:1 methanol-water, then 19:1 methanol-acetic acid).

Acetyl conjugates

Bromoacetyl bromide (350 μL corresponding to 4 mmol) was added to the solution of pantothenic acid in ethyl acetate (4 mmol / 5 mL). The reaction was stopped after 3 min at room temperature using 3 mL 0.05 mol/L phosphate buffer saline (PBS) pH 7. After evaporation of the ethyl acetate, the pH of the aqueous solution was adjusted to 6.5 with 0.5 mol/L Na_2HPO_4.

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BSA was reduced and denatured as follows: 89 mg of BSA in 2 mL of distilled water were mixed with 20 mL of an aqueous solution of 10 mol/L urea and 56 mg of dithiothreitol dissolved in 3 mL of 0.05 mol/L phosphate buffer pH 7. After 30 min, the solution of the bromoacetyl pantothenate was added to the solution of the reduced and denatured protein. The pH was adjusted to 7.3 with NaOH. The mixture was stirred gently for 24 h at room temperature and protected from light.

The conjugate (BSA-Ace-B_5) was purified by dialysis and freeze-dried like the other conjugates.

Using the same procedure, we synthesized conjugates for the coating of the microplates : one using Keyhole Limpet Hemocyanin (KLH) in place of BSA; the other was obtained with human serum albumin (HSA). In contrast with BSA, HSA was neither reduced nor denatured: 45 mg of HSA were dissolved in 11 mL of distilled water and 2 mL of the phosphate buffer. This solution was added directly to the solution of bromoacetyl-pantothenate.

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Coupling Ratio

In order to check the efficiency of the conjugation, the hapten-protein ratio was determined as follows: the freeze-dried conjugates were dissolved in a solution of 8 mol/L urea in PBS. After 4 dialysis cycles against distilled water to remove vitamin not covalently bound to the protein, the conjugates were subjected to an alkaline hydrolysis step (0.01mol/L NaOH) for 30 min at room temperature or to a double enzymatic extraction with alkaline phosphatase and pantetheinase from pigeon liver. The amount of free pantothenic acid was then determined by the microbiological method.

Antibody Production

The immunogens (BSA-Adi-B_5, Thyro-Adi-B_5 or BSA-Ace-B_5) were dissolved in 0.05 mol/L PBS pH 7.4 (1 mg/mL) and emulsified in a same volume of Freund complete adjuvant. Each rabbit was injected subcutaneously at multiple sites on the back with 1 mL of the emulsion. They were boosted every 3 weeks

replacing complete adjuvant by incomplete and blended at the marginal ear vein. After 5 booster injections, the blood was collected by intracardiac puncture and the rabbits euthanasied. The serum was removed after centrifugation and stored at -20°C.

Antibody Purification

According to the result of the antibody production, only the serum anti BSA-Ace-B_5 was purified by immunochromatography in order to minimize cross-reactions of the antibodies.

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A conjugate between BSA and bromoacetyl bromide treated with an excess of dry methanol (BSA-Ace-Me) was synthesized. This conjugate (10 mg) without pantothenic acid was dissolved in 5mL of 0.05 mol/L carbonate buffer pH 9.6 with 0.6 mol/L sodium citrate. After homogenization, 125 mg of the resin (Emphaze ND Biosupport Medium AB 1 Pre-activated Chromatography Beads, 3M, USA) were directly added. The coupling reaction was made at room temperature for 5 h with gentle stirring. After centrifugation (1000 g for 5 min) and removal of the supernatant, 10 mL of 3 mol/L ethanolamine pH 9 (NaOH) was added to block unreacted sites. The resin was stirred overnight in the quenching solution then centrifuged and washed a first time with 0.05 mol/L PBS pH 7.2 for 20 min, with 1 mol/L NaCl and finally with PBS 3 times.

The antiserum diluted to 1/10 in PBS was added to the activated beads and stirred gently for 30 min. After centrifugation and recovery of the supernatant, the antibodies bound to the BSA-Ace-Me conjugate were removed with 4 mol/L

NaCl in PBS for 30 min and the resin was washed 3 times with PBS before another use.

The antiserum was treated 4 times with the immunoabsorbent.

ELISA Protocol

The plates were coated by passive adsorption overnight at +4°C. The antigens for coating were dissolved in 0.05 mol/L PBS pH 7.4.

