A Rapid Determination of the Relative Purity of Vitamin B_{12} (Cyanocobalamin) in Pharmaceutical Products

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A procedure is described involving the determination of absorptivities at 341 and 376 mμ and their ratio. Solutions of crystalline cyanocobalamin gave maximum absorptivities at 341 and 376 mμ of 80.4 and 80.9, respectively. The average ratio was 0.990 with none exceeding 0.998. A number of pharmaceutical preparations of cyanocobalamin injection gave ratios not in excess of 1.02. Several commercial preparations known to contain impure cyanocobalamin gave ratios up to 1.24. Solutions containing cobalamin concentrate N. F. and potential impurities of cyanocobalamin injection gave ratios ranging as high as 1.48. Validity of the procedure was established by comparison with reference cyanocobalamin solutions, and with a "Purity Index" obtained from the results of the Radioisotope Tracer Method for Cobalamins and the U. S. P. Spectrophotometric Method for Cyanocobalamin.

IN THE QUANTITATIVE DETERMINATION of highly purified solutions of cyanocobalamin the spectrophotometric method described in U. S. Pharmacopeia XV (1) has been widely used. This involves measurement of radiant energy absorption at 361 mμ. When it became evident that this method did not differentiate between the biologically active cobalamin compounds and the structurally similar but inactive red pigments, a radioisotope tracer method was devised (2). This method has been adopted by both the U. S. Pharmacopeia (3) and the National Formulary (4) and in the latter for application to cobalamin concentrate N. F. (5).

Application in this laboratory of these two methods to an extensive series of pharmaceutical products offered as vitamin B_{12} injections U. S. P., has provided an estimate of purity of the vitamin used in the preparations tested. Arbitrarily, we have expressed this relationship as "Purity Index," calculated by dividing the amount of cyanocobalamin found by the tracer method by the amount found by the spectrophotometric method. This has been a useful means in determining whether or not the products examined met the U. S. P. specifications.

In a spectrophotometric study of this series it was observed that the radiant energy absorption at the shorter wavelengths was abnormally high for those products known to contain impurities. To use this observation in a quantitative manner the absorption ratio at 341 and 376 mμ was compared for solutions of pure cyanocobalamin and for the impure vitamin preparations. This was found to provide a rapid spectrophotometric method for determining relative purity and to serve as a preliminary sorting procedure for control purposes.

Briefly, the determination involves the measurement of radiant energy absorption at three different wavelengths, 341, 361, and 376 mμ. The ratio of the absorption at 341 and 376 mμ, together with quantitative absorption values at these two wavelengths, gives an index of the purity of the cyanocobalamin. The validity of the method was established by using a solution of crystalline cyanocobalamin as a reference standard, or by comparing results with the Purity Index found for each sample by use of a combination of the tracer and the U. S. P. spectrophotometric methods. It has been shown to be reliable for commercial preparations such as cyanocobalamin U. S. P. XV, cyanocobalamin injection U. S. P. XV, and injections of cobalamin concentrate solution. It is applicable to solutions containing 30 or more mcg./ml. of cyanocobalamin. It is rapid, and requires no special apparatus such as the radioactivity counting assembly of the tracer method.

EXPERIMENTAL

The proposed determination originated in the observation that a number of products containing impure cyanocobalamin gave abnormally high absorption at about 340 mμ. To investigate the impurity absorption spectrum over a definite wavelength range, a standard reference solution of cyanocobalamin was prepared which, according to the value by the tracer method, had the same concentration of cyanocobalamin as the sample under examination. A differential spectrophotometric analysis was then made for each sample using a recording spectrophotometer (Beckman Model DK-1) between 320 and 600 mμ wavelengths. In this procedure the standard solution was placed in the reference beam of the instrument and the sample solution in the sample beam. Then at any particular wavelength, since both standard and sample solutions

