Spectrophotometric Determination of Pyridoxine Hydrochloride (Vitamin B_6) in Multivitamin Preparations

By R. C. SHAH, P. V. RAMAN, and M. M. MEHTA

A spectrophotometric procedure has been developed for rapid and fairly accurate determination of vitamin B_6 in B complex and multivitamin preparations. Vitamin B_6 is determined by the measurement of difference in absorbance value (ΔA) in different solvents at 328 mμ.

THE PRESENT official U.S.P. method (1) for the determination of vitamin B_6 in multivitamin preparations is the colorimetric method, consisting of the coupling of vitamin B_6 with 2,6-dichloroquinone-chlorimide in ammonium chloride–ammonium hydroxide buffer. There are also many other methods for the colorimetric determination of vitamin B_6. Pyridoxine reacts with Folin-Denis reagent to produce a blue color (2). It couples with diazo compounds, such as diazotized sulfanilic acid (3) and diazotized *p*-aminoacetophenone (4, 5). The method of Sweeny and Hall (6) can be used to determine pyridoxine, pyridoxal, and pyridoxamine by suitable modifications.

A survey of literature disclosed little information regarding the ultraviolet absorption characteristics of vitamin B_6. The total concentration of vitamin B_6 can be determined at 325 mμ in an aqueous solution of pH 6.75 (7). The absorption maxima of vitamin B_6 in 0.1 *N* HCl and in 0.1 *N* NaOH are also given. In phosphate buffer of pH 7, two maxima are observed at 324 and 254 mμ, and $E^{1\%}_{1cm.}$ are 345 and 183, respectively (curve 2, Fig. 3). Interference due to other vitamins precluded the emergence of a universal spectrophotometric method for the determination of vitamin B_6 in B complex and multivitamin preparations. A differential spectrophotometric method (8) has been described wherein a procedure for the determination of B_6 in the range of 290–310 mμ has been mentioned. But this procedure calls for specialized apparatus and elaborate instrumentation and calculations. Recently, spectrophotometric absorbance difference (ΔA) methods (9) have come into prominence, where advantage is taken of the difference in absorbance values of the substance in two different solvents at the same wavelength (10), or the absorbance values of the substance in the same solvent are measured at two different wavelengths (11). The present paper offers a method for the assay of vitamin B_6 based on ΔA determination of vitamin B_6 in two different solvents at 328 mμ.

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EXPERIMENTAL

To define the necessary conditions for the proposed analytical technique, the following experiments were performed.

Materials.—Pyridoxine hydrochloride B.P. (Roche), 99.5% pure; phosphate buffer pH 7 U.S.P. (12) containing 1% glycerin v/v; 0.1 *N* HCl (pH 1.12) containing 1% glycerin v/v; ether U.S.P., model H2 Beckman pH meter; model DB Beckman recording-type spectrophotometer; model G 2400 Beckman spectrophotometer; and a set of four 1-ml. matched silica cells were employed in the study.

Ultraviolet spectra were scanned for a concentration of 10 mcg./ml. of vitamin B_6 in water and in 0.1 *N* HCl to investigate the spectrophotometric behavior of vitamin B_6 (Fig. 1). Figure 1 shows evidence that vitamin B_6 exhibits peak absorbance at 326 mμ in water, whereas there is no absorbance up to 330 mμ when 0.1 *N* HCl is employed as solvent. This observation points out that ΔA of the two solutions at 326 mμ can be used for the determination of vitamin B_6. Investigations were continued by recording the curves for solutions of vitamin B_6 in solvents at various pH, and it was observed that, though there was no bathochromic or hypsochromic shift in the peak at 326 mμ, the absorbance was highly dependent upon the pH of the solution. The pH profile is given in Fig. 2, from which it is evident that vitamin B_6 does not absorb in the pH range of 1 to 3, after which, with the increase in pH, the absorbance rises steeply up to pH 6; thereafter, this maximum absorbance is constant up to pH 8. Therefore, vitamin B_6 can be determined by measuring ΔA at 326 mμ of a solution of vitamin B_6 in phosphate buffer pH 7 against 0.1 *N* HCl solution (pH 1.12) containing the same concentration of vitamin B_6. The respective ultraviolet spectra are presented in Fig. 3. The plain phosphate buffer at pH 7 absorbs slightly against plain 0.1 *N* HCl; hence, a correction should be applied while arriving at the final ΔA value. Since solutions of vitamin B_6 are photosensitive, possible variations in the spectrophotometric readings with time were studied, but it was found that ΔA value remained constant for a reasonable period of time (Table I).