After 5 washings with PBS containing 0.05 % Tween 20 (PBS-Tw) to remove unadsorbed conjugate, 50 µL of standard solution or sample and 50 µL of a dilution of antiserum in PBS-Tw were dispensed directly to plate wells. After 1 h on a table top shaker at room temperature, the plates were washed 5 times as above. Next, 100 µL of goat anti-rabbit IgG-horseradish peroxidase diluted 1/2000 in PBS-Tw was reacted for 1 h at room temperature. Then the plates were washed 5 times as before followed by addition to each well of 100 µL of a 0.2 mg/mL solution of substrate (TMB) in citrate-phosphate buffer pH 4 containing 0.01 % urea-hydrogen peroxide. After 10 min at room temperature, the colored reaction was stopped with 100 µL of 0.5 mol/L H_2SO_4 and optical densities were determined at 450 nm with a reference at 620 nm.

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Sample Preparation

Food Sample

The food samples were prepared by the method of Ives and Strong (12) modified by Gonthier et al. (13) using mylase to extract free pantothenic acid.

Each sample was treated with the enzyme (100 mg in 15 mL of 0.5 mol/L acetate buffer pH 4.5) for 2 h at 50 °C. After autoclaving 15 min at 120 °C to destroy any remaining enzyme activity and to coagulate the proteins, the samples were allowed to cool to room temperature, adjusted to pH 7.4 with NaOH, transferred to a 100 mL volumetric flask and made up to volume with distilled water. The contents were mixed thoroughly and filtered through filter paper then stored at -18 °C. The day of the assay further dilution of sample extract was necessary to obtain a total pantothenate concentration of 10 to 50 ng/mL for the microbiological method or of 10 to 1000 ng/mL for the immunoassay.

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Blood and Serum

The alkaline phosphatase (from calf intestine) alone was used for liberation of pantothenic acid in whole-blood (14): 1 mL of a 0.8 % (w/v) alkaline phosphatase in Tris buffer pH 10.3 was added to 1 mL of blood. After one night at 37°C, the sample was autoclaved for 10 min at 120°C and centrifuged at 2000 g for 15 min. The resulting supernatant was then assayed.

The serum was filtered in collodion bags (Sartorius AG, Goettingen, Germany) at 7500 g for 15 min. This filtered sample with lower protein concentration was used for the pantothenic acid assay

Microbiological Method

The sample extract was assayed using the microbiological method of Skeggs and Wright (7) with *Lactobacillus plantarum* and Vitamin pantothenic acid assay

broth for microbiology (Merck) as growth medium. Two mL of the dilution of the sample extract were added to 3 mL of medium, mixed by rotating the tube vigorously in the palm of the hand and sterilized by autoclaving 15 min at 115 °C. After cooling at room temperature, the tubes were aseptically inoculated with one drop of the inoculum and incubated at 37 °C for 20 h.

The turbidity of the growth medium was measured by the optical density at 620 nm and when was plotted against a standard curve of pantothenic acid solution from 0 to 50 ng/mL.

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RESULTS:

Antibody Preparation and Characterization

Coupling Ratio

Both procedures for liberation of pantothenic acid from immunogens (alkaline hydrolysis or enzymatic extraction) gave similar results for the normalized coupling ratio (number of molecules of pantothenic acid by 66000 Da of carrier protein. This expression takes in consideration the molecular weight of the carrier which is very different between BSA, KLH and thyroglobulin) (Table 1). 13 to 17 molecules of pantothenic acid per 66000 Da of the carrier proteins (BSA-Adi-B_5, Thyro-Adi-B_5 and BSA-Ace-B_5) were bound. The enzymatic extraction gave higher values than the alkaline hydrolysis for the acetyl conjugates while the contrary was observed for the adipoyl conjugates.

TABLE 1:

Coupling ratios of the different conjugates according to the extraction procedure. (Hapten-protein ratio : molecules of pantothenic acid / molecule of carrier protein, Normalized coupling ratio : molecules of pantothenic acid / 66000 Da of carrier protein)

		Alkaline hydrolysis		**Enzymatic extraction**	
Conjugates	**M.W (Da) carrier protein**	**Hapten protein ratio**	**Normalized coupling ratio**	**Hapten protein ratio**	**Normalized Coupling ratio**
BSA-Adi-B_5	66000	16.8:1	16.8	10:1	10
Thyro-Adi-B_5	650000	1.8:1	17.5	1.1:1	10.8
BSA-Ace-B_5	66000	13.2:1	13.2	16:1	16
KLH-Ace-B_5	1000000	0.1:1	1.5	0.3:1	4.5
HSA-Ace-B_5	66000	0.6:1	0.6	1.5:1	1.5

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For the capture antigens, the normalized coupling ratios were smaller, 2 to 4 for KLH and 0.6 to 1.5 for HSA.