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contain identical amounts of cyanocobalamin, the reference solution "balances out" or "optically neutralizes" the absorption of cyanocobalamin in the sample solution. With pure solutions a curve of zero absorption was obtained, appearing as a horizontal line on the chart. When impurities were present, however, the result was an absorption curve deviating from the horizontal line. In all samples it was observed that the absorption curve was a horizontal line as the wavelength decreased from 600 mμ to approximately 375 mμ where the line assumed a slight slope which continued to approximately 340 mμ and became approximately horizontal again. The curves showed that in all cases the absorbance of the sample at approximately 340 mμ was higher than that at approximately 375 mμ. In most of the samples examined the curve between 340 and 375 mμ was approximately linear, but in a few it was nonlinear. These observations suggested that the cyanocobalamin solutions contained impurities that absorb strongly in the region of 340 mμ, with little or no abnormal absorption at approximately 376 mμ. Nearly all of the samples showed an approximate proportional relationship between increased absorption at 340 mμ and amount of impurities.

To provide an index of relative purity of cyanocobalamin, wavelengths of 341 and 376 mμ were selected for absorption measurements. At these wavelengths cyanocobalamin gives a ratio of absorptivities of approximately 1.0, and a deviation from this indicates the absorption effect of impurities at lower wavelengths.

Essentially, this ratio is a simplified form of an "Impurity Index" (6) used in many industrial organic analyses to measure quantitatively the amount of unknown absorbing impurities present in a sample being processed. In this report this ratio is used to denote the relative purity; the absolute purity has not been evaluated because it is believed that the commercial samples examined here, especially those of high impurity content, have been manufactured under a variety of conditions and the background impurity absorption curves are not sufficiently uniform to permit accurate estimate of the amount of impurities. In those cases where the absolute purity is needed, the use of the tracer method is indicated. It is emphasized that only where impurities are low or nonexistent is the U. S. P. spectrophotometric method, which measures the absorbance at 361 mμ, satisfactory for quantitative purposes.

In a few of the sample solutions containing impure cyanocobalamin it was observed that the ratio was only slightly in excess of that for cyanocobalamin, but absorption at both 341 and 376 mμ was abnormally high. To detect this type of deviation, the absorptivities at 341 and 376 mμ were calculated from absorbance measurements at 361 mμ and each compared to the corresponding one for cyanocobalamin.

DETERMINATION

Dissolve the solid cyanocobalamin sample in sufficient water or dilute cyanocobalamin injections with sufficient water to give a final concentration of cyanocobalamin of approximately 40 mcg./ml.

Measure carefully the absorbance of the sample solution at 341, 361, and 376 mμ with a Beckman Model DU spectrophotometer, using the tungsten lamp light source and corex filter. Use 1-cm., quartz, matched cells with water as the reference solvent. Calculate the ratio of the absorption at 341 and 376 mμ, using either the absorbance or absorptivity values. Also calculate the absorptivity values at 341 and 376 mμ assuming that the absorptivity at 361 mμ is 207.

It may be of value to include in the determination, measurement of a reference standard solution of cyanocobalamin of high purity. Initially the use of such standard would establish absorbance and ratio values for the spectrophotometer employed. Its use would also improve precision of the determination by minimizing errors from improper setting of the wavelength scale or faulty performance of the instrument.

For this purpose prepare a solution of the reference cyanocobalamin that contains approximately 40 mcg./ml. and make the required absorbance readings at each wavelength, using the undisturbed wavelength setting of the sample measurement. Such reference measurements would be a basis for correction, where necessary.

DISCUSSION OF RESULTS

The absorptivity and ratio values for crystalline cyanocobalamin are shown in Table I. The term "absorptivity," as used here, is defined in the U. S. Pharmacopeia (1) as "The absorbance of a solution containing 1 Gm. per 100 ml. contained in a cell having an absorption path of 1 cm. Symbol: *a*." Here and throughout this report, in order to avoid the necessity of weighing out the crystalline sample or determining the weight of the cyanocobalamin of injections, an absorbance reading at 361 mμ was made as required by the procedure. The absorptivity at this wavelength for cyanocobalamin was assumed to be 207 (1). From the relative absorbance values at the three wavelengths used in the procedure, and with the absorptivity at 361 mμ assumed to be 207, the absorptivity at 341 and 376 mμ is readily calculated.

