of dispersant influenced the amount of drug concentrating on or near the wax particle surface. A glance at the zero intercept values presented in Table III will show that more drug was apparently present on or near the surface of the drug–wax particles produced with the aid of sorbitan monooleate alone than on those prepared with polysorbate 80 USP alone; that increasing the concentration of sorbitan monooleate twofold with constant amount of polysorbate 80 USP gave a marked increase in this portion of drug rapidly released in the alkaline pancreatin solution; and that increasing the concentration of polysorbate 80 USP, while holding constant the amount of sorbitan monooleate in the formula, caused a marked decrease in the amount of SETD representing this value.

For all products except those prepared with use of polysorbate 80 USP singly as dispersant, where a larger portion of the total drug was apparently embedded within the wax matrix (total drug minus apparent surface drug), an increased rate of SETD release in the simulated intestinal fluid was observed.

SUMMARY

SETD–glyceryl tristearate particles were manufactured by a method of aqueous dispersion for prolonged-release application using various concentrations of polysorbate 80 USP and sorbitan monooleate singly and in combination as dispersants. The effects of variation in the type and concentration of surfactant used as dispersant were studied by *in vitro* release tests, physical testing, and visual and microscopic examination. Rates of release of SETD constituting the prolonged dissolution portion were calculated for results in simulated intestinal fluid using square root of time relationships and compared. Percent released square root of time plots gave reasonable linear approximations of prolonged-release SETD dissolution data.

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Keyphrases

Drug release from wax prolonged-release particles
Sulfaethylthiazole–wax particle preparation
Surfactant effect on wax particle formation
Dispersant effect on wax particle formation
In vitro release studies
Colorimetric analysis

Complexation of Sodium Fluorescein with Polyvinylpyrrolidone

By RUSSELL E. PHARES, JR.

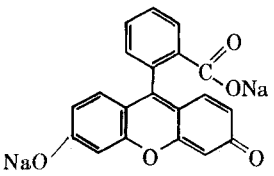
The complexation of sodium fluorescein (NaFluor) with polyvinylpyrrolidone (PVP) was studied at four pH values using a method similar to that of Benesi and Hildebrand. It was found that fluorescein can exist in four forms, each of which is capable of complexing. The results tend to indicate that at low pH values PVP exists in more than one form and that not all forms of the PVP are capable of complexing with NaFluor. When the ratio of NaFluor to PVP is small, a 1 : 1 complex is favored; but as the ratio increases, higher complexes predominate. The stability of the 1 : 1 complex seems to increase as the negative charge on the fluorescein decreases.

IT WAS recently discovered that polyvinylpyrrolidone (PVP), which for a long time has been known to complex with many substances, would form a sodium fluorescein complex possessing some interesting properties (1). By utilizing the unique properties of this complex, Krezanoski (1) has been able to formulate a stable and effective applanation tonometry diagnostic aid[1] con-

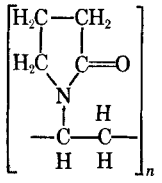
Received April 10, 1967, from the Research Laboratories, Barnes-Hind Pharmaceuticals, Inc., Sunnyvale, CA 94086
Accepted for publication August 23, 1967.
The author gratefully acknowledges the invaluable assistance of Mr. F. A. Caligiuri and Mr. F. D. Dreier.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

[1] Fluress, Barnes-Hind Ophthalmic Products.

taining chlorobutanol, benoxinate HCl, and 0.25% sodium fluorescein in an isotonic boric acid buffer. It seemed desirable to collect more information about this complex in view of its practical applications and the fact that sodium fluorescein is a relatively simple molecule possessing the same basic structure found in many dyes which might be complexed with PVP to give other useful products. The structures of PVP and sodium fluorescein are given in I and II.



