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Spectrophotometric Determination of Vitamin B_6 by Coupling with Diazotized p-Nitroaniline

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ABSTRACT

A spectrophotometric method has been described for the determination of pyridoxine hydrochloride (vitamin B_6) in pure and pharmaceutical preparations. The method is based on coupling reaction of vitamin B_6 with diazotized p-nitroaniline in alkaline solution in the presence of CTAB to form an azo dye which has maximum absorption at 480 nm. Beer's law is obeyed over a range of 5-500 µg/25ml with a molar absorpitivity of 2.7×10^4 l. $mol^{-1}.cm^{-1}$ and Sandell sensitivity index of 0.0076 $\mu g.cm^{-2}$, a relative error +0.22 to + 0.47% and a relative standard deviation of ± 0.11 to ± 0.98%, depending on the concentration level. The proposed method has been applied successfully to the determination of vitamin B_6 in pharmactical preparations.

Keywords : spectrophotometry ; diazo – coupling ; p-nitroaniline ; vitamin B_6 .

التقدير الطيفي للفيتامين B_6 بالاقتران مع الكاشف بارانايتروانيلين المؤزوت

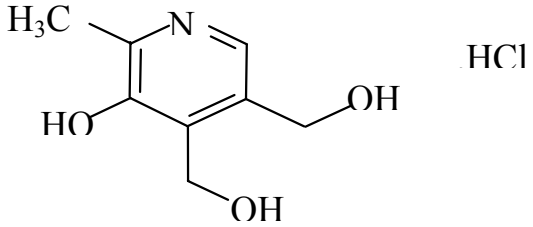
الملخص

تم اقتراح طريقة طيفية لتقدير البيروكسيدين هيدروكلورايـد (الفيتـامين B_6) بـشكله الحـر وفـي مستحضراته الدوائية. تعتمد الطريقة على تفاعل الاقتران لفيتامين B_6 مع 4-نايتروانيلين المؤزوت في وسط قاعدي وبوجود الـ CTAB لتكوين صبغة ازوية تعطي أعلى امتصاص عند 480 نانوميتر. كانت حـدود قانون بير 5-500 مايكروغرام / 25مل. وكانت الامتصاصية المولارية 10^4 ×2.7 لتر. مـول$^{-1}$. سـم$^{-1}$ ودلالة ساندل 0.0076 مايكروغرام . سم$^{-2}$ ، الخطا النسبي يتراوح بين 0.22+ و 0.47 + % وانحـراف قياسي نسبي بين 0.11 ± و 0.98 ± % اعتمادا على مستوى التركيز. تم تطبيق الطريقة المقترحة بنجاح في تقدير الفيتامين B_6 في مستحضراته الصيدلانية .

INTRODUCTION

Vitamins are biologically active organic compounds with a diverse chemical nature. They enter in the human organism with food, in small amounts, and play a major role as biocatalysts in metabolism. Both a lack and an excess of certain vitamins in an organism may cause significant disturbances of various functions of the organism, resulting in serious diseases (Melentyeva and Antonova, 1988). The vitamins of the B_6 group are compounds that contain the pyridine ring in their molecules and they are water soluble. There are six forms of vitamin B_6 : pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their phosphate derivatives : pyridoxal 5-phosphate (PLP), pyridoxine 5-phosphate (PNP), and pyridoxamine 5-phosphate (PMP). Pyridoxine was the first isolated vitamin B_6 (Kaplan and Pesce, 1989).

Pyridoxine hydrochloride (5-hydroxy-6-methylpyridine-3, 4-diyl) dimethanol hydrochloride is a white or almost white, crystalline powder, freely soluble in water, slightly soluble in alcohol. It melts at about 205^{o}C, with decomposition, the molecular structure $C_8H_{11}NO_3$.HCl,M.Wt = 205.6 g/mol (British Pharmacopeia, 2005) is as follows:



Pyridoxine functions as a coenzyme in the metabolism of amino acids, protein and the maintenance of body cells (Lehne, 2001).