Characterization of the Antisera

Although the normalized coupling ratios were similar (Table 1), only the BSA-Ace-B_5 conjugate induced specific antibody production against the vitamin. The adipoyl conjugates (BSA-Adi-B_5 and Thyro-Adi-B_5) have only induced antibodies against carrier protein but none against the pantothenic acid.

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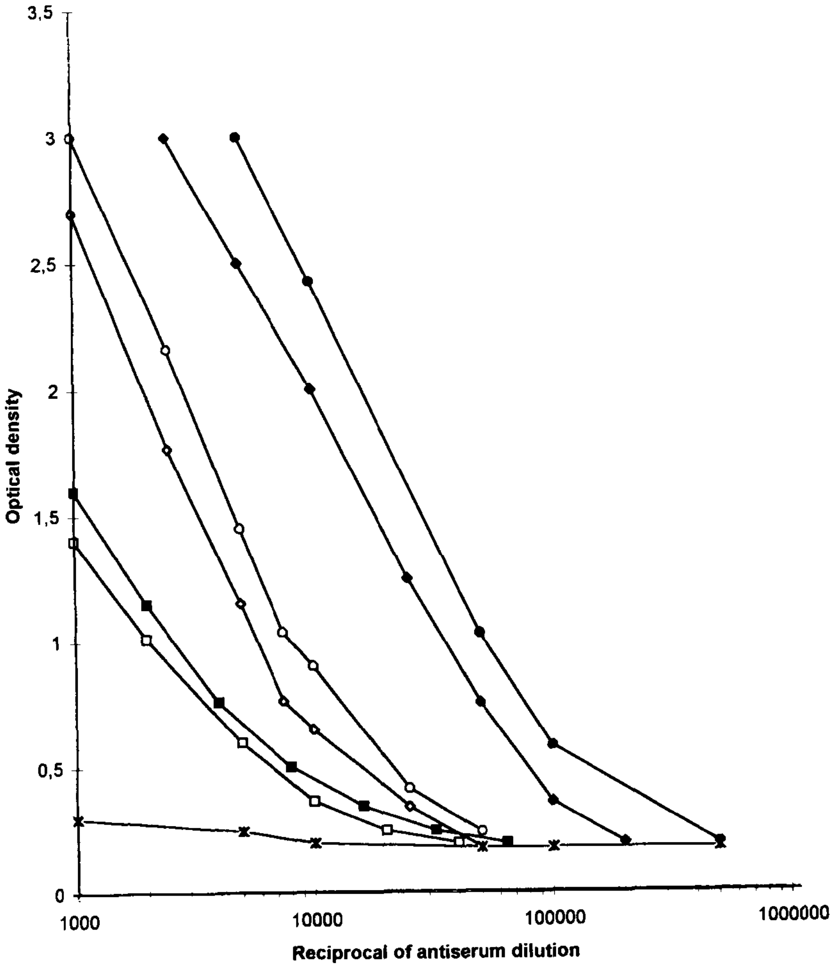

FIGURE 2: Dilution curves of the antiserum obtained with BSA-Ace-B_5 as the immunogen in function of the capture antigen:
Thyro-Adi-B_5 (—■— or —□—), KLH-Ace-B_5 (—●— or —○—) and HSA-Ace-B_5 (—◆— or —◇—) with crude (plain symbols) or purified (open symbols) antiserum.
(—*—: non immune serum).