The four different samples of cyanocobalamin used were manufactured at different times during the period 1950 to 1955. The purity of cyanocobalamin samples *A* and *B* were demonstrated by the use of a countercurrent procedure; this type of analysis is particularly suitable for purity determinations of cyanocobalamin. By use of the procedure "Countercurrent Analysis of Vitamin B_{12}" (7), Sample *A* had a purity of 97.0% and Sample *B* 98.5%. High spectrophotometric purity of each sample has been demonstrated frequently since both have served as spectrophotometric reference standards in our laboratory. Sample *B* was a portion of cyanocobalamin that was used in preparing the U. S. P. Reference Standard. Both samples have been used in our laboratory also as reference standards in the tracer method, and thus all values for Purity Index in this report are related to either Sample *A* or Sample *B*. Samples *C* and *D* were commercially available cyanocobalamin samples and were used for comparison purposes with Samples *A* and *B*.

Water solutions were prepared to serve as references for solid cyanocobalamin samples, while water solutions containing 1.5% benzyl alcohol by

TABLE I.—ABSORPTIVITY OF CRYSTALLINE CYANOCOBALAMIN

Sample	Solvent	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, $a_{341\ m\mu}/a_{376\ m\mu}$
A	Water	80.3	80.7	0.995
A	1.5% Benzyl alcohol	80.4	80.6	0.998
B	Water	79.3	80.3	0.988
B	1.5% Benzyl alcohol	79.4	80.3	0.989
C	Water	79.0	80.3	0.984
C	1.5% Benzyl alcohol	79.4	80.9	0.981
D	Water	79.4	80.3	0.989
D	1.5% Benzyl alcohol	80.0	80.7	0.991
Av.	Water	79.5	80.4	0.989
Av.	1.5% Benzyl alcohol	79.8	80.6	0.990

volume were prepared for comparison with cyanocobalamin injections. A typical cyanocobalamin injection contains isotonic sodium chloride and 1.5% by volume benzyl alcohol. It was observed that isotonic sodium chloride gave no absorption at any of the three wavelengths involved in the determination. However, benzyl alcohol does absorb to some extent, and it was necessary to include benzyl alcohol in the reference solution for injections. The actual average increase in absorption due to the addition of benzyl alcohol, as noted in Table I, was very small. From the data in Table I a given sample of cyanocobalamin dissolved in water, or water containing 1.5% benzyl alcohol, should not give an absorptivity in excess of 80.4 at 341 mμ or 80.9 at 376 mμ. The ratio of absorptivity at 341 mμ and 376 mμ should not exceed 0.998. The presence of typical absorbing impurities will tend to increase both the absorptivity at 341 mμ and the ratio; in some cases, the absorptivity at 376 mμ may also increase.

Table II shows the absorptivity and ratios of samples of cyanocobalamin injections having a purity index of less than 95%. The history of these samples indicated that they were made from some type of oral grade solids, possibly with additional purification. The deviation from U. S. P. standards for injections of crystalline cyanocobalamin was corroborated by the fact that all of these samples contained unaccountable excess solids. A final proof that at least three of these samples contained impurities that absorb abnormally high at 341 mμ was shown by the fact that, after they had been examined by the tracer method (which yields a solution of cyanocobalamin in pure form), the ratios on the final solutions were essentially those for cyanocobalamin. Sample 1, with a ratio initially of 1.086, dropped to 1.000; Sample 4 changed from 1.085 to 0.983; and Sample 5 from 1.242 to 1.025. By the same procedure, it has been demonstrated that samples containing crystalline cyanocobalamin give initial and final ratios that are essentially identical.

The data of Table II show that where impure cyanocobalamin samples having an indicated purity of less than 91.3% (Samples 1 to 4), a ratio of 1.085 or more is obtained as contrasted to the reference ratio of 0.990 for cyanocobalamin solutions (Table I). Sample 5, with an indicated purity of 94.2%, showed an unusually high ratio of 1.242. This sample was of special interest because the impurity absorption curve (resembling a sine curve) was quite different from the other samples of impure cyanocobalamin injections shown in Table II. Between wavelengths 341 and 376 mμ the absorption curve was nonlinear, with negligible absorption at 361 mμ and 376 mμ, but with considerable absorption at 350 mμ and 341 mμ. Samples 6 and 7, with purity indexes of 93.8% and 94.8%, showed ratios of 1.021 and 1.012, respectively. These ratios do not exceed that for cyanocobalamin by too large amounts, but they would be viewed with suspicion because the absorptivities at 341 and 376 mμ exceed those for cyanocobalamin solutions by considerable amounts.

