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Quantitative determination of vitamin B_6 in dietary foods for special medical purposes by microbiological assay method

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"Dietary food for special medical purposes" is defined as the foodstuffs that are specifically produced or formulated and consumed under medical supervision with the aim of establishing diets of patients. These foods labeled and marketed are generally liquid or powder products formulated to meet specific needs. The objective of this research was to quantitative determination vitamin B_6 (pyridoxine) in dietary foods for special medical purposes by the use of microbiological assay method. To this end, the quantity of vitamin B_6 in total 36 liquid dietary foods for special medical purposes was determined by disc inoculum method of microbiological assay using *Neurospora sitophila* ATCC 9276. The response of the organism to pyridoxine was determined by measuring the zone diameter of growth surrounding the inoculum disc, and the amount of vitamin B_6 from these foods ranged from 0.12 mg/100 mL to 0.76 mg/100 mL. In the statistical evaluation, the results were evaluated according to independent samples T-test. It was observed that the values determined for these foods were similar to the values declared on the labels of the foods. The results of the study have indicated that quantitative determination of vitamin B_6 in the foods can be achieved by the use of microbiological assay method. Also, it is approved that the values of vitamin B_6 declared on the labels of foods are accurate.

Key words: Dietary food for special medical purposes, vitamin B_6 (pyridoxine), microbiological method.

INTRODUCTION

Dietary foods for special medical purposes are used to meet and/or support the entire nutritional needs of an individual who cannot receive adequate amount of nutrition by normal feeding procedure (mouth) for various reasons (loss of appetite, various diseases, operations…etc). In Turkey, The Ministry of Health is responsible for the regulations and practices of import, export, production, and inspection of these foods according to the law of (No = 5996) 'production, consumption, and inspection of foods as described in the amended law' (Anon, 2010). Since these foods are not currently produced in our country, they are only inspected for legal import and market controls. Dietary foods for special medical purposes contain vitamins. Vitamins are substances that the body needs to grow and develop normally. Vitamin B_6 is a water-soluble vitamin that exists in three major chemical forms pyridoxine, pyridoxal and pyridoxamine (Wrigth, 1954). The nervous and immune system in the body need vitamin B_6 to function effectively; and it is also essential for blood cell metabolism and protein metabolism (Bender, 1994).

Various methods such as chemometric, electrochemical, spectrophotometric, fluorometric, voltammetric, microbiological and chromatographic have been used to deter-

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Table 1. Composition of the basal medium for pyridoxine assay.

Parameter	Measurement
Sucrose	20.0 g
Ammonium tartarate	5.0 g
Ammonium nitrate	1.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.5 g
Sodium chloride	0.1 g
Calcium chloride	0.1 g
Biotin	4.0 mL
Salt solution	750.0 mL

mine the amount of vitamin B_6 (Mo and Cao, 2007; Ganjali et al., 2008; Liu et al., 2012). This study aimed to show the applicability of microbiological method in determination of the amount of vitamin B_6 (pyridoxine) in dietary foods for special medical purposes.

MATERIALS AND METHODS

The quantity of vitamin B_6 was determined by microbiological assay method using *Neurospora sitophila* ATCC 9276. In assay with the *N. sitophila* ATCC 9276, the disc inoculum method described by Barton-Wright (1963) was used with minor modifications. To test the applicability of the method, 36 imported liquid dietary foods for special medical purposes having valid shelf lives were used.

Test organism and growth media

N. sitophila ATCC 9276 used in our study was obtained from Culture Collections of the Refik Saydam National Public Health Agency. The test organism, *N. sitophila* ATCC 9276 was grown in the media which contained (per L) 5.0 g yeast extract, 5.0 g protease peptone, 40.0 g maltose and 15.0 g agar. The spores from a 48-h culture of the organism at 25±1°C were used.