Sodium Fluorescein
I



PVP
II

EXPERIMENTAL

The sodium fluorescein (NaFluor) used was USP grade, and the PVP was pharmaceutical grade[2] having an average molecular weight of 30,000. All absorbance values were determined using a Beckman DK-2 spectrophotometer. A preliminary experiment was done to determine the spectral absorbance curves of a 1×10^{-5} *M* sodium fluorescein solution at pH values of 0.6 to 12.0. These curves are shown in Figs. 1 and 2. Another preliminary investigation was made using Job's method of continuous variation (2). A 3.7×10^{-5} *M* solution of PVP was mixed in various ratios with a 3.7×10^{-5} *M* solution of sodium fluorescein. Each solution was made in a pH 5.0 buffer adjusted to have an over-all ionic strength of 0.1. The absorbance of each mixture, as well as the absorbances of various concentrations of sodium fluorescein without PVP, was determined at 475 mμ. Figure 3 shows a plot of the differences between the expected and the observed absorbances for the various volume ratios of the PVP and sodium fluorescein solutions.

It was decided, based upon the results of the preliminary experiments, to study the complexation process at pH values of 1.5, 3, 5, and 8, using a method similar to that of Benesi and Hildebrand (3). The experiments conducted were as follows:

pH 1.5:

Sodium fluorescein held constant at 5×10^{-5} *M*, and the PVP varied from 1.66 to 13.28 $\times 10^{-4}$ *M*. Buffer system was HCl and KCl.

pH 3.0:

Sodium fluorescein held constant at 5×10^{-5} *M*, and the PVP varied from 1.66 to 13.28 $\times 10^{-4}$ *M*. Buffer system was phthalate.

PVP held constant at 1.8×10^{-5} *M*, and the sodium fluorescein varied from 1 to 36 $\times 10^{-5}$ *M*.

pH 5.0:

Sodium fluorescein held constant at 5, 25, and 50 $\times 10^{-6}$ *M*, and the PVP varied from 8.3 $\times 10^{-5}$ to 1.33 $\times 10^{-3}$ *M*. Buffer system was neutralized phthalate.

PVP held constant at 6.5×10^{-6} and 1.3×10^{-5} *M*, and the sodium fluorescein varied from 2.0×10^{-4} to 1.4×10^{-3} *M*.

[2] Plasdone C, General Aniline & Film Corp.

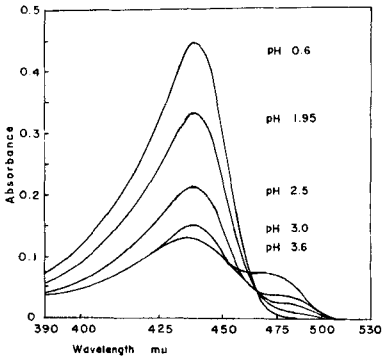


Fig. 1—Effect of pH on the spectral absorbance curve of sodium fluorescein.

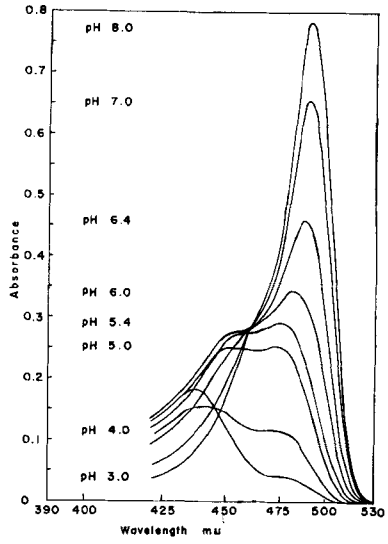


Fig. 2—Effect of pH on the spectral absorbance curve of sodium fluorescein.

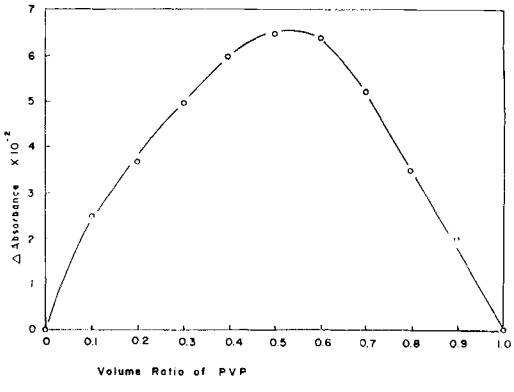


Fig. 3—Job plot of the NaFluor–PVP complex.