TABLE II.—ABSORPTIVITY OF PHARMACEUTICAL PREPARATIONS OF CYANOCOBALAMIN INJECTION U. S. P. HAVING A PURITY INDEX LESS THAN 95%

Sample	Cyanocobalamin Concn., mcg./ml.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, $a_{341\ m\mu}/a_{376\ m\mu}$
1	1,000	89.6	86.8	79.9	1.086
2	1,000	90.2	94.1	86.3	1.090
3	1,000	90.8	96.8	86.5	1.122
4	1,000	91.3	86.5	79.7	1.085
5	1,000	94.2	101.5	81.7	1.242
6	1,000	93.8	83.8	82.1	1.021
7	1,000	94.8	84.5	83.5	1.012

[a] Purity Index is obtained by dividing the cyanocobalamin content determined by the Radioisotope Tracer Method by the cyanocobalamin content determined by the U. S. P. Spectrophotometric Method.

Sample 7 has an especially high absorptivity at 376 mμ indicating a background impurity absorption curve somewhat horizontal, but of high magnitude. Samples 6 and 7 no doubt contain purified oral grade solids, and if encountered in our laboratory on a routine basis, would indicate the use of the tracer method and a solids determination to indicate compliance with the U. S. P. specification for cyanocobalmin injection.

Table III shows the absorptivities and ratios of pharmaceutical preparations found to have a purity index of more than 95%. The quality of the cyanocobalamin used in these samples is unknown. Arbitrarily, a purity index in excess of 95% was chosen to serve as an indication of the presence of crystalline vitamin B_{12} (cyanocobalamin) where the product was so labeled. The 95% criterion was chosen because of the U. S. Pharmacopeia requirement (1) that cyanocobalamin "has a purity of not less than 95% on the dried basis." It should be noted, however, that the two criteria are not identical as illustrated by the following hypothetical cases. One sample, containing 95 parts of anhydrous pure cyanocobalamin and 5 parts of sodium chloride, would assay 95% by the U. S. P. method; since that method compares absorbance at 361 mμ to dry weight of sample and would assay 100% Purity Index as this method compares cyanocobalamin alone to absorbance at 361 mμ. Another sample, containing 95 parts of anhydrous pure cyanocobalamin and 5 parts of "red pigments" (such as Sample 6 of Table IV), would assay 100% by the

TABLE III.—ABSORPTIVITY OF PHARMACEUTICAL PREPARATIONS OF CYANOCOBALAMIN INJECTION U. S. P. HAVING A PURITY INDEX MORE THAN 95%

Sample	Cyanocobalamin Concn. mcg./ml.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, $a_{341\ m\mu}/a_{376\ m\mu}$
1	2,000	101.5	81.4	81.8	0.995
2	1,000	100.5	81.1	82.3	0.985
3	30	100.3	81.8	81.5	1.004
4	1,000	100.2	80.1	79.4	1.009
5	30	98.9	82.0	81.4	1.007
6	1,000	98.9	82.3	82.3	1.000
7	1,000	98.8	81.1	81.2	0.999
8	100	98.8	79.9	79.4	1.006
9	1,000	98.7	82.3	81.8	1.006
10	50	98.7	80.1	80.1	1.000
11	1,000	98.6	83.0	81.6	1.017
12	100	98.5	81.8	81.2	1.007
13	2,000	97.4	79.0	79.9	0.989
14	100	96.5	82.5	81.7	1.010
15	1,000	96.3	80.9	81.2	0.996
16	1,000	95.5	84.0	81.8	1.027
17	2,000	95.5	79.7	81.5	0.978

[a] See footnote—Table II.