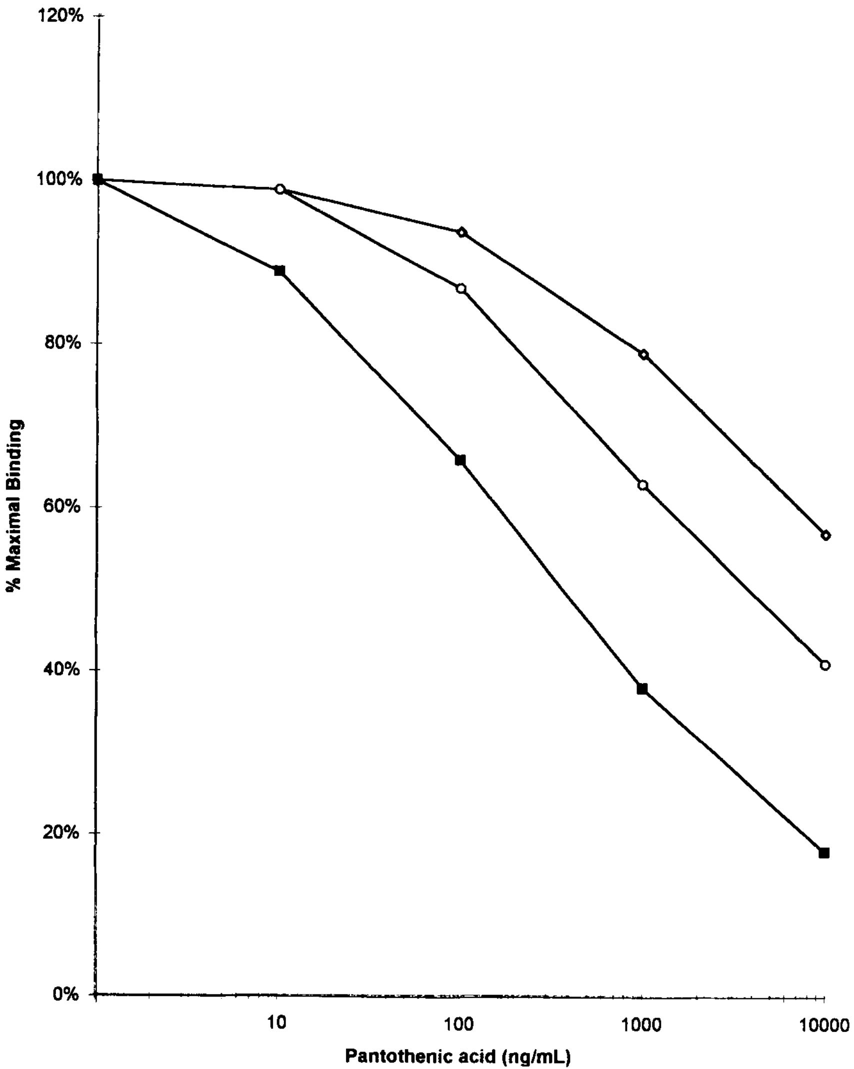

FIGURE 3: Inhibition of the fixation of the antibody by pantothenic acid according to the coating antigen:

HSA-Ace-B_5 10µg/mL (—◇—) KLH-Ace-B_5 0.1 µg/mL (—o—) both with purified serum (1/2500 in PBS-Tw) and Thyro-Adi-B_5 1µg/mL (—■—) with crude serum (1/1000 in PBS-Tw).

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The antiserum obtained after 6 injections using the BSA-Ace-B_5 conjugate was tested with different capture antigens (Figure 2). The dilution of the antiserum giving an optical density equivalent to the blank control (without first antibody) differed according to the capture antigen: 1/50000 on Thyro-Adi-B_5 (1μg/mL), 1/200000 on HSA-Ace-B_5 (10μg/mL) and 1/500000 on KLH-Ace-B_5 (0.1μg/mL). However, a very high quantity of free vitamin was needed to inhibit the fixation of the antibody on a capture conjugate with the same linker as the immunogenic antigen (HSA-Ace-B_5, KLH-Ace-B_5). This is likely due to the greater affinity of the antibody to the coupled pantothenic acid than to the free.

The purification of the immune serum by immunochromatography with a resin coupled with BSA-Ace-Me gave a reduction of the titer on the 2 coatings with acetyl arm (HSA-Ace-B_5, KLH-Ace-B_5) (Figure 2) but with no improvement of the sensitivity (not shown). On the other hand this purification had no significant effect on a coating with Thyro-Adi-B_5 (Figure 2).

The sensitivity of the assay evaluated as half of the maximal binding was higher with Thyro-Adi-B_5 as capture antigen even without purification of the serum (Figure 3). From these different data, Thyro-Adi-B_5 was chosen for the further experiments and the unpurified antiserum used.

ELISA Optimization

Coating Conditions

We have compared plates with high capacity of adsorption (Nunc Maxisorp

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and Costar EIA/RIA plate High Binding) and plates with normal capacity (Nunc Polysorp and Greiner). The first plates needed a lower quantity of capture antigen (0.1 μg/mL of Thyro-Adi-B_5), but they required a saturation step to reduce non-specific fixation. As, for this purpose, milk powder and casein contain pantothenic acid, we have chosen gelatin from cold water fish skin (0.05 % in PBS, 250 μL per well, 1 h) after 3 washes of the plates with PBS without Tween.

We have noticed that the sensitivity of our assay was inversely correlated to the quantity of capture antigen. So, plates with high fixation capacity were not necessary. Thus, we have chosen Nunc Polysorp plates in preference to Greiner plates because lower internal coefficients of variation were observed. Due to the sensitivity of the conjugate to the alkaline hydrolysis, we have used 0.05mol/L PBS pH 7.4 as coating buffer in place of the classical carbonate buffer pH 9.2. The solubility of the Thyro-Adi-B_5 conjugate was enhanced by the addition of urea. So we have dissolved the freeze-dried antigen at 1 mg/mL in 4 mol/L urea in PBS pH 7.4 and diluted this solution at 1 μg/mL in PBS pH 7.4 without urea. The plates were coated with this solution at 250 μL per well overnight at +4°C.

