ELSEVIER

International Journal of Pharmaceutics 172 (1998) 241–253

international journal of pharmaceutics

Spectral properties and ion dissociation behavior of retinoids I. Aqueous solutions

Chien-Hsuan Han [1], Timothy S. Wiedmann *

Department of Pharmaceutics, College of Pharmacy, University of Minnesota, 308 Harvard St. SE, Minneapolis, MN 55455, USA

Received 3 March 1998; received in revised form 26 June 1998; accepted 29 June 1998

Abstract

Purpose: The spectral properties and ionization behavior of four retinoids: retinoic acid; MTTO; TTNN; and TTNPB, have been determined in aqueous solution to provide a better understanding of their aggregation behavior. Methods: The solubility and ultraviolet absorption spectrum were measured in solutions prepared by equilibration with solid and by dilution of an ethanol solution with buffer. The UV spectral shift was measured as a function of pH to quantify the extent of ionization in 5 and 150 mM NaCl. Fluorescence polarization measurements of TTNN were made. Results: The solubility of the triclinic form of retinoic acid was lower than that of the monoclinic indicating the former is the more stable polymorphic form. For the retinoids, the measured solubility increased with the number of phenyl rings. The wavelengths of maximum absorption and absorptivity of the non-ionized forms of the retinoids systematically changed with chemical structure provided that the preferred molecular conformation was considered. High apparent negative logarithms of the ionization constant, $pK_{a_{obs}}$, were observed indicating that the retinoids undergo self-association. The titration curves were fit to the Henderson–Hasselbalch relationship, and the $pK_{a_{obs}}$ was dependent on the retinoid concentration in solution. The value of the pK_a at low ionic strength was slightly larger that that obtained at high ionic strength. The fluorescent polarization suggested that the aggregation number changes with ionic strength and extent of ionization. Finally, the difference between the observed pK_a and the pK_a of model compounds was smaller for retinoic acid than for MTTO, TTNPB, and TTNN. Conclusions: All retinoids under study were self-associated giving rise to relatively high values of the apparent pK_a. The properties of the aggregates are dependent on the concentration and ionization, and as such, the retinoids probably lack a well-defined aggregation number and critical micelle concentration. The nature of the aggregation for retinoic acid appears distinct from that of the arotinoids, MTTO, TTNPB, and TTNN.

Keywords: Retinoids; Ionization; Spectral properties; Acid dissociation; Aggregation

* Corresponding author. Tel.: +1 612 6245457; fax: +1 612 6242974; e-mail: wiedm001@maroon.tc.umn.edu

[1] Present address: Oread, Palo Alto, CA.

0378-5173/98/$ - see front matter
PII S0378-5173(98)00221-X

1. Introduction

Retinoids are derivatives of vitamin A that have been investigated for their potential in cancer chemoprevention and therapy (cf. Weigand et al., 1993). To further develop the use of these compounds in cancer therapy, much effort has been focused on the elucidation of the molecular mechanisms of the control of gene expression by retinoids. In addition, a number of pharmacokinetic studies have been carried out (Regazzi et al., 1997). In these studies, the fraction of dose absorbed of all *trans* retinoic acid has been difficult to assess, since no intravenous formulation is available. Moreover, there have been suggestions that the extent of absorption may change with repeated dosing and the absorption involves active transport or is limited by the solubility in the intestinal tract. As a means to better understand the mechanism of absorption for retinoids, a characterization of the relevant solution properties of retinoids is desired.

For retinoic acid, there is almost an order of magnitude difference in the reported solubilities which may be in part due to the existence of different polymorphic crystalline forms (Szuts and Harosi, 1991; Noy, 1992a). In addition, there is evidence that retinoic acid undergoes self-association in aqueous solution (Noy, 1992a,b), but little information concerning the association of other retinoid derivatives is available. The concentration of retinoic acid in solution was also shown to modulate the ionization behavior of the retinoid which suggests that the aggregation number does not have a unique value (Noy, 1992b). The potentially complicated ionization and aggregation behavior of retinoids when present in aqueous solutions will have a direct impact on its biological activity.