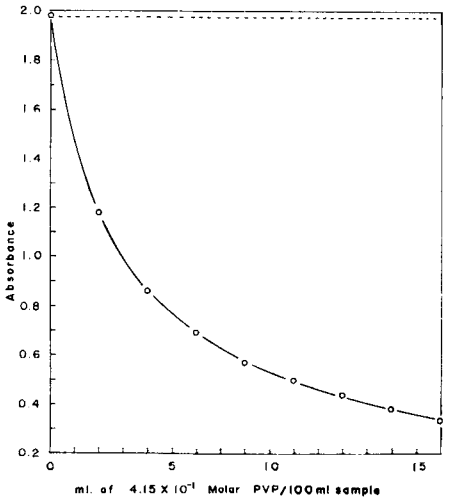


Fig. 4—Effect of PVP concentration on the absorbance of 5 × 10⁻⁶ M sodium fluorescein at pH 1.5.

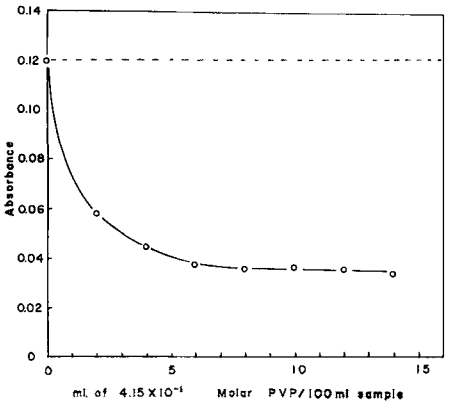


Fig. 5—Effect of PVP concentration on the absorbance of 5 × 10⁻⁶ M sodium fluorescein at pH 5.0.

pH 8.0:

Sodium fluorescein held constant at 5×10^{-5} *M*, and the PVP varied from 1.66 to 13.28 × 10^{-4} *M*. Buffer system was phosphate.
PVP held constant at 1.3×10^{-5} *M*, and the sodium fluorescein varied from 2×10^{-4} to 3.2×10^{-3} *M*.

The absorbances of the above solutions were measured at their spectral peaks, which were 437.5 mμ at pH values of 1.5 and 3.0, 475 mμ at pH 5.0, and 490 mμ at pH 8.0. Figures 4 and 5 are typical of the results obtained from the above experiments, and show the expected absorbances if no complexes were formed and the absorbances which were actually obtained.

DISCUSSION

The data summarized in Figs. 1 and 2 indicate that sodium fluorescein exists in several forms, much the same as phenolphthalein, which is quite similar structurally. Based upon the observed fact that the solubility of sodium fluorescein is much greater at the very low pH values than it is at a pH of about 2.5 to 3.0, and based upon the behavior of phenolphthalein at low pH values, it seems that the absorbance peak at 437.5 mμ is associated with a protonated form of the fluorescein. This form has a pKa of approximately 2.2. At about 465 mμ, there appears to be an isobestic point which is also associated with the protonation. The changes which take place between this isobestic point and the next one, which is also at 465 mμ, are probably associated with the singly charged anion, which appears to have a pKa of about 4.4. The peak at 490 mμ can be attributed to the doubly charged anion, which has a pKa of about 6.7. There were no indications of the formation of a triply charged anion such as is formed by phenolphthalein, even when the pH was raised to 12.0.

The Job plot shown in Fig. 3 has a maximum at about 0.5 parts of PVP by volume, which indicates the preferential formation of a 1:1 complex. It is interesting to note that the side of the curve representing relatively high concentrations of PVP is straight, as would be expected, while the side of the curve representing high concentrations of sodium fluorescein is curved. This curving is presumed to be a result of the formation of higher complexes which give way to the more stable 1:1 complex upon the addition of more PVP.