Using these conditions, the saturation with gelatin was not necessary for antiserum used at higher dilutions than 1/1000.

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Conditions of Incubation

In the studied range, the dilution of the antiserum had no significant influence on the characteristics of our assay. But a higher concentration in antibody gave a

bigger amplitude of the reading of the optical density. This optical density did not depend on the volume added to the well and a preincubation stage had no effect. So, we chose to add 50 µL of the standard dilution or sample and 50 µL of immunserum diluted at 1/1000 in PBS-Tw were added directly to each well. No difference was noted between an incubation of the plates at 37°C or at room temperature. At +4°C, the dynamic range was slightly smaller without modification of the percentage of inhibition (not shown).

The optimal time of the first incubation was 1h. Longer incubation times produced higher blank values without any beneficial effect on the sensitivity of the assay (Figure 4).

A typical dose-response curve obtained with these conditions is shown in Figure 5. The limit of detection defined as the 2 fold standard deviation of the 0 point was 10 ng/mL and the measurement range was established between 10 and 5000 ng/mL of pantothenic acid.

The effects of pH and concentrations of protein on the fixation of the antibody on the capture antigen were studied. Incubation solutions between pH 6 and 9.2 and containing less than 0.5 to 0.1 % of protein did not significantly affect the ELISA system. Stronger acidic or alkaline solution or higher concentrations of protein inhibited the fixation of the antibody or displaced the coating antigen. This resulted to a smaller optical density and an apparent higher pantothenic acid value.

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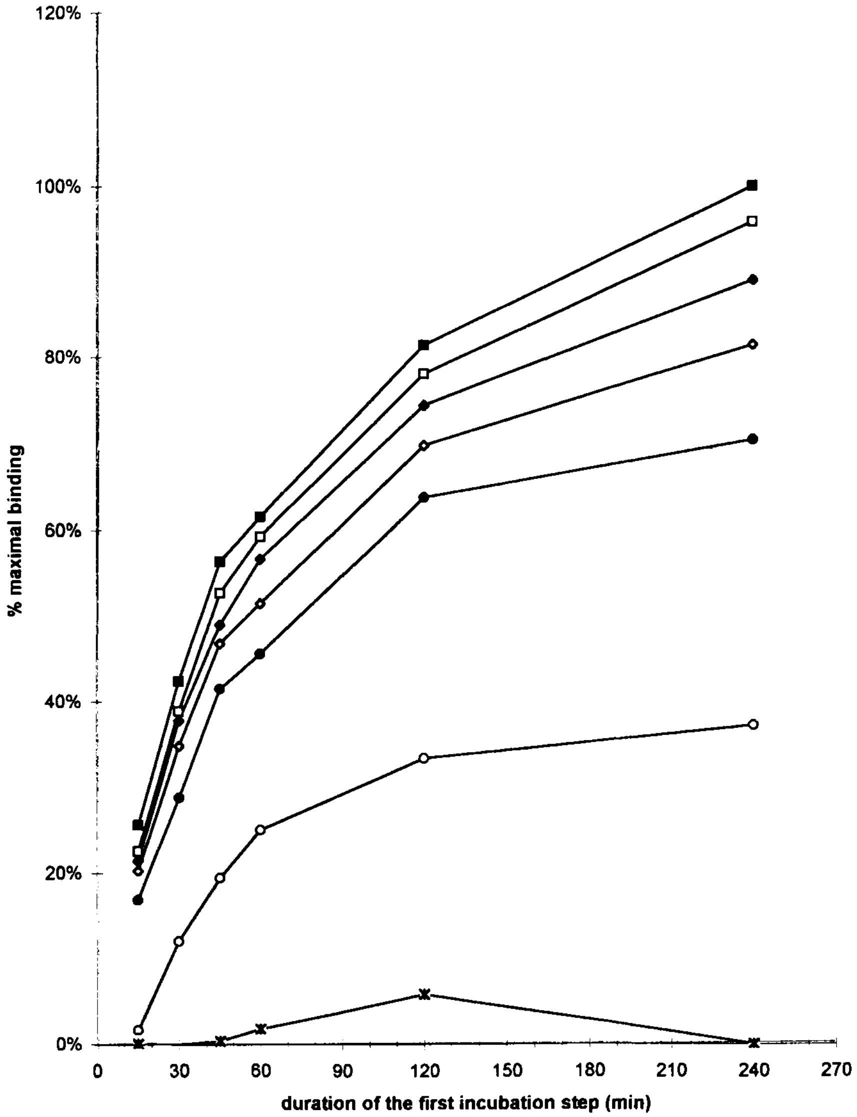