In this study, the solubility, spectral properties and ionization behavior of all-*trans* retinoic acid, MTTO, TTNN, and TTNPB have been determined as a function of pH in aqueous solutions by UV–visible spectroscopy. In the following paper (Han et al., 1998), these compounds have also been studied in bile salt micelles and bile salt/phospholipid micelles in order to provide the physical chemical properties more directly relevant to their intestinal absorption.

2. Experimental

2.1. Materials

Retinoic acid (RA) was purchased from Sigma: MTTO, (all E)-3,7-dimethyl-9-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2,4,6-octatrienoic acid]; TTNPB, r-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid; TTNN, r-(1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-naphthyl)-2-naphthalenecarboxylic acid; and acitretin were received as gifts from Hoffmann-LaRoche. All were used as received. The structures are given in Fig. 1. The water was double-distilled and deoxygenated by repeatedly exposing the solutions to reduced vacuum followed by bubbling with argon. All other chemicals were reagent grade or better.

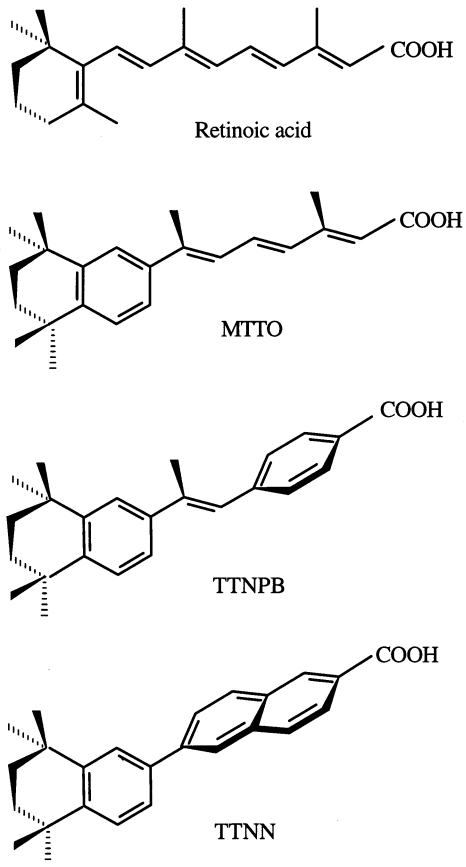

Fig. 1. Structures of retinoic acid, MTTO, TTNPB, and TTNN.

2.2. Methods

2.2.1. Solid state characterization

Differential scanning calorimetry (DuPont 910, Wilmington, DE) was used to measure the melting point and heat of fusion of retinoids both before and after measuring the solubility. The scanning rate was 10°C/min. Hot stage microscopy (M3Z stereomicroscope, Mettler FP80 central processor for programmed temperature control, and a Mettler FP82HT hot stage, WILD Heerbrugg, Switzerland) was used to determine the nature of the phase change.

2.2.2. Aqueous solubility

The aqueous solubilities were determined by dispersing 1–5 mg of solid drug in dialysis membranes (Spectra, molecular weight cut-off: 3500) containing 1 ml of 0.15 M NaCl with the pH adjusted to 3.5–3.7 with HCl. Prior to use, the dialysis bags were soaked in water until there was no measurable absorbance in the UV region of the spectrum (36 h). The dialysis membranes were then placed in Pyrex test tubes with Teflon-lined screw caps, and the tubes were filled with about 10 ml of buffer. After equilibration in a shaking water bath for 24, 48 or 72 h at 19 ± 2°C in the dark, the bags were removed. The remaining solution volume was determined by weight. Acitretin was added as the internal standard (20 μl) to each sample which was then extracted twice with 1 ml of hexane/isopropanol (2/1 v/v). The organic solution was dried under nitrogen and reconstituted with 1 ml of 95% ethanol and placed into amber autoinjection vials for assay.

The analysis of the retinoids was based on a modification of a previously reported method (Thongnopnua and Zimmerman, 1988). The high performance liquid chromatography (HPLC) system consisted of a Waters (Milford, MA) Model 6000A pump, a WISP 710B autoinjector module, and a Shimadzu (Kyoto, Japan) Chromatopac C-R6A integrator. A reversed-phase C_{18} Supelcosil, 4.6-mm I.D., 5 μm column along with a guard column containing LC-18 pellicular packing (both from Supelco, Bellefonte, PA) was used with UV detection at 350 nm. The mobile phase consisted of 75% acetonitrile and 0.3% acetic acid in water which was delivered at a flow rate of 1.5 ml/min.

