Chapter II

Materials and Methods

Preparation of the Vitamin B-Complex Containing Vitamin C Injection.

General formula in local market :

each ml. contains :

Vitamin B_1	5.0	mg.
Vitamin B_2	2.0	mg.
Vitamin B_6	5.0	mg.
Vitamin B_{12}	0.3	mg.
Niacinamide	7.5	mg.
Ca-pantothenate	2.5	mg.
Vitamin C	50.0	mg.
Benzyl alcohol	1 % V/V[21]	

Phosphoric acid or di-sodium hydrogen phosphate is added to adjust the designed pH

Method of preparation for the basic formula.

1. Accurately weigh all ingredients in dried form for 100 ml. solution.
2. Mix them together in a 100-ml. volumetric flask.
3. Accurately weigh each of stabilizer at the amount required.
4. Mix together, add half of the distilled water and mix until all vitamins are dissolved.

5. Pipet Benzyl alcohol into the volumetric flask.
6. Adjust the pH at 3.3[18]
7. Adjust to the volume with distilled water.
8. Keep in amber-color vials with cap-sealed at room temperature.

List of stabilizers and their concentrations.

I. Amino acids group :	1. Glycine	2 %
	2. Histidine	2 %
II. Sugar alcohols group :	3. Mannitol	5 %
	4. Sorbitol	5 %
	5. Xylitol	5 %
III. Xanthines group :	6. Caffeine	0.2 %
	7. Theophylline	0.2 %

Method of assay for vitamin B_{12} in the mixture[22]

Equipment :

1. Glass chromatography column 400 mm. high, diameter 20 mm. with stop-cock.
2. Spectrophotometer, 1 cm. glass cell (Pye Unicam Model SP. 1800)

Reagent :

1. Amberlite CG-50, type 2 (Rhon and Hass Philadephia) treated as follow :
   - Amberlite CG-50 is shaken with distilled water and the mixture is left to stand overnight.

- The turbid supernatant liquid is decanted and the resin is shaken again with distilled water. The washings are repeated until the supernatant liquid becomes almost clear. After decanting the last wash water, a solution of N/1 NaOH is added to the resin and left in contact for approximately half an hour. Then the soda is decanted and the resin is washed again with water until the reaction of the wash liquid is neutral to litmus.
- After decanting the last wash liquid, the resin is left to stand for approximately half an hour with the buffer solution at pH 4. The resin is then ready for use.

2. N/1 NaOH Solution.
3. N/1 HCl
4. Dioxane HCl mixture (Mix 60 ml. dioxane for chromatography with 10 ml. of N/1 HCl and 30 ml. of distilled water)
5. Buffer solution at pH 4, prepared as follows :
   - dissolve 65 gm. of Sodium Citrate G.R. and 60 gm. of Citric Acid in distilled water and made up to 1000 ml. If necessary, the solution is adjusted to pH 4 (glass electrode) by addition of Sodium Hydroxide of Citric Acid .
6. Dilute buffer solution prepared as follow :

   Mix 40 ml. of buffer solution at pH 4 with 60 ml. of

distilled water.

7. 85 % Acetone in distilled water
8. Cotton wool.

Method of Preparation of the Column.

1. Place a plug of cotton wool at the base of the column to support the resin.
2. Place pretreated Amberlite in the column, in sufficient quantity to ensure after sedimentation, the formation of a 10 cm. thick resin layer.
3. After running almost all the liquid, add further buffer solution at pH 4, leaving to settle slowly. The eluate must be approximately at pH 4. If necessary, add the buffer solution until this value is reached.
4. After all the liquid pressent in the column is allowed to run out, leaving a 1 cm. layer above the resin.
   The column is ready to use.

Chromatographic Separation of Vitamin B_{12}

1. Pipet the tested mixture 1 ml.
2. Transfer in the previously prepared column.
3. Leaving the liquid run out until 1 cm. layer left above the resin
4. Wash the column with 10 ml. portions of dilute buffer solution until the eluate becomes almost colourless.

5. Successively wash the column with 50 ml. of N/10 HCl and then with 50 ml. of 85 % Acetone.
6. Finally with N/10 HCl, until the eluate appears completely colourless.

(All the washings are discarded.)

Elution

Beside the reddish colour ring of vitamin B_{12} which has been absorbed (colouring which appears only in the upper part of the column), the column must not show any other foreign colour. Elution will be started when this condition occurs. Leaving 1-2 cm. of N/10 HCl above the resin, 25 ml. of dioxane-HCl mixture are added.