There are various analytical procedures for the assay of pyridoxine, the most important included flow injection systems (Pons *et al.*, 2000; Portela *et al.*, 2004), high performance liquid chromatography methods for the determination of pyridoxine and isoniazid in pharmaceutical formulations (Kompantseva *et al*., 2005) and in flavored milk mixes (Agostini – Costa *et al*., 2007), high performance thin layer chromatography (Argeka and Sawant, 1999), other methods such as a capillary electrophoresis method has been developed that allows the separation and estimations of melatonin and pyridoxine in pharmaceutical preparation using electrochemical detection (Chen *et al.*, 2000).A voltammetry (Solange, 2009) and cyclic voltammetry (Teixeira *et al*., 2003) have also been proposed..

Many spectrophotometric methods have been described for the determination of pyridoxine hydrochloride; these methods included first derivative spectrophotometry to estimate pyridoxine HCl combined with other drugs in tablet (Pathak and Rajput ,2008), colorimetric method based on the oxidation-reduction reaction of vitamin B_6 with cerium (IV) ion has also been described to determine vitamin B_6 in serum and it's pharmaceutical preparations (Qadir and Mosa, 2008). The diazotized sulphanilic acid (Pons *et al*., 1999) reagent has also used for determine B_6 vitamins in N-cetylpyridinium chloride medium.

The oxidative coupling reaction has been used to determine vitamin B_6 by coupling of pyridoxine with 4-aminoantipyrine in the presence of ammonium persulphate; the product shows maximum absorption at 420 nm (Nirmalchandar *et al*., 1987).

In the present work, diazotized p-nitroaniline (D-PNA), along with cetyltrimethyl ammonium bromide (CTAB), is used as a reagent for the determination of pyridoxine hydrochloride (vitamin B_6) in alkaline medium. The method offers the advantages of sensitivity, simplicity and rapidity without the need for extraction or heating.

EXPERIMENTAL

Instruments :

Spectrophotometric measurements are performed using Shimadzu UV-visible Recording Spectrophotometer UV-160, with 1-cm matched glass cells.

The pH measurements are performed on pH meter type HANNA 211 pH - Ion meter.

Reagents :

Chemicals used are of analytical reagent grade.

Working pyridoxine hydrochloride (Vitamin B_6) solution, (100 μg/ml). A 0.01 g of pyridoxine hydrochloride (NDI-Iraq) is dissolved in distilled water and the volume is completed to 100 ml in a volumetric flask. The solution is kept in a brown bottle, where it is stable for at least one week.

Sodium carbonate solution, (0.1 N). This solution is prepared by dissolving 1.325 g of sodium carbonate (BDH) in 250 ml of distilled water.

Diazotized p-nitroaniline reagent solution (5mM). A 0.1727g of p-nitroaniline (Fluka) is dissolved in about 50 ml distilled water. Then 20 ml of 1M HCl is added and the solution is heated, the clear mixture is then transferred to 250-ml volumetric flask and is cooled to 0-5^{o}C in an ice-bath. A 8.65 ml of 1% $NaNO_2$ (BDH) is added and the mixture is stirred vigorously. After 5 min., the solution is made up to volume in 250 ml volumetric flask with cold water. The solution is kept in a brown bottle in a refrigerator and is stable for at least one month (Othman , 2001) .

Cetyltrimethylammonium bromide (CTAB) solution,(1×10^{-3}M). This solution is prepared by dissolving 0.911 g of CTAB in distilled water then the volume is completed to 250 ml with distilled water .

Vitamin B_6 Tablets solution. Five tablets were powdered accurately and weighed and a weight of powder equivalent to one tablet was dissolved and transferred into a 100-ml calibrated flask and made up to volume with distilled water. The solution was filtered and the clear filtrate used for the determination. An appropriate volume of the sample solution was diluted further with water so that the concentration of vitamin B_6 in the final solution was within the working range. **Ampoule of vitamin B_6 solution, (100 μg/ml)**. The 100 μg/ml B_6 solution is prepared by diluting 0.2 ml of ampoule (100 mg B_6/2ml- Troge Medical GMBH, Germany), content to 100 ml with distilled water in a volumetric flask.