2.2.3. Stability analysis

The stability of the retinoids in the spectrophotometer was determined by monitoring the absorbance at the wavelength of maximum absorption as a function of time in a Beckman DU Series 70 Spectrophotometer (Fullerton, CA). In some samples, aliquots were also taken and subjected to HPLC. The aqueous solutions of the retinoids were made by diluting 95% ethanol stock solutions with 0.15 M NaCl. The final concentration of ethanol in the solutions was typically about 0.3%, which was low enough not to perturb the absorption spectrum. Whenever possible, procedures were carried out under yellow light to minimize photo-degradation of the retinoids. Three ml cuvettes were used which had a 1.0 cm pathlength, and the sample was irradiated at a distance of 14 cm by a deuterium lamp.

2.2.4. Titrations

Titrations were conducted by initially determining the absorption spectra of the retinoids under acidic and basic conditions. Two wavelengths were selected for measuring the absorbances as a function of pH. These wavelengths corresponded to the maximum differences in the absorptivities of the ionized and nonionized forms of the retinoid, typically corresponding to the wavelengths of maximum absorption. For most of the titrations, the concentrations were 1.83 μM for RA, 1.41 μM for MTTO, 1.04 μM for TTNN, and 1.55 μM for TTNPB.

For titration, 2 ml of sample was placed into a quartz UV cell which contained a small Teflon-coated stir bar. As in the stability study, a small volume of the retinoid in 95% ethanol was added to a basic solution. Thereafter, incremental volumes of a solution of HCl were added directly to the cuvette to change the pH. The pH of solution was measured by a Corning pH meter (Model 125, Corning Glass Work, Medfield, MA) with a calomel combination pH electrode which had a 3-mm optical density (o.d.) pH bulb and 3.5-mm (o.d.) for the stem (Aldrich Chemical, Milwaukee, WI). The pH meter was initially calibrated by the

two point calibration method and was periodically checked during the experiment. After measuring the pH, the absorbances at the four wavelengths were determined. One wavelength was at 500 nm which corresponded to a region where none of the species had measurable absorbance, one was at the isosbestic point, and the remaining two were at the wavelengths indicated above. The latter two were used for the estimation of the ionization constant. The temperature was maintained at 19 ± 2°C.

In analyzing the data, the apparent negative logarithm of the ionization constant, $pK_{a_{obs}}$, was obtained by nonlinear regression (KaleidaGraph, Abelbeck Software) of the ratio, R, of the absorbances, A, at the two wavelengths, λ_1 and λ_2, $R = A_{\lambda 1}/A_{\lambda 2}$, given as a function of the pH as follows (Albert and Serjeant, 1971):

$$pH = pK_{a_{obs}} + \log[(R - R_{min})/(R_{max} - R)] \quad (1)$$

where R_{max} was chosen as the ratio of the absorbances at low pH and R_{min} as the ratio of the absorbances at high pH. Selecting the ratio of absorbances in this way yielded plots of the residuals as a function of the predicted ratio where the variance was randomly distributed.

2.2.5. Fluorescence

For the fluorescence measurements, the concentration of TTNN was 1.04 μM. The emission spectra were determined at low and high pH in 5 and 150 mM NaCl. A quartz fluorescence cuvette was used in a FluoroMax™ Spectrofluorometer (SPEX, Edison, NJ). The slit width was 1-mm for both the excitation and emission light. The excitation and emission spectrometers were calibrated by measurement of the xenon lamp intensity, maximum peak at 467 ± 0.2 nm, and water Raman spectra with a peak at 397 nm. The light exiting the emission spectrometer was monitored by a photomultiplier detector which operated in the photon-counting mode at 950 V. The temperature of the sample chamber was maintained at 20.0 ± 0.5°C. Before the sample compartment, a beam splitter directed 8% of the light to the reference photodiode. The observed intensity was then obtained as a ratio of the sample and the reference signal to correct for variations in light intensity as a function of wavelength. The spectrofluorometer was operated by a built-in DM3000F computer.

