Spectrophotometric Study of Aqueous Solutions of Warfarin Sodium

By C. F. HISKEY, E. BULLOCK, and G. WHITMAN

The pKa of the enolic form of warfarin has been determined by a spectrophotometric technique and found to be 5.05 ± 0.1. It was found that if its solutions were prepared with an alkaline buffer they could be kept stable and free of precipitation for several weeks when stored at refrigerator temperatures.

SINCE the discovery by Link (1) of the hypoprothrombinemic properties of warfarin sodium,[1] the use of this agent both in a parenteral as well as in the oral form, has become widespread. The oral method of administration is the preferred one, but there are occasions when this route is contraindicated. This occurs, for example, in instances where the patient with a thromboembolic episode cannot swallow because of vomiting or of being comatose. In such cases it is necessary to administer the drug intravenously or intramuscularly. There is usually an initial larger dose of 25 to 50 mg., which is subsequently followed by 5 or 10-mg. daily maintenance doses.

The warfarin sodium used in the parenteral preparation is supplied in the form of a lyophilized powder, containing with it sufficient sodium chloride. When an appropriate amount of sterile water is introduced into the vial a clear isotonic solution containing about 25 mg. of the drug per ml. is obtained. It became of interest to determine whether the lyophilized warfarin powder could be reconstituted to give stable solutions, which on the succeeding days following the start of therapy, could be used for the maintenance doses.

While investigating this problem some difficulties were experienced with such solutions. Precipitates were occasionally found and, consequently, it was decided to make a systematic study of the absorption spectra of warfarin sodium solutions in order to determine the acid ionization constant of the enol form. Because of the very low solubility of the enol form, the more common potentiometric method could not be applied. The data obtained were useful in setting up the conditions for producing stable parenteral solutions and, being of general interest, are therefore presented here.

EXPERIMENTAL

Effect of pH on the Absorption Spectra of Warfarin Solutions

Warfarin powder corresponding in its properties to those described in U.S.P. XVI was used in all of these studies. Two solutions were prepared at a concentration of 6.06×10^{-5} M with respect to warfarin. One was made 0.01 N with hydrochloric acid and the other was made 0.01 N with sodium hydroxide. The absorption spectra of these two solutions were determined using a DK-2 recording spectrophotometer with 1-cm. silica cells and using water as the reference solvent.

The spectra so obtained are presented in Fig. 1 where A represents that of the enolic form and B that of the enolate form. It will be observed that the enolic form has absorption peaks at 274, 282, and 305 mμ. In addition, a peak in the vicinity of 319 mμ emerges as a shoulder on the 305 mμ peak. The enolate spectrum shows a main peak at 308 mμ with a shoulder in the vicinity of 293 mμ. There is, as would be expected, a general shifting of this spectrum toward the visible. Isosbestic points are observed at 289 and 251 mμ. A summary of the molar absorptivities is given in Table I.

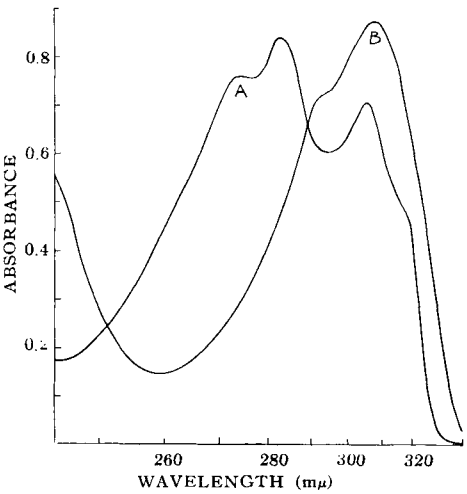

Figure 1

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[1] Warfarin is the coined generic name for the synthetic hypoprothrombinemia-inducing agent 3-(alpha-acetonyl benzyl)-4-hydroxycoumarin. Coumadin sodium is the registered trademark of Endo Laboratories for this chemical.