FIGURE 4: Kinetic studies of the first incubation step in function to the concentration of pantothenic acid: 0 ng/mL (—■—), 10 ng/mL (—□—), 20 ng/mL (—◆—), 50 ng/mL (—◇—), 100 ng/mL (—●—), 1000 ng/mL (—○—) and 10000 ng/mL (—*—).

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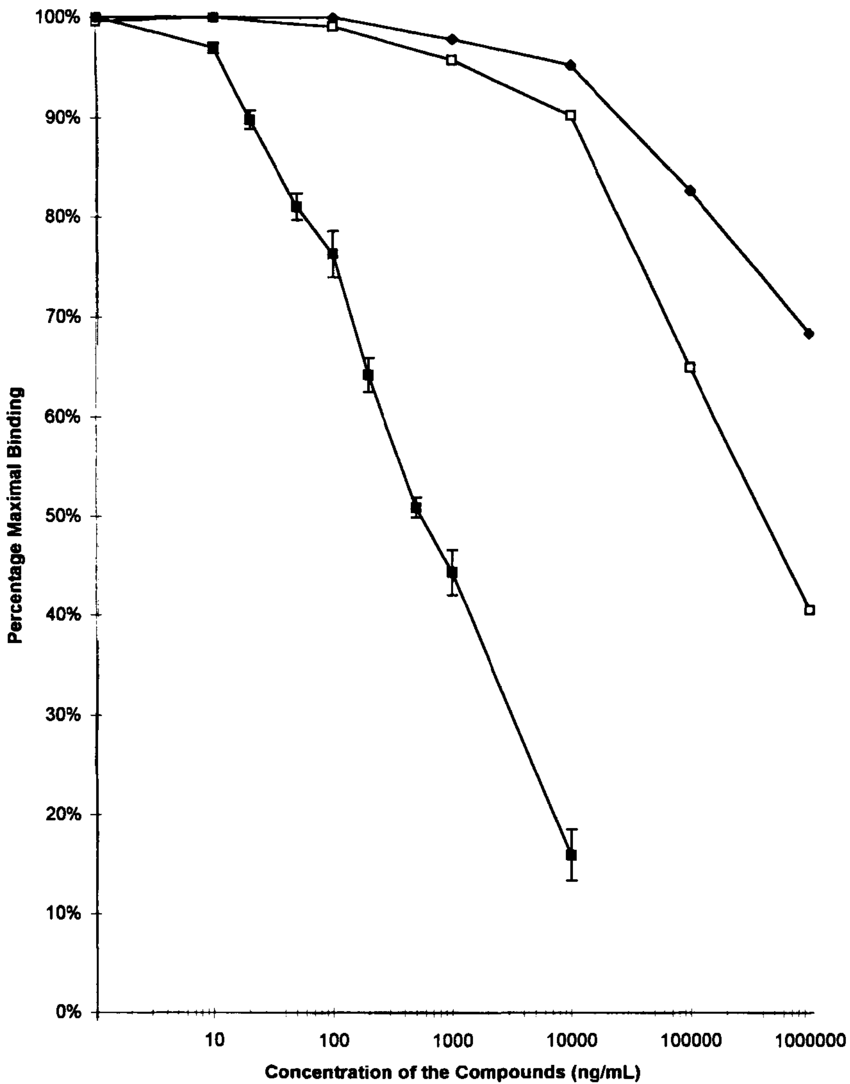

FIGURE 5: Inhibition of the fixation of the anti-pantothenic acid antibody on the coated antigen (Thyro-Adi-B_5) by pantothenic acid (—■—), coenzyme A (—□—) or pantothenol (—◆—). (the bares correspond to the SD on the percentage of maximal binding on 4 wells).

Cross-reactions of the antisera with coenzyme A and pantothenol were tested. The fixation of the antibodies was inhibited only with high quantities of CoA and pantothenol. The amounts of CoA and pantothenol needed to displace 50% of maximal binding were respectively ca 1000 and more than 10000 fold that of free pantothenic acid (Figure 5). These concentrations are higher than those present in biological fluids.