For measurement of the polarization, the polarizers were rotated to obtain the four different values of emitted light: ***VV***; ***VH***; ***HH***; ***HV***; where the first and second letters refer to the polarization vectors of excitation and emission, ***V*** refers to a vertical polarization vector, and ***H*** refers to a horizontal polarization vector. The polarization values, P, were calculated according to the following relationship (Canter and Schimmel, 1980; Lakowicz, 1986):

$$P = (I_{VV} * I_{HH} - I_{HV} * I_{VH})/(I_{VV} * I_{HH} + I_{HV} * I_{VH}) \quad (2)$$

3. Results

3.1. Solid state characterization of retinoids

The differential scanning calorimetry (DSC) scans for retinoic acid, MTTO, TTNPB, and TTNN are shown in Fig. 2. For retinoic acid (Fig. 2A), a small endothermic peak was observed at about 145°C followed by a large endothermic peak at 185°C. From hot-stage microscopy, the solid was observed to change from yellow to an orange color. Upon heating to 185°C, the solid liquefied. Another sample was scanned after initially heating the solid to 165°C and rapidly cooling down. As evident in Fig. 2B, there was only a peak at 185°C with reheating which indicates that irreversible conversion had occurred. Using this approach, it was possible to prepare the high and low melting point polymorphic forms and determine the solubility and apparent solubility of retinoic acid.

The remaining arotinoids, MTTO, TTNN, and TTNPB, underwent sharp melting as shown in Fig. 2C–E. The melting points and enthalpy of fusion, ΔH, are listed in Table 1. Both the melting point and enthalpy of fusion increased with increasing number of benzene rings in the structure.

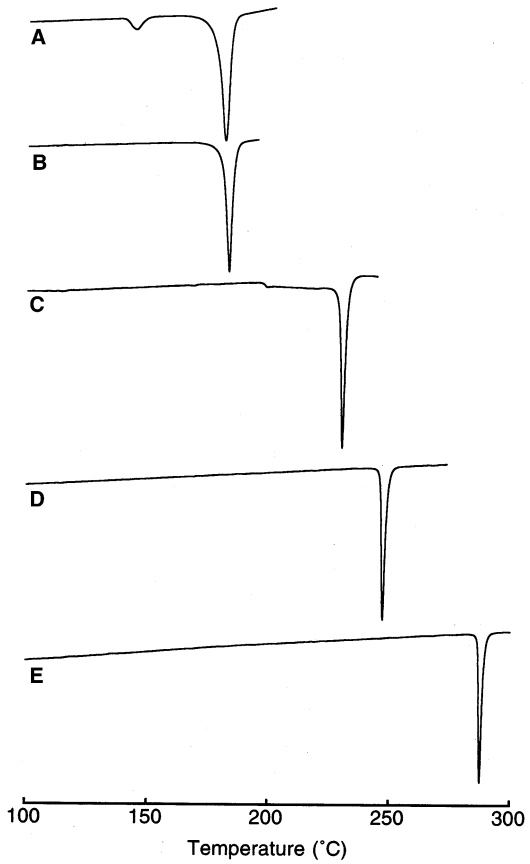

Fig. 2. Differential scanning calorimetry of: (A) retinoic acid as received; (B) rescan of retinoic acid after heating to 165°C; (C) MTTO; (D) TTNPB; and (E) TTNN.

3.2. Aqueous solubility of retinoids

The measured aqueous solubilities are summarized in Table 1. Very little decomposition occurred. The greatest decomposition occurred with retinoic acid, which was less than 0.3% as determined by HPLC. Except for the low melting point form (monoclinic) of retinoic acid, it appears that the system achieved equilibrium after 24 h, since there was no significant difference between the solubility values obtained at equilibration times of 24 and 48 h. The solubility of retinoic acid equilibrated with the high melting point of solid (triclinic) was found to be 1.20 ± 0.06 μM. This value is significantly less than the 1.9 ± 0.04 μM observed at 24 h with the lower melting point solid. At 48 h, the concentration of the latter had fallen to 1.29 ± 0.13 μM, which was comparable to the high melting point equilibrated concentration. The melting point of the solid remaining in the test tube after the solubility measurement was also measured by DSC, and no changes were detected. Overall, the solubility increased as the number of rings in the structure increased from 1.2 for RA to 5.7 μM for TTNN.