oral grade, defined as Cobalamin Concentrate N. F. Sample 2 contained, in addition, 200 mcg./Gm. of a specially prepared red pigment. The relationship between purity index and high ratio is apparent in these two samples. Sample 3 was a cobalamin concentrate which gave a high ratio and high absorptivities at both 341 and 376 mμ. Sample 4 was a high concentration vitamin B_{12} soluble solids sample which, according to the purity index and the ratio, contained a relatively large amount of impurities. Sample 5 was pseudo vitamin B_{12}, isolated from an anaerobic fermentation process in crystalline form and reported present in a commercial vitamin B_{12} feed supplement (8). Sample 6 was a sample of "red pigments" which occurred in the waste stream of a commercial process for preparing cyanocobalamin. The "red pigments" accompany cobalamins rather closely through certain manufacturing steps and are separated only with difficulty, usually on a chromatographic column (2). They are present, apparently, in all cobalamin concentrates N. F., but are removed from cyanocobalamin U. S. P. As poor manufacturing or incomplete processing may not remove these "red pigments" from cyanocobalamin to be used in injections, it is important that any proposed method detect this type of contamination. It is believed the proposed determination which gives a ratio of 1.478 with accompanying high absorptivities at 341 and 376 mμ is sufficiently sensitive to detect small amounts of these "red pigments" in cyanocobalamin preparations. Thus, on a theoretical basis, using this sample as a typical pigment, a synthetic mixture of 95% cyanocobalamin and 5% of "red pigments" would give (assuming Beer's law applies) a ratio of about 1.015. In contrast, the tracer method would give a purity index of 95.2%.

TABLE IV.—ABSORPTIVITY OF COBALAMIN CONCENTRATE PREPARATIONS AND POTENTIAL IMPURITIES OF CYANOCOBALAMIN

Sample	Vitamin B_{12} Activity, mcg./Gm.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, $a_{341\ m\mu}/a_{376\ m\mu}$
1. Collaborative Sample 1	1,076	75.6	108.9	88.5	1.231
2. Collaborative Sample 2	1,067	67.6	106.2	88.3	1.203
3. Cobalamin Conc. N. F. X	500	93.0	90.6	86.9	1.043
4. Vitamin B_{12} Soluble Solids	34.000	91.6	99.3	90.0	1.103
5. Crystalline Pseudo Vitamin B_{12}	..	1	87.4	63.1	1.385
6. "Red Pigments"	..	4	156.7	106.0	1.478
7. Crystalline Red Pigment	..	0	80.2	82.1	0.977

[a] See footnote—Table II.

U. S. P. method and 95% by the purity index method.

Inspection of Table III shows that with the exception of Sample 16, all samples have a maximum ratio of 1.017 and maximum absorptivities of 83.0 and 82.3 at 341 and 376 mμ, respectively. With this exception, it would seem logical to select for sorting purposes the following maximum values for injections made from cyanocobalamin, U. S. P.: Ratio $a_{341\ m\mu}/a_{376\ m\mu} = 1.020$; Absorptivity at 341 mμ = 83.0; Absorptivity at 376 mμ = 82.5. Inspection of the ratio values of Table III shows that 15 out of 17 samples do not exceed the reference ratio for cyanocobalamin (0.990) by more than 2% (i. e., not in excess of 1.010). It appears that in view of the conformity of these samples with the reference samples, the ratio selection of a maximum of 1.020 is justified in a rapid determination for sorting purposes.

The absorptivities and ratios of cobalamin concentrate preparations and some potential impurities of cyanocobalamin are shown in Table IV. In all samples, aqueous solutions were prepared for measurements by removing insoluble material with filtering or centrifuging. Samples 1 and 2 were used in the collaborative study "Cobalamin Assay by the Radioisotope Tracer Method" (2), and contained commercially prepared vitamin B_{12} solids of

Sample 7 of Table IV was a red crystalline substance isolated from the "red pigments" fraction of a commercial vitamin B_{12} fermentation process. It is considered a definite chemical compound, while "red pigments" represented by sample 6 may contain several similar compounds. This red pigment was used in the collaborative study mentioned above, having been added to sample 2 of Table IV. It was used because its ultraviolet absorption spectrum in water was somewhat similar to that of cyanocobalamin, so that spectral differentiation was difficult. In the present studies its absorptivities and ratios were essentially the same as for cyanocobalamin; thus it cannot be differentiated by this means. On studying this pigment further, by a dif-

ferential spectrophotometric analysis using cyanocobalamin as the reference sample, a straight horizontal line was obtained on the absorbance curve, indicating, within experimental error, that this red pigment and cyanocobalamin have identical absorption curves between 600 and 320 mμ. It is interesting to note that this sample which showed no biological activity did not interfere in the tracer determination of sample 2 in the aforementioned collaborative study. From a practical viewpoint, the inapplicability of the proposed determination is not too serious because it is unlikely that cyanocobalamin will be contaminated with only one such specially purified fraction from "red pigments."