Noninterfering Vitamins and Other Substances.—Vitamin B_1, ascorbic acid, nicotinamide, calcium pantothenate, sodium pantothenate, and panthenol did not absorb in the range 360–330 mμ, having only slight absorbance in the range 330–320 mμ. Normally, in a multivitamin preparation these vitamins

are present in high concentrations compared to vitamin B_6; hence, any possible interference when these vitamins are present 100 times in excess of vitamin B_6, individually or together, was investigated. At 326 mμ, a slight interference was noticed; but no interference was observed at 328 mμ. As the peak at 326 mμ is sufficiently broad not to make the wavelength setting highly critical, the absorbance at 328 mμ can be used safely. Ingredients like DL-methionine, choline chloride, choline dihydrogen citrate, and L-lysine hydrochloride, when present 100 times in excess of vitamin B_6, did not interfere at 328 mμ. Fungicides, like sodium salts of methyl and propyl parabens and bactericides like phenol, chlorobutanol, and benzyl alcohol also did not interfere. Vehicles and sweetening agents like glycerin, sugar syrup, propylene glycol, sorbitol, saccharin, sodium cyclamate, and nonionic emulsifying agents like polysorbates and sorbitans also did not interfere. Common excipients used for tablet formulations like starch, lactose, mannitol, talc, gum acacia, gum guar and lubricants like magnesium stearate, calcium stearate, and stearic acid also did not interfere.

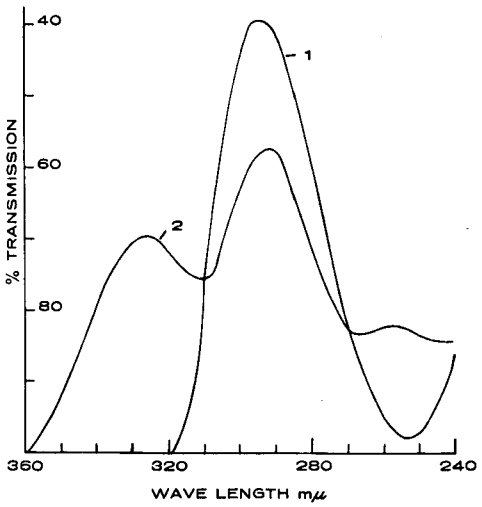

Fig. 1.—Ultraviolet spectra for 10 mcg./ml. of vitamin B_6 in [1] 0.1 *N* HCl and [2] water.

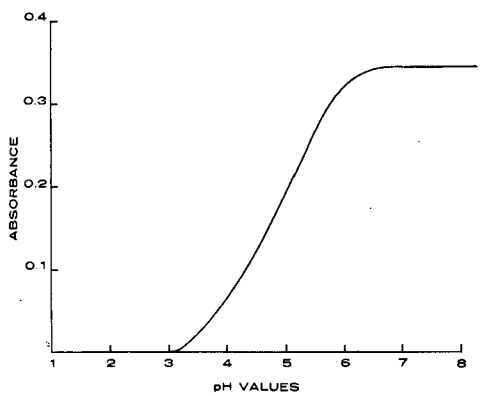

Fig. 2.—Absorbance of 10 mcg./ml. of vitamin B_6 at 326 mμ in solvents of different pH.

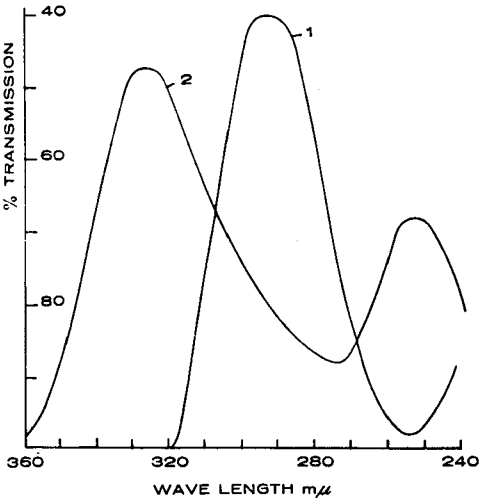

Fig. 3.—Ultraviolet spectra for 10 mcg./ml. of vitamin B_6 in [1] 0.1 *N* HCl and [2] phosphate buffer pH 7.