Procedure and calibration curve

To series of 25-ml volumetric flasks, aliquots of pyridoxine hydrochloride (B_6) solution are transferred to cover the range of 5-600 µg/25ml B_6, i.e., 0.2-24 ppm. Then 1.5 ml of diazotized p-nitroaniline (5 mM) reagent solution and 3 ml of (1×10^{-3}m) CTAB is added . The mixture are shaken , then 3 ml of (0.1 N) sodium carbonate solution is added and the volumes are made up to the mark with distilled water . The mixtures are shaken and the absorbances are measured at 480 nm against the corresponding reagent blank , using 1-cm glass cells. Fig.1 shows the calibration curve obeyed Beer's law over the concentration range 5-500 µg/25ml , i.e., 0.2-20 ppm and a concentration above 500µg/25 ml gives a negative deviation. The molar absorptivity is 2.7×10^4 $1.mol^{-1}.cm^{-1}$

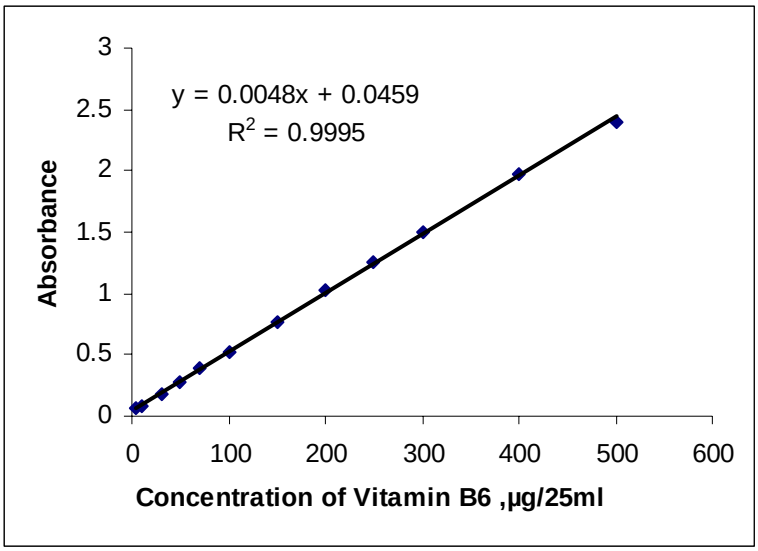


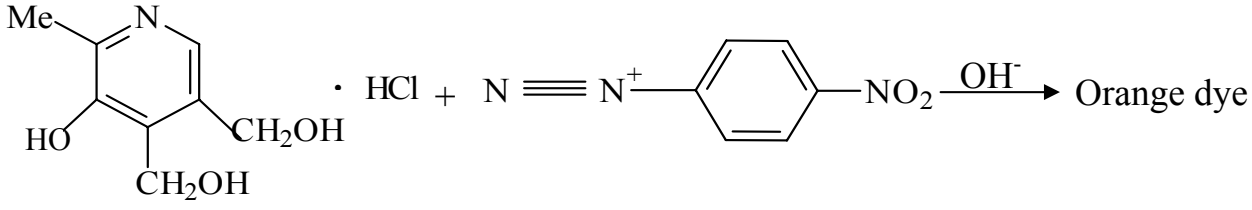
Fig. 1 : The Calibration curve for vitamin B_6 determination using coupling reaction with diazotised p-nitroaniline.

RESULTS AND DISCUSSION

For the following experiments, 100 µg of vitamin B_6 is taken in 25 ml final volumes and absorbance measurements are performed at 480nm.

Principle of the method

The method involves the coupling of the determinand vitamin B_6 with diazotized p-nitroaniline to form, in basic medium and in the presence of CTAB,an intense coloured dye:



Vitamin B_6

Diazatized p- nitroaniline reagent

Optimum reaction conditions

The effect of various parameters on the absorption intensity of the coloured dye is investigated and the reaction conditions are optimized.