Assuming a 1:1 complex in dilute solutions of sodium fluorescein and recognizing the fact that any and possibly all forms of sodium fluorescein might be complexing with the PVP, the following equations were derived to describe the deviations of absorbances from those that would be predicted if no complexation were taking place.

The following equilibria are assumed:

$$\frac{[H^+][Fluor^-]}{[H\ Fluor^-]} = K_3 \text{ and } [H\ Fluor^-] = \frac{[H^+]}{K_3}[Fluor^-] \quad \text{(Eq. 1}a\text{)}$$

$$\frac{[H^+][H\ Fluor^-]}{H_2\ Fluor} = K_2 \text{ and } [H_2\ Fluor] = \frac{[H^+]^2}{K_3 K_2}[Fluor^-] \quad \text{(Eq. 1}b\text{)}$$

$$\frac{[H^+][H_2\ Fluor]}{H_3\ Fluor^+} = K_1 \text{ and } [H_3\ Fluor^+] = \frac{[H^+]^3}{K_3 K_2 K_1}[Fluor^-] \quad \text{(Eq. 1}c\text{)}$$

$$\frac{[C_1]}{[PVP][H_3\ Fluor^+]} = k_1 \text{ and } [C_1] = [PVP]k_1[H_3\ Fluor^+] \quad \text{(Eq. 2}a\text{)}$$

$$\frac{[C_2]}{[PVP][H_2\ Fluor]} = k_2 \text{ and } [C_2] = [PVP]k_2[H_2\ Fluor] \quad \text{(Eq. 2}b\text{)}$$

$$\frac{[C_3]}{[PVP][H\ Fluor^-]} = k_3 \text{ and } [C_3] = [PVP]k_3[H\ Fluor^-] \quad \text{(Eq. 2}c\text{)}$$

$$\frac{[C_4]}{[PVP][Fluor^-]} = k_4 \text{ and } [C_4] = [PVP]k_4[Fluor^-] \quad \text{(Eq. 2}d\text{)}$$

The following equation describes the measured absorbance (A_M) obtained when the sodium fluorescein is held constant and the absorbance due to PVP is negligible.

$$A_M = [C]_T Q_1 + [\text{Na}_2\text{ Fluor}]_{TF} Q_2 \quad \text{(Eq. 3)}$$

Q_1 and Q_2, defined in Eqs. 5 and 6, are constants for a given pH. The absorbance which would be expected (A_E) if no complex was formed should be:

$$A_E = [\text{Na}_2\text{ Fluor}]_T Q_2 \quad \text{(Eq. 4)}$$

The subscript TF stands for total free, T stands for total, and C stands for complex.

$$Q_1 = \frac{\epsilon_1 k_1[H^+]^3 + \epsilon_2 k_2 K_1[H^+]^2 + \epsilon_3 k_3 K_1 K_2[H^+] + \epsilon_4 k_4 K_1 K_2 K_3}{k_1[H^+]^3 + k_2 K_1[H^+]^2 + k_3 K_1 K_2[H^+] + k_4 K_1 K_2 K_3} \quad \text{(Eq. 5)}$$

$$Q_2 = \frac{\epsilon_5 K_1 K_2 K_3 + \epsilon_6 K_1 K_2[H^+] + \epsilon_7 K_1[H^+]^2 + \epsilon_8[H^+]^3}{K_1 K_2 K_3 + K_1 K_2[H^+] + K_1[H^+]^2 + [H^+]^3} \quad \text{(Eq. 6)}$$

where ϵ_1, ϵ_2, ϵ_3, ϵ_4, ϵ_5, ϵ_6, ϵ_7, and ϵ_8 are the absorptivity coefficients for C_1, C_2, C_3, C_4, H_3 Fluor$^+$, H_2 Fluor, H Fluor$^-$, and Fluor$^=$, respectively.