Basal medium for pyridoxine assay

The basal medium determined by Barton-Wright (1963) was used. The composition of the basal medium is given in Table 1. The pH of the medium was adjusted to 4.6 to 4.8 with 0.01 M HCl and 0.01 M NaOH. Finally, the washed agar was added in the basal medium.

Preparation of washed agar

Twenty gram agar was weighed and completed to 1000 mL distilled water. Four grams of crystalline sodium sulphide was added. A few drops of concentrated hydrochloric acid were added until there was a strong smell of SO_2. The mixture was allowed to stand for 5 to 6 h and the supernatant liquor was poured off. The agar was repeatedly washed over 24 h with several changes of 1000 mL distilled water, acidified with hydrochloric acid and allowed to stand for 2 to 3 h between each change.

Preparation of biotin

10 mg of anhydrous *d*-biotin (Sigma) was diluted to 100 mL with 0.2 N acetic acid. Then, 1.0 mL quantity of this solution was diluted to 100 mL with distilled water.

Table 2. Composition of the salt solution.

Parameter	Measurement
Boric acid	5.72 mg
Ammonium molybdate	25.75 mg
Copper sulphate	2.50 mg
Ferrous sulphate	10.00 mg
Manganese sulphate	6.20 mg
Zinc sulphate	9.00 mg
Distilled water	1000.00 mL

Salt solution

The composition of the salt solution is given in Table 2.

Vitamin B_6 standard solution

10 mg of pyridoxine hydrochloride standard (Sigma) was diluted in 100 mL distilled water, and 1.0 mL of this solution was diluted in 100 mL distilled water. Then, 2.0 mL of the solution was diluted in 100 mL distilled water and final volume was 0.02 µg/mL.

Preparation of inoculum

4 mL of the vitamin B_6 standard solution was added 1.0 mL distilled water and autoclaved at 121°C for 15 min. Then, a loopful of spores was taken from a slope culture, placed in the solution, well-dispersed by shaking and poured into a sterile Petri dish. A tube of basal medium which melted in a boiling water bath was poured into the dish. All were well mixed and allowed to set. The dish was incubated for 18 to 20 h at 25±1°C. At the end of the incubation, inoculum agar discs with a diameter of 8 mm were cut from the Petri dish where a well-distributed mycelia growth was obtained.

Preparation of samples

A suitable quantity of the liquid dietary foods for special medical purposes to be assayed was weighed, added 4% NaOH and autoclaved for 1 h at 121°C. Then, the pH of the samples was adjusted to 4.5 with 0.1 N HCl and the samples were filtered with Whatman-41 filter paper. 0.5, 1.0, 2.0 and 4.0 mL of the filtrate were added in flasks, respectively. The total volume in each flask was made up to 5.0 mL with distilled water. Similarly, 0.02 µg/mL vitamin B_6 standard solution was prepared in flasks containing solutions from 0.5 to 4.0 mL and the final volume was made up to 5.0 mL with distilled water. The standard and all the samples were analyzed in duplicate. In addition, blank was prepared with 5.0 mL distilled water. Blank, standard and samples were autoclaved at 121°C for 15 min and poured into sterile Petri dishes. The requisite number tubes of containing basal medium were melted in a water bath and poured into the dishes.

All were mixed and allowed to set. When the medium was set firm, the inoculum agar discs, which were prepared were placed in the center of each dish. The dishes were incubated at 25±1°C for 18 to 20 h.

Calculation

The response of the organism to pyridoxine was determined by

Table 3. The amounts of vitamin B_6 from dietary foods for special medical purpose (mg/100 mL).