The solubility of each retinoid in the diluted ethanol solution was also estimated from plots of the absorbance as a function of added retinoid concentration by determining the intersection of the rising regression line of the absorbance as a function of concentration with the horizontal line where the absorbance reached a plateau. Above 3 μM, time dependent precipitation of retinoic acid was observed. For the arotinoids, the remaining values obtained from ethanol solutions were much closer to values obtained by equilibrium measurements (Table 1).

3.3. Absorption spectra

The absorption spectra obtained from aqueous solutions at low and high pH are given in Fig. 3A–D. The results for retinoic acid are in good agreement with previous published spectra, where peaks were observed at 340 nm at high pH and 380 nm at low pH (Noy, 1992a). The spectral change with pH was reversible with titration and did not undergo further changes with additional acid or base. Analogous changes were observed with the three other retinoids. The wavelengths of maximum absorbances, λ_{max}, are given in Table 2 along with those obtained from ethanol solutions. In each case, the λ_{max} of the non-ionized form was higher than the λ_{max} of the ionized form. The λ_{max} obtained from an ethanol solution always fell between that obtained at high and low pH. Finally, there was no significant effect of ionic strength on the λ_{max} (data not shown).

3.4. Apparent acid dissociation constants

The results from the determination of the absorbance ratio as a function of pH are given in Fig. 4A–D. Each plot represents three or four

Table 1
Properties of retinoic acid, MTTO, TTNPB, and TTNN

Property	RA Monoclinic	RA Triclinic	MTTO	TTNPB	TTNN
Melting point (°C)	145.23 ± 0.64[a]	181.46 ± 0.52[a]	229.02[b]	246.8[b]	287.6[b]
Enthalpy change (J/g)	8.95 ± 0.56[a]	85.2 ± 5.3[a]	94.3[b]	107.7[b]	140.6[b]
Cs[c] at 24 h (μM)[a]	1.90 ± 0.04	1.20 ± 0.14	1.25 ± 0.03	3.19 ± 0.15	5.42 ± 0.60
Cs at 48 h (μM)[a]	1.29 ± 0.13	1.20 ± 0.06	1.24 ± 0.03	3.31 ± 0.42	5.70 ± 0.53
Ethanol ppt value (μM)[a]	2.22 ± 0.15		1.34 ± 0.08	4.26 ± 0.09	5.58 ± 0.12
Cs reported by Noy (μM)	≈ 3				
Cs reported by Szuts (μM)	0.21 ± 0.02				

[a] Mean $\pm$ S.D. with $n = 3$.
[b] Represents the mean of two determinations.
[c] Aqueous solubility.

independent titrations. In Fig. 4A, the titration results for retinoic acid are given. At low pH, there is a plateau in the absorbance ratio of A_{380}/A_{340} with a value of about 1.5. At a pH above 4, the ratio falls in a sigmoidal fashion with another plateau evident at pH values above 8. At high pH, the absorbance ratio has a value of about 0.4. The solid line in the figure represents the titration curve obtained from nonlinear regression to Eq. (1) of the pooled data from the individual titrations.

In the corresponding panels Fig. 4B–D, the experimental results for the titrations of MTTO, TTNPB, and TTNN are given along with the best fit titration curves. For MTTO, the ratio was about 1.4 at low pH and fell to about 0.4 at high pH. TTNPB had a smaller ratio of 1 at low pH and at the high pH, the ratio was about 0.6. For TTNN, the data were more scattered. This is due to the relatively small difference in the absorption spectra of the ionized and non-ionized forms of TTNN.

The observed pK_a values of the retinoids are given in the Table 3. The pK_a values in infinitely dilute solutions of model compounds are also given in the Table. A few values of the pK_a were determined at lower concentrations with the result that smaller values were obtained. No limiting value of the pK_a was observed at lower concentrations. In 150 mM NaCl, MTTO had the highest p$K_{a_{obs}}$ with a value of 7.18. This was followed by TTNN at 6.54, TTNPB at 6.45 and RA had the smallest p$K_{a_{obs}}$ of 6.05. Decreasing the salt concentration from 150 to 5 mM NaCl resulted in higher values of the pK_a. The increase in the p$K_{a_{obs}}$ for retinoic acid was about 0.44, whereas an increase of about 0.73 was obtained for the arotinoids.