It can also be shown that when the above assumptions are true:

$$[A_E - A_M] = \Delta A = [C]_T(Q_2 - Q_1) \quad \text{(Eq. 7)}$$

and

$$\Delta A_{\max.} = [C]_{T\max.}(Q_2 - Q_1) = [\text{Na}_2\text{ Fluor}]_T(Q_2 - Q_1) \quad \text{(Eq. 8)}$$

An apparent stability constant (K_a) can be calculated as follows:

$$K_a = \frac{[C]_T}{[\text{PVP}][\text{Na}_2\text{ Fluor}]_{TF}} = \frac{\Delta A}{[\text{PVP}]_{TF}(\Delta A_{\max.} - \Delta A)} \quad \text{(Eq. 9)}$$

Equation 9 can be rearranged to give Eq. 10, which is more convenient for plotting. In plotting Eq. 10, it is assumed that $[\text{PVP}]_{TF} \simeq [\text{PVP}]_T$.

$$\frac{[\text{PVP}]_T}{\Delta A} = \left(\frac{1}{K_a\,\Delta A_{\max.}}\right) + \left(\frac{1}{\Delta A_{\max.}}\right)\left([\text{PVP}]_T\right) \quad \text{(Eq. 10)}$$

The data for each experiment in which sodium fluorescein was held constant are plotted in Figs. 6–9. The calculated K_a and the $\Delta A_{\max.}$ for each experiment are given in Table I. Although it appears that the stability constant for the complex at pH 5.0 may change as the amount of substrate changes, the variation may actually be a result of the inaccuracy associated with determining the absorbance values of small amounts of substrate. It is interesting to note that when the expected absorbance for each level of substrate is plotted *versus* the predicted $\Delta A_{\max.}$ a straight line which goes through zero, as would be expected, is obtained.

It can be shown that:

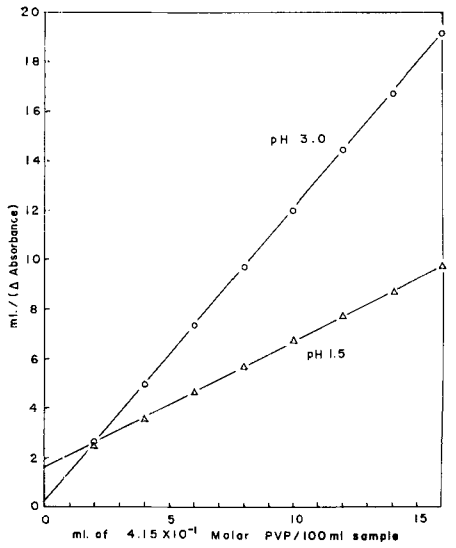


Fig. 6—The pH 1.5 and 3.0 data plotted according to Eq. 10.

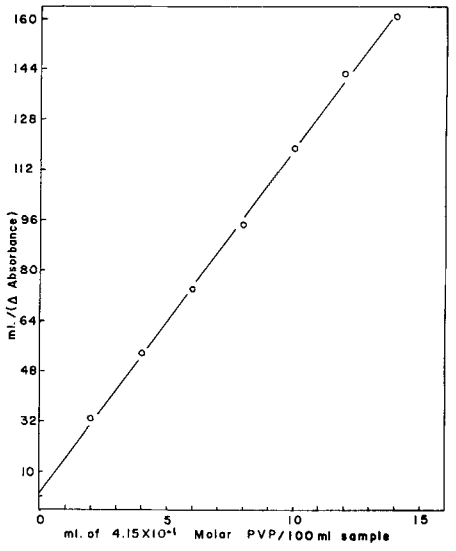


Fig. 7—The pH 5.0 data for 5 × 10^{-6} M substrate plotted according to Eq. 10.

$$K_a = \frac{k_1[H^+]^3 + k_2 K_1[H^+]^2 + k_3 K_1 K_2[H^+] + k_4 K_1 K_2 K_3}{[H^+]^3 + K_1[H^+]^2 + K_1 K_2[H^+] + K_1 K_2 K_3} \quad \text{(Eq. 11)}$$

By knowing the individual dissociation constants for sodium fluorescein and the apparent stability constants calculated at four different pH values, it should be possible to calculate the stability constant for the complex produced by each form of fluorescein. A large negative value was obtained for k_1 when the K_a values in Table I and the approximate dissociation constants for sodium fluorescein of 2.2,