TABLE I.—ΔA VALUES FOR 10 MCG./ML. OF VITAMIN B_6 AT VARIOUS INTERVALS OF TIME

Time, hr.	Absorbance
0	0.335
1	0.335
2	0.335
3	0.330
24	0.310

Different concentrations—*viz.*, 5, 10, 15, 20, and 25 mcg./ml. of vitamin B_6—were prepared in 0.1 *N* HCl and phosphate buffer at pH 7, and the ΔA values were read at 328 mμ. After applying the correction for the plain buffers, the above concentrations were found to obey Beer's law.

Interfering Vitamins.—Vitamins A and B_2 absorb at 328 mμ. Solutions containing 15 units/ml. of vitamin A were prepared in phosphate buffer at pH 7 and in 0.1 *N* HCl with the help of polysorbate 80.[1] Ultraviolet spectra were traced on the same graph paper for each solution using the respective solvent as blank. Both the spectra were identical. Graphs for vitamin B_2 for the two solvents were also identical. Vitamin A and vitamin B_2 therefore, will not contribute to any ΔA value at 328 mμ when they are present in the same concentration in 0.1 *N* HCl and phosphate buffer at pH 7.

Since vitamins B_2 and B_6 are highly photosensitive, it was observed that ΔA values for the mixture of these two vitamins decreased with time when exposed to light but remained fairly constant when kept in the dark or when protected with amber glass. Use of glycerinated buffers also stabilized the spectrophotometer readings (Table II). Instability in ΔA values for vitamin A, when alone or in the presence of vitamin B_6, was also observed. Use of glycerinated buffers helped to stabilize the readings appreciably. To obtain high accuracy of the assay when vitamin A is present, it is imperative that the ΔA value be read immediately after the preparation of the final dilutions in glycerinated buffers.

[1] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

Both folic acid and vitamin B_{12} absorb at 328 mμ, but ΔA values for both of them were zero. Tocopheryl acetate (vitamin E) has no absorbance at 328 mμ, and no interference was noticed when menadione (vitamin K) was present in a proportion likely to be encountered in commercial preparations.

Interference Due to Flavoring Essences.—The commonly used commercial soluble flavoring essences—*viz.*, vanilla, orange, pineapple, lemon, and raspberry—were examined for their interference. Of these, vanilla and orange were found to absorb significantly at 328 mμ with high ΔA values. The remaining three flavors—*viz.*, pineapple, lemon, and raspberry—did not interfere. Hence, in a given multivitamin oral liquid where one does not know the nature of the flavor used, it is imperative that the flavor is removed before proceeding to determine the ΔA value. This can be effected by repeated extractions with ether, usually about four times. This extraction cannot be carried out when flavors and vitamin A are present together in the oral liquid, due to the emulsifying agent used for dissolving vitamin A.

Method

Tablets.—20 tablets were weighed, finely powdered, and mixed thoroughly. Two portions of this powder, each containing 1 mg. of vitamin B_6, were weighed separately and transferred into two 100-ml. volumetric flasks. Glycerinated phosphate buffer at pH 7 was added to one flask, and glycerinated 0.1 *N* HCl to the other. The flasks were shaken vigorously for about 10 min. then made up to volume with the respective solvents. The solutions were filtered through ordinary filter paper, rejecting the first 10 ml. of the filtrate. The clear filtrates were transferred immediately to 1-ml. silica cells, and absorbance at 328 mμ was measured using 0.1 *N* HCl solution as blank. Vitamin B complex and multivitamin tablets were assayed for vitamin B_6. In multivitamin tablets where the absorbance was high, the final concentration containing 10 mcg./ml. of vitamin B_6 was reduced to 5 mcg./ml. to obtain an accurate response from the instrument.