Effect of Base

The preliminary experiments have shown that the coloured dye can be developed only in alkaline medium. Many (strong and weak) bases with different amounts are examined and the results indicated that 3 ml of 0.1 N Na_2CO_3 produces the highest intensity of the dye and better colour contrast ($\Delta\lambda$). So, it has been selected in the subsequent experiments (Table 1).

Table 1 : Effect of type and amount of base.

0.1 N of Base	Variable	Absorbance / ml of Base Used				
		1	2	3	4	5
NaOH	A	0.102	0.308	0.299	0.217	0.207
	$\Delta\lambda^*$,nm	89.5	91.5	90.0	92.0	91.5
	pH	2.90	11.15	11.95	12.10	12.26
KOH	A	0.074	0.499	0.493	0.368	0.328
	$\Delta\lambda$	85.5	87.5	93.0	39.0	94.0
	pH	2.77	6.85	11.40	12.05	12.17
Na_2CO_3	A	0.074	0.495	0.521	0.496	0.491
	$\Delta\lambda$	104.0	105.5	110.0	102.05	90
	pH	2.60	6.10	7.01	8.00	9.10
$NaHCO_3$	Turbid					
CH_3COONa	A	-	-	0.042	0.093	0.159
	$\Delta\lambda$	-	-	92.0	93.0	93.5
	pH	2.63	3.10	4.28	4.62	4.81

$\Delta\lambda^* = \lambda_{max}$ (sample) - λ_{max}(blank)

Effect of Diazotized p-Nitroaniline Reagent Amount

The effect of the diazotized p-nitroaniline reagent amount on the color intensity of the dye has been studied. The results are shown in Table 2.

Table 2 : Effect of p-nitroaniline reagent amount on absorbance.

ml of Diazotized p-nitroaniline solution(5mM)	Absorbance / μg of vitaminB_6								r^2 (determination coefficient)
	10	30	50	70	100	150	200	300	
0.25	0.074	0.147	0.206	0.275	0.308	0.352	0.418	0.489	0.9056
0.50	0.079	0.174	0.276	0.37	0.507	0.711	0.828	0.983	0.9480
1.0	0.082	0.182	0.278	0.385	0.529	0.776	1.012	1.502	0.9999
1.5	0.084	0.180	0.278	0.387	0.526	0.772	1.011	1.499	0.9999
2.0	0.083	0.180	0.276	0.371	0.517	0.750	0.996	1.409	0.9990
3.0	0.078	0.167	0.272	0.372	0.515	0.763	1.004	1.416	0.9984
5.0	0.081	0.165	0.240	0.312	0.453	0.648	0.800	1.309	0.9956

From the values of r^2 and absorbance, 1.5 ml of 5 mM diazotized reagent has been recommended for the subsequent experiments **.**

Effect of Surfactant

The effect of surfactant on the intensity and stability of azo dye was studied by adding 3 ml of various types of surfactant to the medium of reaction. The result in Table 3 showed that cationic surfactant addition causes the highest stability of azo dye with no effect on the intensity of the dye. Therefore, 3 ml of 10^{-3}M CTAB solution is used in the subsequent experiments.

The selected surfactants are :

Cetyltrimethylammonium bromide (CTAB) (cationic).
Cetylpyridinium chloride monohydrate (CPC) (cationic).
Sodium dodecyl sulphate (SDS) (anionic).
Tween 80 (neutral) .

Table 3 : Effect of surfactant**.**

Surfactant used	λ max, nm	Absorbance at zero time	Absorbance after 1 hour
CTAB (10^{-3}M)	480.0	0.535	0.533
CPC (10^{-3}M)	480.5	0.520	0.517
SDS (1%)	476.5	0.527	0.519
Tween 80 (1%)v,v	477.0	0.530	0.523
Without	476.0	0.537	0.408

Order of Surfactant Addition

The three orders of CTAB addition are tried and results are given in Table 4

Table 4 : The order of CTAB addition.