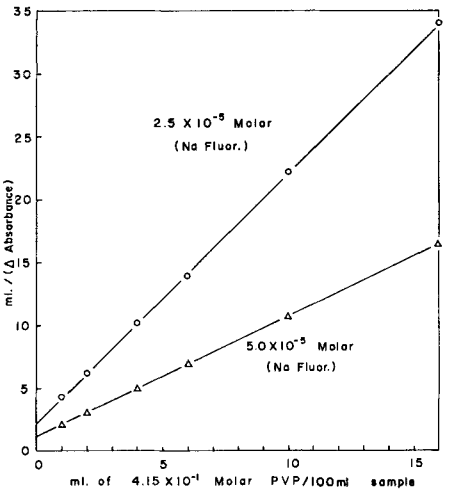


Fig. 8—The pH 5.0 data for 2.5 and 5.0 × 10^{-5} M substrate plotted according to Eq. 10.

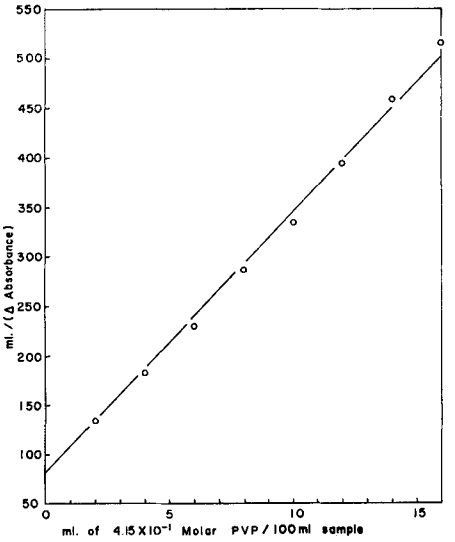


Fig. 9—The pH 8.0 data plotted according to Eq. 10.

TABLE I—SUMMARY OF BINDING DATA

pH	K_a × 10^{-4}	$\Delta A_{max.}$	ΔA_∞[a]	Concn. of Substrate × 10^{+5}
1.5	0.377	1.940	0.048	5.0
3.0	5.37	0.848	0.038	5.0
5.0	1.51	0.095	0.025	0.5
5.0	1.08	0.502	0.102	2.5
5.0	1.04	1.039	0.176	5.0
8.0	0.384	0.038	0.293	5.0

[a] ΔA_∞ is the measured absorbance at infinitely high concentrations of PVP and is obtained when all of the sodium fluorescein is complexed.

4.4, and 6.7 were used to calculate k_1, k_2, k_3, and k_4. This negative value was a result of the unusually

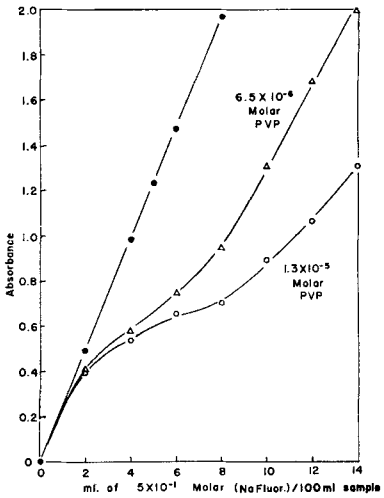


Fig. 10—Effect of sodium fluorescein concentration on the absorbance of 6.5 × 10^{-6} M PVP at pH 5.0.

small K_a observed at pH 1.5. A low value for K_a would result if the concentration of PVP free to complex were lower than expected. This would be the case at pH 1.5 if the complexing form of PVP was in equilibrium with a significant amount of a noncomplexing form of PVP. The literature states (4) that a PVP solution has its minimum viscosity at a pH of about 1.5, a statement which suggests that the PVP is in more than one physical form at this pH.