When the vitamin B_6 content of each tablet was 1 mg. or more, the process was modified to make the assay more accurate. An aliquot of finely crushed powder, equivalent to 5 mg. of vitamin B_6, was transferred to a 50-ml. volumetric flask. Distilled water was added, and the flask was shaken for 5 min. Volume was made up to the mark with distilled water and the contents mixed thoroughly. The mixture was filtered, and 5-ml. aliquots of the filtrate were transferred to two 50-ml. volumetric flasks. The volumes were made up to the mark with 0.1 *N* HCl and phosphate buffer pH 7. A slight haziness that may be observed in phosphate buffer when calcium pantothenate is present can be removed by simple filtration.

Table II.—Spectrophotometric Stability of ΔA Readings with Time for a Mixture of 10 mcg./ml. of Vitamin B_6 and 20 mcg./ml. of Vitamin B_2 under Different Conditions

Time, hr.	In Light	In Light but Amber Glass	In Dark	In Light but with 1% Glycerinated Buffer
0	0.330	0.330	0.330	0.330
1	0.302	0.327	0.330	0.330
40	0.050	0.300	0.312	0.275

Soft Gelatin Capsules.—A counted number of capsules, equivalent to about 5 mg. of vitamin B_6, were crushed without appreciable loss. About 5 Gm. of polysorbate 80 was added and the contents mixed thoroughly. The mixture was warmed on a water bath for about 3 to 4 min., mixing the contents during heating. Ten milliliters of glycerin was added to this and mixed well; 10 ml. of distilled water was added and mixed until a clear solution was formed. The contents were transferred to a 50-ml. volumetric flask and made up to volume with distilled water. Five-milliliter portions of this solution were transferred to two 50-ml. volumetric flasks and made up to volume with glycerinated 0.1 *N* HCl and phosphate buffer pH 7, and shaken vigorously; the absorbance was read at 328 mμ using 0.1 *N* HCl solution as blank.

Injectables.—Aliquots containing 1 mg. of vitamin B_6 were transferred to two 100-ml. volumetric flasks. Glycerinated 0.1 *N* HCl and glycerinated phosphate buffer at pH 7 were added to each flask, shaken, and the volume made up to the mark with the respective solvent. After thorough shaking, the solutions were transferred to 1-ml. silica cells, and the absorbance at 328 mμ was read using 0.1 *N* HCl solution as blank. Any slight haziness that may be observed in the phosphate buffer at pH 7 when calcium pantothenate is present (instead of sodium pantothenate or panthenol) can be removed easily by simple filtration.

Oral Liquids.—Aliquots of the liquid containing 1 mg. of vitamin B_6 were transferred into two 100-ml. volumetric flasks, made up to volume with glycerinated 0.1 *N* HCl and glycerinated phosphate buffer at pH 7, and shaken thoroughly. Any haziness that may develop in phosphate buffer pH 7 solution can be removed by simple filtration. Fifty milliliters of each were extracted four times, using 20 ml. of ether for each extraction to remove the flavors. The aqueous solutions were transferred to 1-ml. silica cells, and the absorbance at 328 mμ was read using 0.1 *N* HCl solution as blank.

Correction for absorbance of plain buffers was applied in all the cases in arriving at the final ΔA value. The percentage of vitamin B_6 in the sample is given by the formula $[(x - z)/(y - z)] \times 100$, where x is the ΔA value for the sample solutions containing 10 mcg./ml. or 5 mcg./ml. of vitamin B_6, y is the ΔA for standard solutions containing 10 mcg./ml. or 5 mcg./ml. of vitamin B_6, and z is the absorbance for plain phosphate buffer pH 7 against plain 0.1 *N* HCl as blank. (Buffer correction.)

All the preparations also were assayed by the official U.S.P. method (1). The results are given in Table III. The compositions of all the products assayed are given also. Samples *1* to *6* are products marketed by this firm, whereas samples *7* to *12* are commercial samples manufactured by other firms. The validity of the proposed method was confirmed by submitting a multivitamin preparation (sample *12*, Table III) not containing vitamin B_6.