Soon after a sharply outlined red ring forms and migrate towards the bottom of the column, the dioxane-HCl eluate is then collected in a 25-ml. or 10-ml. volumetric flask and left to run until the fractions dripping into the flask becomes colourless. During the whole elution, a layer of dioxane-HCl must always remain above the column (if the initial 25 ml. is not enough, some more should be added.)

If a volume is not reached with the eluate, the eluate is diluted to volume with the dioxane-HCl mixture.

Measurement

The solution that has been made up to the mark in volumetric flask is measured against water or dioxane HCl mixture. In this experiment, using dioxane-HCl mixture as blank and measures

in a spectrophotometer, using 1 cm. cell, at 361 nm. max., 550 nm. max.

Calculation

The value of $E^{1\%}_{1\ cm.}$ is used to calculate for the percentage of vitamin B_{12}, the formula is :

$$\% \text{ Vitamin } B_{12} = \frac{A_{361}}{0.207} \times \frac{0.01}{\text{sample conc.}} \times 100$$

A_{361} = absorbance of the solution at 361 nm.

0.207 = Extinction at 361 nm. of 100 mcg. cyanocobalamin in 10 ml. solution, measured in a 1 cm. cell.

$E^{1\%}_{1\ cm.}$ = 207 at 361 nm.
= 63 at 550 nm.

Purity Ratio of the B_{12} Fraction[22]

If the maximum absorbtion of the sample solution does not lie at 361 nm. and doubt arises as to the purity of vitamin B_{12} fraction measured, it is rechecked by determining the absorbance at the maximum of 550 nm. and 361 nm. The ratio $E_{361} : E_{550}$ should lie between 3.0 and 3.5

If it falls within the given limits, the vitamin B_{12} fraction is considered satisfactory. If it does not fall in

the limits, the fraction is not sufficiently pure.

Impurities in the sample solution.

It is not always possible to purify the vitamin B_{12} on the column because the interfering impurities, whether decomposition products or other cobalamins from the products, often have absorption characteristics similar to those of vitamin B_{12}. Their effect on the slope of the absorption curve of the vitamin B_{12} sample solution is usually minor.

Procedure for the method of assay for Vitamin B_{12} by spectrophotometric method using column chromatography.

1. Prepare the vitamin B_{12} solution alone in water, in different concentrations, as in Table 1.
2. Using the method of assay for Vitamin B_{12} in the mixture to determine the percentage of vitamin B_{12}, read the absorbance at 361 and 550 nm.
3. Directly read the absorbance of vitamin B_{12} solution against water at 361 nm and 550 nm.

   Calculate the percentage of vitamin B_{12} purity.
4. Calculate the percentage recovery of vitamin B_{12}.
5. Determine the purity of vitamin B_{12} fraction from the ratio of 361/550 in Table 2.

Procedure for determination for the best stabilizer.

1. Prepare of the sample solutions by following the method of preparation for the basic formula

   Sample No. 1 to 7 contains the stabilizers from the list and sample No. 8 is a control (without stabilizer).

2. Dissolve vitamin B_{12} solution in water (pure crystalline form) in the concentration range of 0.01-0.03 mg/ml. and read the absorbance at 361 nm. for the percentage of vitamin B_{12} at the initial period.

3. Keep all samples at the room temperature.

4. Determine the percentage of vitamin B_{12} by using the method of assay of vitamin B_{12} in the mixture; at the first, third, fourth and eighth week.

5. Calculate the percentage of vitamin B_{12} for the percentage of recovery by using the percentage of vitamin B_{12} purity, in Table 3.

6. Determine the purity of vitamin B_{12} fraction from the ratio of 361/550 as shown in Table 4.

Procedure for determination for the minimum concentration of the best stabilizer.

1. Prepare the sample solutions by using the method of preparation for the basic formula

2. The best stabilizer is added in the sample solution for comparison in the suitable concentrations.

3. Determine the percentage of vitamin B_{12} in the initial

sample for the percentage of vitamin B_{12} purity.

4. Determine the vitamin B_{12} in the sample solutions in the 1^{st}, 3^{rd}, 4^{th} and 8^{th} week by the method of assay for vitamin B_{12} in mixture.

5. Calculate the parcentage of vitamin B_{12} recovery, in Table 6. and the purity of vitamin B_{12} fraction in Table 7.