Table V shows the absorptivity and ratio of seven commercial samples of injections made from cobalamin concentrate solution, preparations now available for clinical use. They appear to contain cyanocobalamin of higher purity than that in cobalamin concentrate N. F., but not as pure as cyanocobalamin U. S. P. The results in Table V show that all samples gave a purity index of 94.8% or more. With the exception of Sample 1, the ratio range was between 0.992 and 1.023. Thus most commercial samples can be expected to give ratios below 1.025, which is slightly higher than the maximum ratio of 1.020 expected from injections made from cyanocobalamin. It is interesting to note that Sample 7, with an indicated purity of 99.4%, gave an average ratio of 0.994 which is very close to the cyanocobalamin reference ratio of 0.990.

Because of the fact that the ratio of the absorptivity at 341 and 376 mμ is a rather sensitive index for determining relative purity of cyanocobalamin in various preparations, a brief study was made of reproducibility of results. Table V lists, for each sample, results of two separate determinations. It can be seen that maximum deviation in the ratio was for Samples 1 and 2, approximately 1.3%. The average difference between duplicates of all the samples was 0.6%.

This degree of reproducibility is not considered excessive when the following experimental facts are considered: *first*, the duplicate determinations in Table V and all other determinations in this report were made in essentially the same manner as routine analyses in a typical control laboratory. Four clean, matched silica cells were used interchangeably for all determinations in this report. *Second*, the reproducibility and accuracy of the method is probably largely dependent on the performance of the spectrophotometer. In a typical analysis where the concentration of the solution is about 40 mcg./ml. cyanocobalamin, the absorbance reading at 341 mμ and 376 mμ is about 0.325, and at 361 mμ about 0.82. The slit widths used (sensitivity control of midpoint) at 341, 361, and 376 mμ were approximately 0.15, 0.095, and 0.08 mm., respectively. A frequently occurring reproducibility of a particular Beckman Model DU may, in individual samples being measured for absorbance at the three wavelengths, be of the magnitude of about 0.003 absorbance unit. If 0.003 error is made in both the 341 and 376 mμ readings, and in opposite directions, it is apparent that the calculated ratio of a typical sample might contain an error of 1.8%. Thus, the reproducibility of about 1.3% with samples 1 and 2 appears to be reasonable and probably arises from the performance of the Beckman rather than defects

TABLE V.—ABSORPTIVITY OF COMMERCIAL INJECTIONS MADE FROM COBALAMIN CONCENTRATE SOLUTIONS

Sample	Cyanocobalamin Concn. mcg./ml.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, a_{341} mμ/ a_{376} mμ
1	1,000	96.5	87.3	81.3	1.074
	1,000	96.5	87.2	82.3	1.060
2	1,000	95.3	81.4	80.5	1.011
	1,000	95.3	81.0	81.2	0.998
3	1,000	96.4	82.8	80.9	1.023
	1,000	96.4	83.1	81.8	1.016
4	100	94.8	83.6	82.6	1.012
	100	94.8	84.0	83.1	1.011
5	1,000	96.1	81.7	81.9	0.998
	1,000	96.1	82.0	82.0	1.000
6	1,000	96.9	82.5	82.0	1.006
	1,000	96.9	83.1	82.5	1.007
7	1,000	99.4	82.0	82.7	0.992
	1,000	99.4	82.3	82.6	0.996

[a] See footnote—Table II.