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Applications of the assay

Food Products

Pantothenic acid (1 μg) treated by the mylase extraction procedure and assayed with the ELISA system gave a mean of 1095 +/- 222 ng (n=4). This extracted standard and supplemented food (chocolate powder) gave curves of dilution identical to the standard curve (Figure 6) . For the analysis of food products, the intra and inter assay coefficients of variation including both the extraction and measurement steps were below 20% (13).

We have compared the immunological method to the microbiological method for the determination of the pantothenic acid in foods. For 12 different foods (chocolate powder, infant formula, supplemented cookies...) we have obtained a good correlation between the 2 methods. The linear regression equation was y=1.02x-33 and the correlation coefficient was r=0.986.

Pantothenic acid (200 ng) added to each food before the extraction step gave recoveries of 209 +/- 31 ng (n=4) with extremes of 158 and 249.

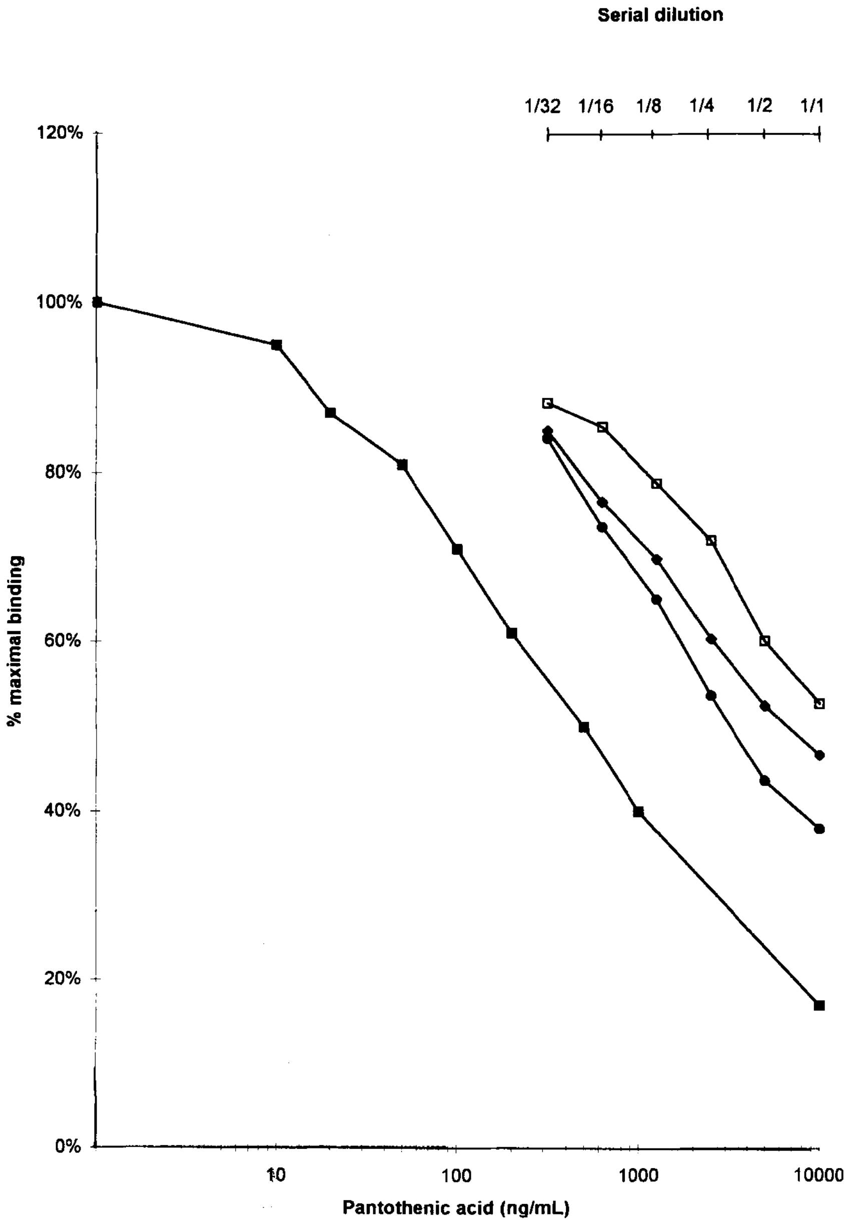

FIGURE 6: Application of the ELISA method to food products and blood: Standard curve for pantothenic acid (—■—) and serial dilution curves of an extracted standard (—□—), a food product (—●—) or an extracted sample of human blood (—◆—).

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Blood and Serum.

In contrast with blood for which extraction is required, no enzymatic hydrolysis was needed in serum prior to the analysis as it contains free pantothenic acid only.