Order	Absorbance	λ max,nm
I	0.534	480.5
II	0.531	480.5
III	0.530	476.5

I = vitamin B_6 (S) + Diazotized p-nitoaniline (R) + CTAB(C) + Base (B)
II = S + C + R + B
III = S + R + B + C

From the results above, order I has been recommended for the following experiments.

Effect of time and amount of vitamin B_6 on absorbance

The effect of time on the development and stability of the dye obtained from three different amounts of vitamin B_6 has been investigated under the optimum experimental conditions described above. The formation of coloured dye being complete immediately, and the absorbance of the coloured species remained constant for at least 1 hour.The results is shown in Table 5.

Table 5 : Effect of time and vitamin B_6 on absorbance.

µg of vitamin B_6 /25ml	**Absorbance / minute standing time**							
	0	**5**	**10**	**20**	**30**	**40**	**50**	**60**
10	0.092	0.092	0.091	0.090	0.090	0.091	0.092	0.092
100	0.531	0.527	0.529	0.532	0.532	0.534	0.532	0.530
200	1.021	1.020	1.020	1.019	1.019	0.018	1.020	1.020

Final absorption spectra

The absorption spectra of the coloured dye formed from coupling vitamin B_6 with diazotized p-nitroaniline in alkaline medium, in the presence of CTAB, against its corresponding reagent blank show maximum absorption at 480 nm in contrast to reagent blank which shows no absorbance at the wave length of maximum absorption (Fig 2) .

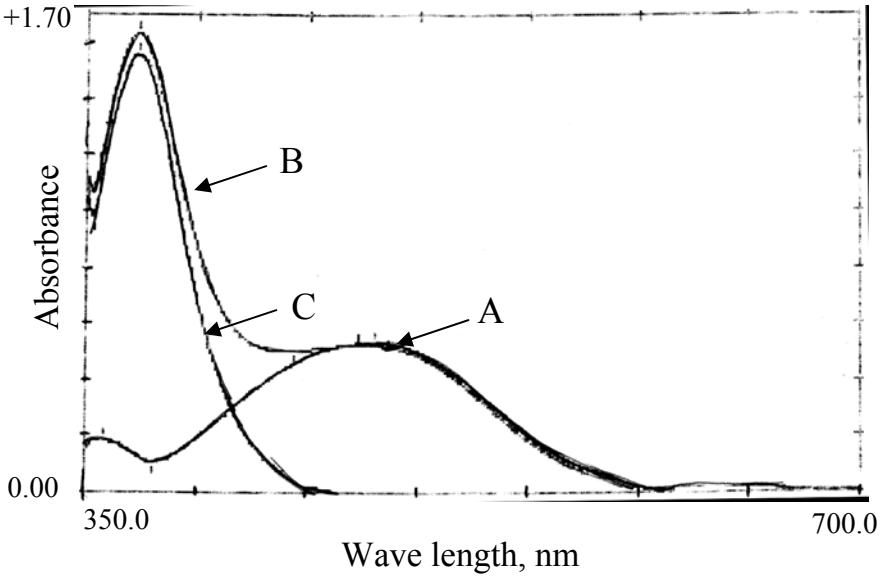


Fig. 2 : Absorption spectra of 100 µg vitamin B_6/25 ml treated according to the optimum conditions and measured against (A) blank, (B) distilled water and (C) blank measured against distilled water.

Accuracy and precision

The accuracy and precision of the calibration curve are checked by determining vitamin B_6 at three different concentrations. The results shown in Table 6 indicate that the method is quite satisfactory.

Table 6 : Accuracy and precision.

Amount of Vitamin B_6 taken, µg/25ml	**Amount of Vitamin B_6 found, µg/25ml**	**Relative error*,%**	**Relative standard deviation *,%**
10	10.04	+0.47	±0.98
100	100.22	+0.22	±0.24
200	199.53	-0.23	±0.1

*Average of five determinations.