in the method. A word of caution, however, should be considered in making absorbance readings on the Beckman Model DU in this determination. The readings at 341 and 376 mμ are taken on a rather steep portion of the curve where the absorbance is changing rapidly with wavelength, making it imperative that the wavelength setting be made with care. In order to obtain the best precision, the following procedure is recommended: in addition to making an absorbance reading at 341 or 376 mμ on a sample, after the wavelength dial has been carefully adjusted, also make a reading on a reference sample of cyanocobalamin and compare the difference in ratios. This actual difference will mean much more than individual absolute values because it will be compensated not only for an improper setting of the wavelength dial, but also for small permissive errors in the wavelength scale of the instrument. The absorbance reading at 361 mμ caused no difficulty and it is doubted if the reproducibility of most samples in this report exceeded 0.5%. *Third*, since the validity of the proposed determination is based on comparison with the purity index procedure, it was considered desirable to determine the reproducibility of the latter, which is dependent largely upon the variation of the tracer method with little variability being contributed by the U. S. P. spectrophotometric method. To determine the variability of the indicated purity procedure, duplicate determinations were made on five of the samples under study. Sample 3 of Table II gave a difference between duplicates of 1.1; Sample 5 of Table II, 1.0; Sample 6 of Table II, 1.4; Sample 16 of Table III, 0.4; and Sample 1 of Table V, 1.1. On a percentage basis, the average reproducibility was 1.1; and the maximum, 1.5. Thus, the purity index or the ratio of absorptivity at 341 and 376 mμ have approximately the same degree of reproducibility.

SUMMARY

A rapid reproducible spectrophotometric procedure is described for determining the relative purity of cyanocobalamin that is applicable to commercial preparations. It involves measuring

the absorptivity values at 341 mμ and 376 mμ, and calculation of the ratio of the absorptivities at these two wavelengths. The procedure can be used on solutions containing 30 or more mcg./ml. of cyanocobalamin. The validity of the procedure was affirmed by comparison with solutions of cyanocobalamin of known purity, or a purity index calculated from the results of the Radioisotope Tracer and the U. S. P. Spectrophotometric Methods. The procedure was used on a variety of samples and the relative purity of cyanocobalamin estimated was generally in agreement with that given by the purity index. The procedure serves as a rapid sorting device, but until broader experience is available, final reliance must be placed in the use of the purity index involving the Radioisotope Tracer Method.

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The Metabolic Fate of a Hexamethylenimine Analgesic*†

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The fate and metabolism of 4-carbethoxy-1-methyl-4-phenylhexamethylenimine hydrochloride (Wy-401) a synthetic analgesic, was studied in animals with the aid of the C^{14}-labeled drug. Six degradation products were found in the urine; the result of hydrolysis, demethylation, and hydroxylation. Following injection, liver and kidney were found to contain the greatest concentrations of the labeled drug. Highest blood levels were reached within one hour of intra-abdominal injection and within 48 hours the urine was void of all traces of the drug or its metabolites.

IN A RECENT STUDY on the metabolism and excretion rates of meperidine (1) the demethylated and de-esterified derivatives recovered in the urine accounted for one-third of the administered dose. N-demethylation was previously established (2) on the basis of the labeled respiratory CO_2 obtained from animals injected with meperidine tagged in the N-methyl group with carbon 14. A number of phenyl-substituted seven-membered heterocyclic compounds (3, 4) related to meperidine were investigated pharmacologically (5–7) and found to possess analgesic activity.

The present investigation was undertaken to ascertain excretion patterns and products of biotransformation following intra-abdominal administration of one of these drugs, 4-carbethoxy-1-methyl-4-phenylhexamethylenimine hydrochloride (Wy 401). Unlabeled Wy 401 and the drug labeled in the 4- position with carbon 14 were used in this study. Methods were developed for assay of de-esterified metabolites and for a hydroxylated derivative of Wy 401 found in urine.

MATERIALS AND METHODS

Tissue level and excretion pattern studies of Wy 401 and its metabolites were conducted on 14 dogs, 10 rats, and 4 rabbits. The dogs and rabbits were injected intra-abdominally with 35 mg./Kg. and the rats with 50 mg./Kg. of the drug. The urine of dogs and rabbits was collected by catheterization, that of rats in metabolism cages.

Assay of Wy 401 and Its Metabolites.—The determinations of Wy 401 and hydroxy Wy 401 and their de-esterification products were carried out successively on a single 1-ml. urine sample. For every set of analyses, urine collected immediately

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† Ethoheptazine, Wyeth Laboratories.