DISCUSSION

The results in Table III show close agreement between the assay figures for the proposed method

TABLE III.—DETAILS OF PRODUCTS INVESTIGATED AND ASSAY FIGURES FOR VITAMIN B_6

Sample	Description	Active Ingredients[a]	Vitamin B_6		Overage of Vit. B_6, %
			Proposed Method, %	U.S.P. Method, %	
1	B Complex tablets (coated yellow)	Each tablet contains B_1, 5; B_2, 0.5; B_6, 2; cal. pant., 1; nic., 25; folic acid, 0.5	96	96	Nil.
2	Multivitamin tablets (coated orange)	Each tablet contains A, 5000; D, 1000; B_1, 1.5; B_2, 2; ascorbic acid, 37.5; B_6, 0.1; E, 2; cal. pant., 1; nic., 20	118	119	20
3	B Complex elixir	Each 30 ml. contains B_1, 10; B_2, 4; B_6, 1; nic., 50; B_{12}, 50 mcg.; sod. glycerophosphate, 200; L-lysine HCl, 500; choline dihydrogen citrate, 100	123	125	25
4	B Complex injection	Each ml. contains B_1, 10; B_2, 0.5; B_6, 1; nic., 25; sod. pant., 1; lidocaine HCl, 5; phenol, 0.5%	106	105	5
5	B Complex forte injection	Each ml. contains B_1, 25; B_2, 1; B_6, 2.5; nic., 50; sod. pant., 5; lidocaine HCl, 10; phenol, 0.5%	104	105	5
6	B Complex super forte injection	Each ml. contains B_1, 50; B_2, 4; B_6, 2.5; nic., 100; sod. pant., 10; lidocaine HCl, 10; phenol, 0.5%	105	105	5
7	Multivitamin tablets (coated green)	Each tablet contains A, 2000; B_1, 1; B_2, 1.2; B_6, 0.08, B_{12}, 1 mcg.; *d*-panthenol, 2; nic., 10; C, 30; D, 200; E, 0.2; menadione, 0.067	109	110	Unknown
8	Multivitamin injection	Each ml. contains A, 5000; D, 1000; B_1, 5; B_2, 1; nic., 30; panthenol, 2; B_6, 1; C, 25; E, 1; chlorbutol, 0.5%	106	107	Unknown
9	B Complex tablets (uncoated)	Each tablet contains B_{12}, 5 mcg.; B_1, 3; B_2, 1; nic., 30; cal. pant., 1; B_6, 0.5	100	101	Unknown
10	Multivitamin forte soft gelatin capsules	Each capsule contains B_{12}, 5 mcg.; A, 10,000; D, 1000; B_1, 20; B_2, 5; nic., 100; cal. pant., 10; B_6, 2.5; C, 100; E, 10	97	96	Unknown
11	B Complex liquid	Each 5 ml. contains B_{12}, 6.25 mcg.; B_1, 3.75; B_2, 1.25; nic., 37.5; cal. pant., 1.25; B_6, 0.62	100	98	Unknown
12	Multivitamin tablets	Each tablet contains A, 5000; B_1, 3; B_2, 2; nic., 20; C, 30; D, 1000; calcium phosphate, 250	2	2	...

[a] Cal. pant. and sod. pant., calcium pantothenate and sodium pantothenate, respectively. Nic., nicotinamide. Figures for vitamins A and D are in I.U.; the rest of the figures denote quantity in milligrams.

and the official U.S.P. method. The proposed method is simple and less time consuming, compared to the official U.S.P. method. All the samples tested were not older than 4 months, and further studies are in progress for investigating 1 and 2 years old and date expired samples for assessing interference, if any, due to the decomposition products. Even though the incorporation of 1% glycerin in the buffer stabilizes ΔA values, it is advisable to complete the entire procedure of dilution, filtration, etc., as quickly as possible and to read ΔA values immediately. In multivitamin preparations where the vitamin B_6 content is small compared with other vitamins in the formula, the final sample dilution in 0.1 *N* HCl containing 10 mcg./ml. of vitamin B_6 will have high absorbance value. In such cases where the absorbance of 0.1 *N* HCl sample solution is high, it is necessary to reduce the final dilution to 5 mcg./ml. to obtain an accurate response from the instrument. It is suggested to compare the samples with standard vitamin B_6 solution to eliminate differences in instrumental parameters from time to time. When vitamin A is present in the preparation, it is imperative that the filtration, if any, should be carried out in the dark. The method, being a ΔA method, is highly critical regarding correct weighing and accurate measurements of volumes. The more critical these factors are, the higher the absorbance. In conclusion, this technique will be helpful for control in pharmaceutical manufacturing since it is rapid.