Nature of the dye

To estimate the composition of the coloured dye species formed between vitamin B_6 and diazotized p-nitroaniline reagent under the optimum conditions. Job's method of continuous variations is applied. The obtained results (Fig. 3) showed that the combining ratio of vitamin B_6 with diazotized p-nitroaniline reagent is found to be 1:1 .

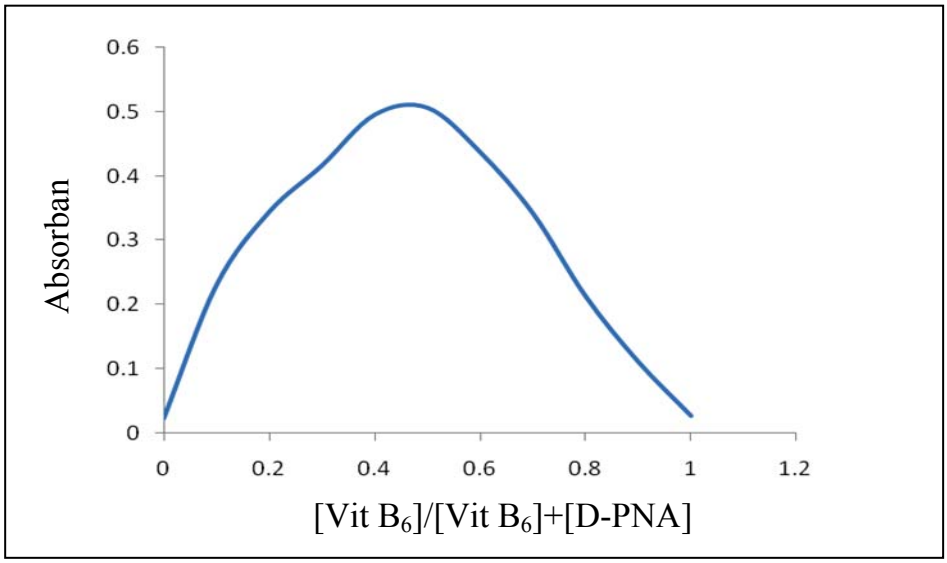
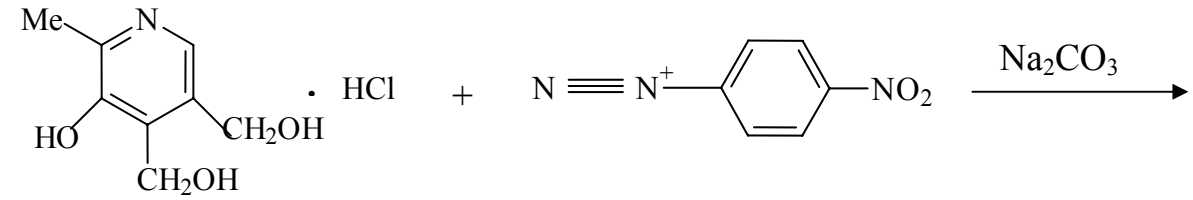
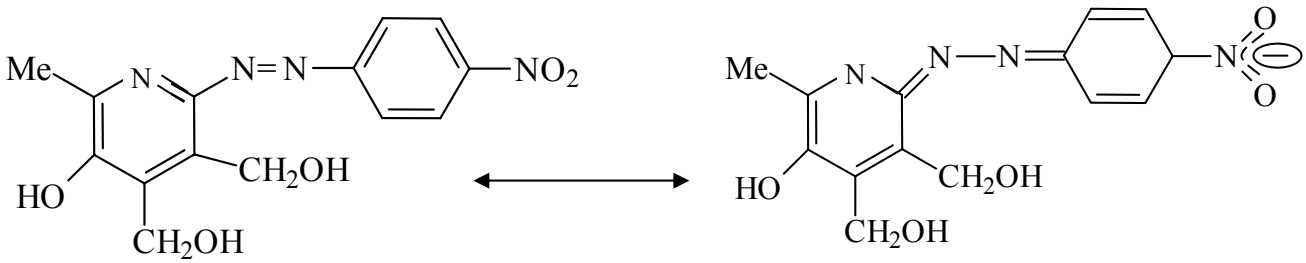


Fig. 3: Job's plot for vitamin B_6-p-nitroaniline dye.