TABLE I.—SUMMARY OF MOLAR ABSORPTIVITIES

Form	mμ	Molar Absorptivity
Enol	274	12,500
	282	13,900
	305	11,600
Enolate	308	14,400
	293	11,900

In order to determine the acid ionization constant of this compound, a group of solutions was prepared with identical molar concentrations of the anticoagulant, i.e., 5.5×10^{-5} *M*, but with a pH varying between 2 and 10. These were prepared by adding disodium hydrogen phosphate in an amount sufficient to give a 0.1 *M* solution. The pH was adjusted in a preliminary way to the desired value by adding either hydrochloric acid or sodium hydroxide, after which the solutions were brought to volume. The spectrum was then recorded for each solution, after which a final pH measurement was made. Some of the spectral curves that were obtained are shown in Fig. 2.

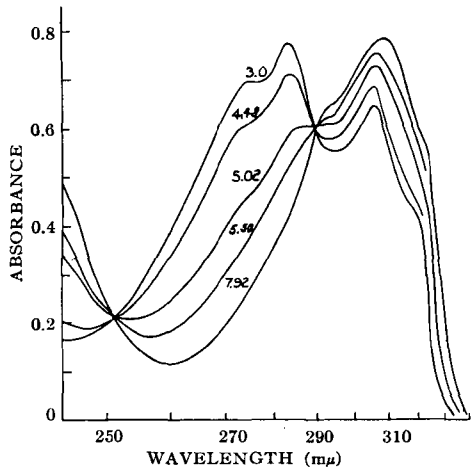

Figure 2

The data were solved for the pKa by assuming an enol-enolate equilibrium given by the simple proton transfer reaction shown in Eq. 1. From the Nernst relation we may write Eq. 2

$$\text{Enol} + H_2O \rightleftharpoons \text{Enolate}^- + H_3O^+ \quad \text{(Eq. 1)}$$

$$\text{pH} = \text{pKa} + \log \frac{(\text{Enolate})}{(\text{Enol})} \quad \text{(Eq. 2)}$$

where the terms in the brackets represent the concentration of the acid and base forms. Inasmuch as the spectrophotometric method involves a concentration determination rather than an activity determination, the expression in Eq. 2 is only approximate.

By examining the curves obtained in Fig. 2, it will be observed that the largest spectral change occurs in the 274 mμ region. This was the region chosen for our calculations since it would give us the greatest change per pH unit. The concentrations of the enolate and enol forms were found by fitting the data into an equation of the form shown in Eq. 3

$$A = a_1bc \frac{h}{h + \text{Ka}} + a_2bc \frac{\text{Ka}}{h + \text{Ka}} \quad \text{(Eq. 3)}$$

where A, is the absorbance of the solution at 274 mμ, a_1 and a_2 are the absorptivities of the enol and enolate species, respectively, b is the path length of the absorption cell, c is the concentration of the anticoagulant in the solution, and h is the activity of the hydrogen ion in these solutions. Having determined these concentration values it can be seen that substituting them into Eq. 2, a linear plot of the data should be obtained with a slope of unity. In Fig. 3 this has been done and reasonable agreement with Eq. 2 is observed. The pKa determined graphically is 5.05 ± 0.1. Thus, it is seen that the enol is an acid nearly as strong as acetic acid. At a pH of 8 or higher the warfarin will be completely in the enolate form. Consequently, clear stable solutions may be anticipated at that pH.

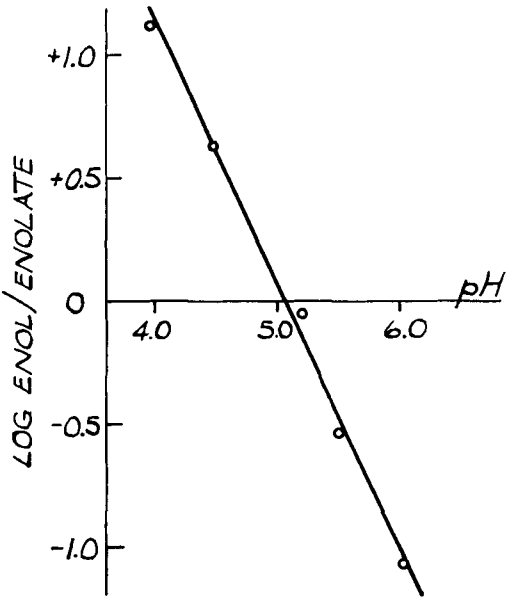

Figure 3

This study was performed in order to determine whether reconstituted vials of lyophilized warfarin sodium injection are stable enough to be used over a period of time as a multidose vial. Samples from three lots of 50-mg. vials and three lots of 75-mg. vials were reconstituted with 2 and 3 ml., respectively, of sterile water. In another study similar vials were reconstituted with similar amounts of disodium hydrogen phosphate buffer at a concentration of 10 Gm. per L. Daily withdrawals were made which were assayed for the warfarin sodium content. The vials were examined each day for the appearance of crystals and for discoloration of the solution. The reconstitution and withdrawals were made under simulated clinical conditions and the vials were stored in a refrigerator between withdrawals.