SUMMARY

A rapid and accurate ΔA spectrophotometric method has been devised for assaying vitamin B_6 in B complex and multivitamin preparations. Solutions containing 10 mcg./ml. or 5 mcg./ml. of vitamin B_6 are prepared in glycerinated 0.1 *N* HCl and phosphate buffer at pH 7, and the absorbance is read at 328 mμ using 0.1 *N* HCl solution as blank. Results are evaluated after applying correction for absorbance of plain buffers. The method is not applicable when flavors and emulsifying agents are present together.

REFERENCES

(1) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, pp. 194–197.
(2) Kuhn, R., and Low, I., *Ber.*, **72**, 1453(1939).
(3) Swaminathan, M., *Nature*, **145**, 780(1940).
(4) Bina, A. F., Thomas, J. M., and Brown E. B., *J. Biol. Chem.*, **148**, 111(1943).
(5) *Ibid.*, **158**, 455(1945).
(6) Sweeny, J. P., and Hall, W. L., *J. Assoc. Offic. Agr. Chemists*, **38**, 697 (1955).
(7) Higuchi, T., and Brochmann-Hanssen, E., "Pharmaceutical Analysis," 1st ed., Interscience Publishers, Inc., New York, N. Y., 1961, p. 669.
(8) Tardif, R., THIS JOURNAL, **50**, 693(1961).
(9) Second Panel Report, *Analyst*, **89**, 385(1964).
(10) Elvidge, D. A., and Peutrell, B., *J. Pharm. Pharmacol., Suppl.*, **13**, 111T(1961).
(11) Shah, R. C., Raman, P. V., and Shah, B. M., THIS JOURNAL, **52**, 167(1963).
(12) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 929.

Interfacial Properties of Antimicrobial Long-Chain Quaternary Ammonium Salts I

Soluble Films at the Air–Water Interface

By NORMAN D. WEINER and GEORGE ZOGRAFI*

The surface tension of solutions containing selected long-chain quaternary ammonium compounds, having the same chain-length and counterion but differing in their polar group, was measured using the drop-volume method. The Gibbs adsorption equation was applied to obtain the surface concentration for each bulk concentration used. Data obtained in very dilute solutions conform to the equation of state for an ideal two-dimensional "gaseous" monomolecular film; whereas at higher concentrations, the films are in a "liquid-condensed" state. The standard free energy, entropy, and enthalpy for adsorption were calculated from data obtained in the ideal region. It appears that the change in entropy is the major thermodynamic factor involved in the adsorption process under study. Differences in adsorption for these compounds are discussed on the basis of the electronic and steric configuration of the polar groups.

ALTHOUGH surface-active quaternary ammonium compounds are used widely as antimicrobial agents, few detailed studies of their surface properties have been reported. Those studies which have been conducted have dealt primarily with the determination of critical micelle concentrations (CMC) and the effect of chain length and counterions on adsorption at the air–water interface (1–7).

It was the purpose of this investigation to gain insight into the role of the polar group on adsorption of quaternary ammonium compounds at the air–water interface and to quantify these differences by use of surface pressure *versus* area curves, equations of state, and thermodynamic functions of adsorption.

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EXPERIMENTAL

Materials.—Three quaternary ammonium compounds, having the same chain length and counterion but differing in their polar group, were selected. These were dodecylpyridinium chloride (DPC), dodecyltrimethylammonium chloride (DTAC), and dodecyldimethylethylammonium chloride (DEAC).

DPC (obtained from the Richardson-Merrell Pharmaceutical Co.) was purified by the addition of charcoal to a methanol solution of the sample. DTAC was prepared by the condensation of methyl chloride (Matheson Chemical Co.) and *N,N*-dimethyldodecylamine (Eastman Organic Chemicals); the latter reactant was purified according to Hinsberg (8). The reaction was carried out in a Paar hydrogenator for 12 hr. using ether as a solvent. DEAC was prepared by the condensation of *N,N*-dimethyldodecylamine and ethyl chloride (Gebauer's Chemical Co.) in a bomb at 100° for 6 hr. All three compounds were washed thoroughly with petroleum ether to remove any unreacted materials and were recrystallized three times from acetone.