The dye structure, therefore, may be suggested as follows :



Pyridoxine Hydrochloride Diazotized p- nitroaniline



azo - dye

Study of interferences

The effect of some excipients which often accompany pharmaceutical preparations were studied by adding different amounts to 100 μg vitamin B_6 in final volume 25 ml (Table 7).

Table 7 : Effect of foreign compounds for assay of vitamin B_6 .

Foreign compound	Interference (%) of 100 μg vitamin B_6 per μg foreign compound added		
	50	100	500
Glucose	+ 0.18	+0.20	+ 1.28
Sucrose	+ 1.3	+ 0.75	+ 0.94
Lactose	+0.73	+ 1.65	+ 1.84
Dextrose	- 0.92	+ 0.37	- 0.55
Starch	+ 0.74	+ 1.37	+ 2.50
Gum Arabic (Acacia)	+ 0.92	+ 2.20	+4.41
Glycerol	+1.66	+ 2.14	+ 0.92

The above data show that the method is very selective to determine vitamin B_6 in pharmaceutical preparation .

Application of the method

To test the applicability of the present method, it has been applied to the determination of vitamin B_6 in pharmaceutical preparation. On applying the proposed procedure, good recovery is obtained as shown in Table 8.

Table 8 : Determination of vitamin B_6 in pharmaceutical preparation.

Drug	Pharmaceutical preparation	Manufacturing Company	μg B_6 present /25 ml	μg B_6 measured / 25 ml	Recovery (%)
Samavit B6	Tablets	SDI, Iraq	10	10.18	101.83
			100	100.56	100.56
			200	196.78	98.39
Vitamin B6	Injection Ampoule	Torge Medical GMBN,Germany	5	5.0	100.00
			100	100.55	100.55
			200	199.21	99.60

Comparison of the methods and t-test

A comparison between the present method and British pharmacopia standard method (British Pharmcopia, 2000) for the determination of vitamin B_6 in tables, is based on the t-test to show the ability of using the present method in the determination of vitamin B_6 in pharmaceutical preparation. The results shown in Table 9.

Table 9: Analysis of vitamin B_6 by proposed and standard method.

Drug	Recovery * %		± t_{exp}
	Present method	British pharmacopia method**	
Samavit B_6 (tablets)*	98.638	99.628	0.9927
Vitamin B_6 injection**	100.290	100.264	1.0144

* Average of five determinations .
** (UV also instead of HPLC).

The comparison between some of analytical variables obtained from present method and another spectrophotometric method is shown in Table 10.

Table 10 : Comparison of the methods.

Analytical parameters	Present method	Literature method (Qadir and Mosa, 2008)*
pH	9.84	3.02
Temperature (^{0}C)	Room temperature	Room temperature
Development time (minutes)	Direct measuring	5
λ max (nm)	480	716
Medium of reaction	Aqueous	Aqueous
Reagent used	Diazotized p-nitroaniline	Arsenazo III
Beer's law range (ppm)	0.2-20	0.04 -0.56
Molar absorpitivity ($l.mol^{-1}.cm^{-1}$)	2.7×10^4	1.12×10^5
Stability of the color(minutes)	60	25
RSD (%)	≤ ± 0.98	≤ ± 2.76
Color of the dye	Orange	Green
Application of the method	Determination of vitamin B6 in tablets and injection ampoule	Determination of vitamin B6 in tablets, injection ampoule and serm

*The most recent spectrophotometric publication

The results in Table 10 show that the suggested method for the determination of vitamin B_6 have a good stability and range comparing with the other method.

CONCLUSION

A simple, rapid, accurate and precise spectrophotometric method is evaluated for determination of pyridoxine hydrochloride (vitamin B_6) in pure and pharmaceutical formulation. The short analysis time and low cost are the main advantage of this method for routine analysis in quality control.

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