Before reconstitution, the metal caps were removed and the vials were weighed with the rubber caps and seals in place. The sterile water and disodium hydrogen phosphate solution were injected into each vial with a 5-ml. syringe and the vials were weighed to determine the precise amount of solution that had been added. The buffer solution had pre-

TABLE II.—RESULTS OBTAINED WITH A PHOSPHATE BUFFER

Daily Withdrawal Schedule, 0.2 ml.

Size	75-mg. Vial			50-mg. Vial		
Endo Lot No.	70028	70096	60307	01146	01144	01219
Anticipated concn.	23.1	23.5	23.1	23.3	22.6	23.57
Days after reconstitution						
0	23.1	23.3	23.2	23.9	22.99	23.2
1	23.5	23.7	23.4	23.9	23.2	22.9
2	23.6	23.5	23.3	23.6	23.0	22.9
3	23.9	23.8	23.4	23.9	22.7	23.0
4	23.9	23.7	23.4	23.6	23.2	22.9
7	23.6	23.6	23.45	23.9	22.5	22.8
8	23.8	23.9	23.6	24.0	23.1	22.4
9	23.8	23.8	22.8	24.2	23.2	23.1
10	23.6	23.8	23.4	...	22.9	23.1
11	23.6	23.7	23.4	...	...	...
14	23.8	23.4	23.2	...	...	...
28	23.8	23.7	23.3	...	...	...
35	...	23.8	23.5	...	...	...

There was no discoloration of solution or formation of precipitate.

Daily Withdrawal Schedule, 0.3 ml.

Size	75-mg. Vial			50-mg. Vial		
Endo Lot No.	70028	70096	60307	01146	01144	01219
Anticipated concn.	24.5	24.2	22.05	24.45	27.3	24.9
Days after reconstitution						
0	25.6	24.6	22.5	25.0	...	24.6
1	...	24.0	22.4	25.1	28.0	24.3
2	25.0	24.3	22.0	...	28.3	25.0
6	25.2	24.5	22.5	25.0	28.3	24.2
7	25.2	24.3	22.4	25.2	27.8	24.2
8	24.7	24.0	22.2	25.1	...	24.4
9	25.0	24.1	22.3	...	...	...
12	24.9	24.1	22.4	...	...	...
26	25.2	24.1	22.5	...	...	...
33	...	...	22.5	...	...	...

There was no discoloration of solution or formation of precipitate.

Daily Withdrawal Schedule, 0.4 ml.

Size	75-mg. Vial			50-mg. Vial		
Endo Lot No.	70028	70096	60307	01146	01144	01219
Anticipated concn.	24.05	23.4	23.2	23.4	23.9	23.2
Days after reconstitution						
18	...	23.8	23.4	23.9	24.8	23.8
25	24.4	23.4	23.6	24.0	24.9	23.8
26	24.8	23.7	23.5	24.1	24.6	23.6
27	24.7	23.8	23.6	24.2	...	23.8
29	24.6	23.8	23.6	24.0	24.2	23.7
32	24.3	23.6	23.7	...	...	...
33	24.5	23.5	23.6	...	...	...

There was no discoloration of solution or formation of precipitate.

viously been placed in 10-ml. bottles, sealed with rubber caps and metal seals, and autoclaved at 248° F. and 15 p.s.i. for 15 minutes.

The anticipated concentration could be computed by using the original assay value for each lot. A 1-ml. tuberculin syringe was used to remove a 0.2-ml. portion of solution immediately after reconstitution, and daily thereafter. The amount of solution withdrawn was determined by injecting the solution into small, tared, stoppered test tubes which were weighed and transferred quantitatively to a suitable volumetric flask. Dilutions were made with freshly prepared 0.01 *N* sodium hydroxide to give a concentration of warfarin sodium equal to 1 mg./100 ml.