Code	Zon diameter* (mm)	Label value	Determined value
E1	26±0.00	0.17	0.17
E2	25±0.00	0.13	0.13
E3	25±0.00	0.15	0.13
E4	24±0.00	0.13	0.13
E5	24±0.00	0.14	0.13
E6	25±0.00	0.19	0.18
E7	26±0.00	0.18	0.18
E8	26±0.00	0.19	0.18
E9	41±0.00	0.56	0.50
E10	26±0.00	0.18	0.16
E11	25±0.00	0.17	0.16
E12	26±0.00	0.20	0.18
E13	25±0.00	0.18	0.18
E14	25±0.00	0.18	0.18
E15	31±0.00	0.35	0.35
E16	30±0.00	0.33	0.33
E17	33±0.00	0.37	0.35
E18	32±0.00	0.35	0.33
E19	31±0.00	0.35	0.33
E20	33±0.00	0.37	0.35
E21	24±0.00	0.18	0.17
E22	33±0.00	0.37	0.35
E23	30±0.00	0.35	0.35
E24	24±0.00	0.18	0.17
E25	25±0.00	0.21	0.20
E26	27±0.00	0.26	0.26
E27	40±0.00	0.65	0.65
E28	30±0.00	0.35	0.34
E29	39±0.00	0.52	0.50
E30	25±0.00	0.15	0.15
E31	24±0.00	0.13	0.12
E32	39±0.00	0.53	0.54
E38	26±0.00	0.16	0.17
E34	26±0.00	0.19	0.18
E235	42±0.00	0.75	0.76
E36	27±0.00	0.28	0.29

*Values are means ± standard deviations of duplicate measurements.

measuring the zone diameter of growth surrounding the inoculum disc with calipers as mm. The relationship between 0.5, 1.0, 2.0 and 4.0 mL of 0.02 µg/mL vitamin B_6 standard solution and the zone diameters determined for each of these concentrations were calculated by using potent regression feature of calculator. The quantity of vitamin B_6 in the sample was determined by the concentration corresponding to the zone diameter and dilution factor.

Statistical analysis

In the statistical evaluation, the results were evaluated according to independent samples T-test. The difference between the mean vitamin B_6 values determined in the samples and the mean values declared on the food label was not statistically significant ($p>0.05$).

RESULTS

In this study, the quantity of vitamin B_6 in total 36 liquid dietary foods for special medical purposes was determined by microbiological assay method using *N. sitophila* ATCC 9276. In the assay with the *N. sitophila* ATCC 9276 in our laboratory, the disc inoculum method, a former method described by Barton-Wright (1963) was used. In our study, the response of the organism to pyridoxine was determined by measuring the diameter of growth surrounding the inoculum disc, and the amount of vitamin B_6 from these foods ranged from 0.12 mg/100 mL to 0.76 mg/100 mL. The blank exhibited no growth. The diameters of growth surrounding the inoculum discs in the foods, and the amounts of vitamin B_6 are given in Table 3. In addition, the growths of the organism surrounding the inoculum discs in the vitamin B_6 standard solution and the food (E25) as the response of the organism to pyridoxine are shown in Figure 1. For Table 3 statistical