It was found that higher than 1:1 complexes were formed when the PVP was held constant and the sodium fluorescein varied. Although the stoichiometry and the stability constant of the higher complexes could not be determined, there was no doubt about their existence. The minimum number of moles of complexed sodium fluorescein required to produce the observed ΔA values were much larger than the number of moles of PVP which were present. The results at all three pH values were essentially the same as those shown in Fig. 10 for pH 5.0. None of the systems could be properly evaluated because of solubility problems, extremely high absorbances, or the need to make assumptions with questionable validity.

CONCLUSIONS

Based upon spectrophotometric data and qualitative solubility results, it appears that fluorescein can exist as a singly charged anion, a noncharged molecule, and a singly charged or doubly charged anion. Although each form of fluorescein has characteristic absorption properties, all four forms have a similar yellow color in solution. Each form of fluorescein can complex with PVP, but with varying strengths. This is probably a charged transfer type of complex. It appears that the higher the positive charge on the fluorescein, the weaker is the complex. When the ratio of fluorescein to PVP is about equal, a 1:1 complex is formed, but as the amount of fluorescein is increased, the order of the complex also increases. At low pH values anomal-

ous results are obtained which might well be due to an interaction of PVP with itself.

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Keyphrases

Complex formation
Sodium fluorescein–polyvinylpyrrolidone system
pH effect on complex formation
Colorimetric analysis

Comparative Pharmacokinetics of Coumarin Anticoagulants I

Unusual Interaction of Bishydroxycoumarin with Plasma Proteins—Development of a New Assay

By RENPEI NAGASHIMA, GERHARD LEVY*, and EINO NELSON†

A widely used method for the determination of bishydroxycoumarin (BHC) in plasma, which involves extraction of BHC from acidified plasma (pH <1) into an organic solvent, fails at high concentrations of BHC. Evidence is presented to show that this is not due to a limited solubility of the drug in the organic phase but rather to a concentration- and pH-dependent interaction of plasma protein with BHC. Complete extraction of BHC from the blood plasma of rats, guinea pigs, dogs, monkeys, and man is obtained readily only in the narrow pH range of 3.0 to 3.5. A new method of analysis for BHC in plasma has been developed on the basis of these findings and its specificity is demonstrated. It is shown that the development of analytical methods for drugs in plasma or serum, which involve extraction of the drug into an organic phase, cannot be based solely upon a consideration of the effect of pH on the distribution of the drug between the organic and aqueous phases. It is necessary also to consider the effect of pH (and possibly of other factors, such as the type of organic solvent, the buffer system, and ionic strength) on the physical-chemical properties of plasma proteins as they affect the type and magnitude of interaction of these proteins with the drug.

THE PHARMACOKINETICS of the coumarin anticoagulants has been and is the subject of intensive investigation in many laboratories (1–24). Studies have been concerned with the kinetics of elimination of various coumarin anticoagulants in several animal species (2–11) and in man (12–17), as well as with the interaction of these anticoagulants with various other drugs (*e.g.*, 18–23) and with plasma proteins (24). Of particular interest is the unusual, dose-dependent pharmacokinetics of bishydroxycoumarin (BHC) elimination in man, as well as the biologic fate of this drug which so far has eluded adequate characterization (25, 26).

The most widely used method for the determination of BHC is that of Axelrod and his associates (27), which involves extraction of the drug from plasma acidified with 3 *N* hydrochloric acid to pH <1 into *n*-heptane, followed by re-extraction into 2.5 *N* aqueous sodium hydroxide solution, and spectrophotometric measurement of BHC in the latter phase. In the course of studies in this laboratory on the comparative pharmacokinetics of BHC elimination, which will be described in subsequent reports (28, 29), it was noted that the assay method of Axelrod and his associates fails at relatively high concentrations of BHC. Therefore, a new analytical method for the determination of BHC in plasma or serum has been developed. This method and studies concerning its specificity are described in detail in view of the extensive interest in and continuing research related to the pharmacokinetics of the coumarin anticoagulants.

It will be shown that the recovery of BHC from plasma of various species, including man, is un-

Received August 14, 1967, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication October 19, 1967.
* To whom requests for reprints should be directed.
† Deceased.