3.5. Fluorescence

The fluorescent emission intensities of TTNN determined at low and high pH and at two different ionic strengths are summarized in Table 4. With the same excitation wavelength of 258 nm, the emission peak occurred at 450 nm at low pH but occurred at 420 nm at high pH. Thus, the spectra were blue-shifted with an increase in pH. The shape of the spectra was also changed with pH but not with ionic strength. The intensity at high pH was about ten times larger than that at low pH. At high ionic strength, the intensity was reduced relative to the values obtained at low ionic strength by 10 and 30% at low and high pH, respectively.

The polarization values of TTNN at low and high ionic strength are listed in Table 4. The polarization values at low pH were ten times larger than those at high pH. At high pH, the polarization in 150 mM was twice as that observed in 5 mM NaCl. However, there was no significant effect of ionic strength on the polarization at low pH.

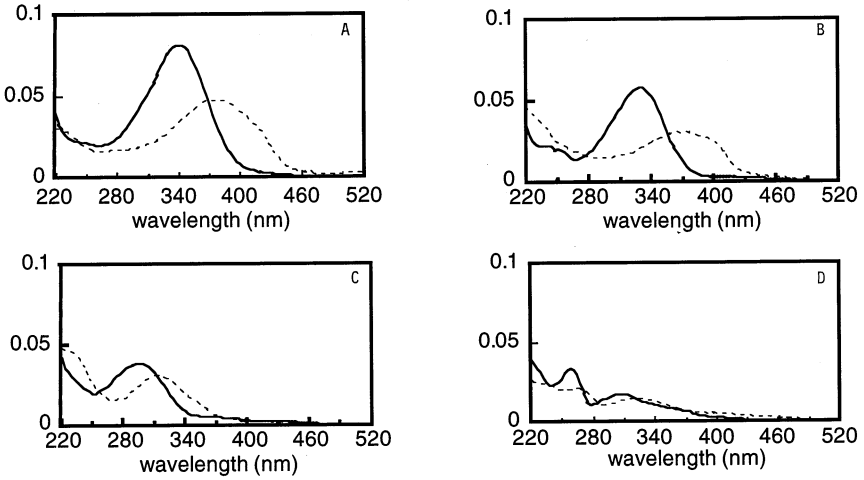

Fig. 3. Absorbance given as a function of wavelength at (- - - - -) low pH and (——) high pH in 0.15 M NaCl for (A) retinoic acid, (B) MTTO, (C) TTNPB, and (D) TTNN.

4. Discussion

Retinoids have interesting possibilities in prevention of cancer; however, they pose a number of difficulties for development as oral dosage forms. In particular, they have very low aqueous solubilities and are prone to chemical decomposition. In this study, retinoic acid, MTTO, TTNN, and TTNPB were examined for the purpose of quantitatively determining the properties of these compounds in solution. They were chosen such that the number of restricting rings would systematically vary, and each would have a carboxylic acid and a cyclohexadiene moiety. These chemical features lead to low water solubility and self association and thus relatively complicated solution behavior. Understanding this complicated solution behavior is the first step in characterizing their oral bioavailability, since the solution behavior must be considered in determining the fundamental driving force for absorption as well as for biological activity.

Table 2
Wavelength (nm) of maximum absorbances

Solvent	RA	MTTO	TTNPB	TTNN
Ethanol	355	342	301	261, 308
Low pH	380	370	309	261, 322
High pH	340	325	286	258, 296

The solid state properties of retinoic acid were in good qualitative agreement with Stam and MacGillavry (1963). They found that with crystallization from methanol, monoclinic crystals were obtained. Upon heating, the metastable monoclinic crystals were irreversibly converted to triclinic crystals, and the conversion temperature occurred at 120°C. However, in this study, the solid–solid conversion appeared to occurred between 140 and 145°C. This temperature difference represents a relatively large discrepancy for assignment of the solid–solid transition to the monoclinic to triclinic conversion; however, because the monoclinic form is metastable, the melting temperature may depend on the specifics of the crystallization process.