The absorbance was determined on the Beckman DU spectrophotometer at 308 mμ, using 0.01 *N* sodium hydroxide in the reference cell. Computation of concentration was as follows:

$$\text{mg./ml.} = \frac{11.62 \times A_{308} \times W_2}{\text{ml. solution} \times W_1}$$

where W_1 = weight of sample, W_2 = total weight of container contents after addition of phosphate solution, W_1/W_2 is the fraction of the total contents of the vial taken for assay, and ml. solution refers to the volume of phosphate solution added to the vial.

Daily withdrawals of 0.3 and 0.4 ml. from other vials of these lots were made employing similar methods. Sterile conditions were maintained. All syringes were thoroughly washed, wrapped in brown paper, autoclaved at 248° F. and 15 p.s.i. for 15 minutes, and dried in an oven at 105° C. Before

each entry into a vial, the caps were rubbed with alcohol.

The results obtained with the phosphate buffer are presented in Table II. With sterile water, similar results were obtained but it was noticed after a few days that about half of the vials showed precipitation of the enol form. When precipitates were observed the vials were discarded. Since this would be unsatisfactory from a manufacturers as well as a clinical viewpoint, use of sterile water for reconstitution and multiwithdrawals is not recommended.

The data in Table II indicate that a vial of reconstituted warfarin sodium solution with disodium hydrogen phosphate buffer is quite stable over a reasonable period of time and can be used as a multidose vial.

REFERENCE

(1) Link, K. P., *Circulation*, **19**, 97(1959).

Oral *versus* Subcutaneous Potency of Codeine, Morphine, Levorphan, and Anileridine as Measured by Rabbit Toothpulp Changes

By FLOYD E. LEADERS and H. H. KEASLING†

The ratio of oral *vs.* subcutaneous potency of codeine, morphine, levorphan, and anileridine has been obtained using rabbit toothpulp threshold changes. Values for codeine, morphine, and levorphan were approximately equal. The value for anileridine was approximately one-half that of the other agents.

MORPHINE is generally recognized to be inactive when administered orally (1). Likewise, oral codeine in doses up to 60 mg. is ineffective as an analgesic agent (2, 3). Levorphan (4) and anileridine (6), however, have been reported to have approximately equivalent analgesic potency by either oral or subcutaneous routes of administration. The differences in reported efficacy of these compounds by these routes prompted this study. The change in toothpulp thresholds in the rabbit was chosen to compare the oral *vs.* subcutaneous potency of codeine, morphine, levorphan, and anileridine.

METHODS

Toothpulp threshold changes were measured as reported by Yim, *et al.* (5), using 0.7 to 1.5 Kg. rabbits. Fresh drug solutions were prepared daily in 0.9% saline and administered to the rabbit subcutaneously or orally by stomach tube.

The drugs utilized in this study were codeine phosphate, morphine sulfate, levorphan tartrate, and anileridine dihydrochloride. All doses were given in terms of μm./Kg. of the base.

The values for total area under the time-response curve were approximated as described by Winter and Flataker (7). These figures were obtained from the percentage changes of each individual rabbit. The peak percentage change during the first 90 minutes post drug was utilized in the analysis of peak responses.

A two by two parallel line bioassay was performed on all data except that of levorphan. A two by three parallel line bioassay was used in this instance. Both peak effect and total area under the curve responses were statistically analyzed by the method of Finney (8), using log response and log dose as effect and dose metameters, respectively.

RESULTS

The results of the bioassay of oral *vs.* subcutaneous doses of codeine phosphate, morphine sulfate, levorphan tartrate, and anileridine dihydrochloride are presented in Figs. 1 and 2. Log dose was plotted against both the log of the total area under the response curve and the log of the peak response. The relative potencies with fiducial limits are summarized in Table I.

Table II illustrates the results of the analysis of variance for each of these groups.

DISCUSSION

Our data demonstrate that codeine, levorphan, and morphine have subcutaneous-oral relative potency values of approximately equivalent magnitudes as measured by elevation of toothpulp thresholds in rabbits. This would be expected since they are close chemical congeners. Absolute potency, however, did vary from drug to drug. These data would thus be in agreement with the literature which

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