Dilution curve of blood extracted with alkaline phosphatase was identical with the standard curve (Figure 6). In serum, the high concentration of protein was interfering to our ELISA system. This interference was eliminated by filtration of the serum by the collodion membrane. Four samples of the same serum from a pool of human healthy adult filtered separately and assayed on the same plate gave a result of 79.7 +/- 9 ng/mL (mean+/-SD). The percentage of recovery of pantothenic acid added before the filtration varied from 96 to 112 %

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DISCUSSION

The development of dietetic food products increases the demands of vitamin assays for nutritional labeling, process controls and other nutritional studies. This requires more rapid and specific method than the classical microbiological assay. The immunological assays and particularly the enzyme-linked immunosorbent assay appear well-suited to reach these goals (15).

For this purpose we prepared antibodies to pantothenic acid coupled to a carrier protein with different linkers.

The coupling ratios obtained with the bromoacetyl bromide as linker were lower to those observed by Wyse et al. (8) (24 molecules of pantothenic acid / molecule of BSA) or by Morris et al. (9) (20 molecules/molecule of BSA). But,

they were determined with ^{14}C pantothenic acid. The yield of the conjugation was quite good according to the number of coupling sites of the BSA: a single cysteine residue and 34 half-cystine residues forming 17 disulfide bonds (16).

The fixation of the Adipoyl chloride-Pantothenate conjugate occurs on the NH_2 group of the lysine residues. Thyroglobulin contains 150 to 200 lysine residues (17) and BSA 59 (18) with molecular weight of 650000 and 66000 Da respectively. Despite this difference of potential coupling sites, we observed similar normalized coupling ratio with this arm : ca 17 molecules of pantothenic acid per 66000 Da of carrier protein.

Regarding the quite flat slope of the standard curve, the affinity of the antibody we obtained with BSA-Ace-B_5 to the free hapten is likely to be low. Using the same immunogen, Wittwer et al. (19) reported a weak association constant of ca 10^7 L/mol. An increase of this association constant might be obtained using another immunogens. Unfortunately, we have not been able to produce antibody using adipoyl dichloride as linker although the hapten protein ratio appeared sufficient to obtained immunogenic conjugates. So, the real influence of the linker on the affinity of the antibody to free pantothenic acid could not be determined. We have noticed that the thyroglobulin conjugate induced weaker inflammatory reactions on the back of the rabbit than the BSA conjugates. However, there were no difference in this respect between the two BSA conjugates (BSA-Adi-B_5 and BSA-Ace-B_5).

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The sensitivity of our assay was very low when we used KLH-Ace-B_5 and HSA-Ace-B_5 as capture antigen even with purified antibody. This sensitivity was better after we have changed the linker of the capture antigen (Thyro-Adi-B_5). The use of this capture antigen allowed us to obtained greater sensitivity than those reported by Song et al. (11) or Morris et al. (9). There are two possible explanations. Although the immunogen carrier protein (reduced and denatured BSA) was different from the capture carrier protein (reduced and denatured KLH or HSA in native form), the use of the same conjugation procedure might induce antigenic cross-reactions. The other explanation is that the adipoyl dichloride arm decreased the affinity of the antibody to the bound vitamin by comparison to the free. So the competition between free and immobilized pantothenic acid toward the site of the antibody reached an equilibrium by contrast to what Song et al. (11) had noticed. The 1 hour competition step increased the dynamic range but did not modify the percentage of inhibition.

As Morris et al. (9) had reported, the sensitivity of the ELISA system was inversely correlated to the quantity of the coating antigen. Using Thyro-Adi-B_5 at 1 μg/mL as capture antigen the slope of the standard curve of our assay was steeper than that of the two another ELISA systems (9, 11) with similar other characteristics. The limit of detection was 10 ng/mL corresponding to a quantity of 500 pg of calcium pantothenate per well. The standard curve was log-linear between 10 and 5000 ng/mL.

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The measurement of pantothenic acid in food and blood depend on the extraction procedure. In red blood cell and food the vitamin occurs as well on free form (pantothenic acid) and combined forms (CoA or different metabolites). These combined forms must be hydrolyzed by extraction procedure before measurement of pantothenic acid. This extraction is done by endogenous or added enzymes. In serum, this extraction is not necessary. The serum contain only the free form of the vitamin B_5. But it must be obtained rapidly by centrifugation to avoid the migration of free vitamin from red blood cell. These aspects will need further studies which are in progress.

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In conclusion we have developed an ELISA easier to perform and less time-consuming than the microbiological assay. This immunological assay will be tested in further studies with the aim to use it in routine analysis of pantothenic acid in foods and blood.

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