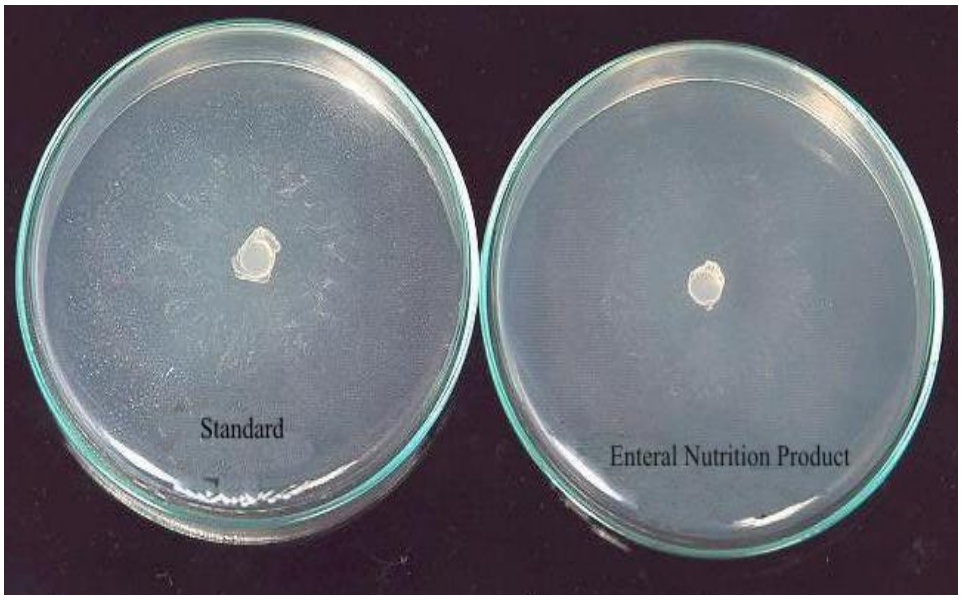

Figure 1. The growths of the organism surrounding the inoculum discs in 4.0 mL of the vitamin B_6 standard solution and 4.0 mL of the dietary food for special medical purpose (E25).

evaluations, the independent sample T-test was used.

There were no statistically significant differences between the mean values determined for liquid foods for special medical purposes and the mean values declared on the labels of the foods ($p > 0.05$).

DISCUSSION

This study examined the quantity of vitamin B_6 in liquid dietary foods for special medical purposes by the use of microbiological assay method. Various studies have been conducted to determine the quantity of vitamin B_6 in different samples as foods, food supplements, drugs, energy drinks, pharmaceutical preparations (Sather and Verning, 2011; Muszalska et al., 2011; Thi Viet Do et al., 2012; Kirilov et al., 2012). Short and Fairbairn (1962) observed that their study has been made of the application to urine of two microbiological methods using *Saccharomyces carlsbergensis* and *N. sitophila*, mutant 299 as the test organisms for the assay of vitamin B_6. It is determined that modifications have been made to the method using *N. sitophila*, mutant 299, which enables it to be applied satisfactorily to assays of total vitamin B_6 in urine; although, the method using *S. carlsbergensis* is unsuitable.

In our research, it is observed that the microbiological assay method using *N. sitophila* ATCC 9276 as the test organisms for determination of vitamin B_6 in liquid dietary foods for special medical purposes can be applied successfully. Also, it was determined that the amount of vitamin B_6 from dietary foods for special medical purposes ranged from 0.12 mg/100 mL to 0.76 mg/100 mL (Table 1). Many researches described for determination of vitamin B_6 in infant formula represented special dietary for infants (Mann et al., 2001, 2005). In a collaborative study by Bergaentzlé et al. (1995), different food samples and solution for tube feeding containing various amounts of vitamin B_6 (form 0.6 to 32.8 µg g^{-1} of pyridoxol) were analyzed. Kall (2003) reported that amount of vitamin B_6 (pyridoxine) in different food samples determined through HPLC analysis and a microbiological analytical method using *Sacchoramyces uvarum* ranged between 0.006 and 0.0124 mg/100 g and 0.02 and 0.04 mg/100 g (pyridoxine, HCl), respectively. Markopoulou et al. (2002) determined that pyridoxine hydrochloride (B_6) in multivitamin tablets was 50-300 µg/mL. In our study, the values determined from the liquid dietary foods for special medical purposes and the values declared on the labels of the foods were compared. It was found that the values determined for liquid dietary foods for special medical purposes were similar to the values declared on the labels of the foods.

In conclusion, the results of this study showed that the quantitative determination of vitamin B_6 (pyridoxine) in the liquid dietary foods for special medical purpose can beapplied to disc inoculum method of microbiological assay using *N. sitophila* ATCC 9276. Also, it is approved that the values of vitamin B_6 declared on the labels of foods are accurate. However, comparison of these method and HPLC for determining the amount of vitamin B_6 in dietary foods for special medical will be the focus of our future study.

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