For the aqueous solubility, a number of the technical problems were circumvented by the judicious experimental conditions. The dialysis method allowed relatively rapid equilibrium and solved the inherent wetting problem of the retinoids. The results from the HPLC confirmed that the equilibration process did not lead to decomposition of the retinoids ($< 1\%$). Therefore, the time dependent solubility of the monoclinic form of retinoic acid most likely reflects the metastability of the solid. Specifically, the drop in

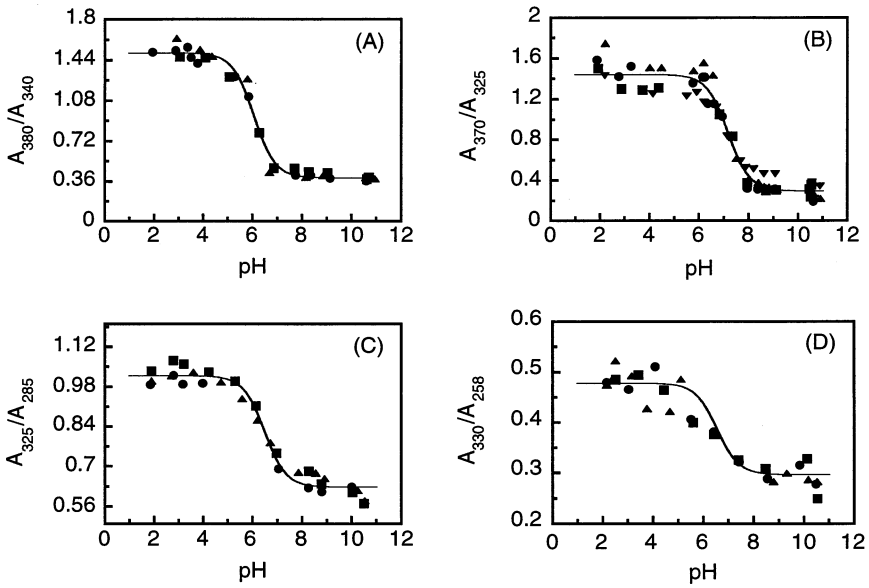

Fig. 4. Absorbance ratio plotted as a function of pH in 0.15 M NaCl for (A) retinoic acid, (B) MTTO, (C) TTNPB, and (D) TTNN. The data were derived from three individual determinations as indicated by the different symbols but the solid line represents the best fit of the pooled data to equation 1.

concentration at 48 h suggests that there is conversion of the solid from the monoclinic to triclinic phase. The absence of changes in the thermal properties of the recovered solid indicates that the monoclinic–triclinic conversion is primarily a surface phenomenon, and the bulk of the solid remains in the monoclinic phase. The triclinic form yielded the lowest value of the solubility consistent with it being the more stable polymorphic form of retinoic acid.

The solubility obtained in this study still exceeds that of Szuts and Harosi (1991) who determined the solubility by equilibration of an aqueous solution with a solid film deposited by ethanol. The low value may be the result of equilibration of yet a more stable solid polymorph or may reflect an inadequate equilibration time which was 30 min. Precipitation from ethanol solution as carried out here and by Noy (1992a) yielded the highest value of RA where metastable solutions may be formed. In contrast to retinoic acid, the solubility values of the arotinoids determined by precipitation were in reasonable agreement with the values determined by equilibrating solid with the aqueous solution. The variability in the thermal properties and observed solubility indicate that a careful characterization of the solid state properties of retinoic acid is necessary when examining the intestinal absorption of solid dosage forms of retinoic acid. This does not appear to be the case for the arotinoids used in this study.

As expected, the melting points and enthalpies of fusion of the retinoids increased with increasing number of restricting benzene rings in the structure (Grant and Higuchi, 1991). A decrease in solubility was also expected with increasing the number of benzene rings, however, the opposite trend was observed (Grant and Higuchi, 1991). This result appears to be the consequence of the aggregation of the retinoid molecules in solution. Moreover, the extent of aggregation may be greater with more benzene rings present in the structure. In the absence of electrostatic repulsion, the exposed hydrophobic area of the molecule is the main driving force for the formation of aggregates in aqueous solution (Mukerjee, 1974). Thus, there is tendency toward larger aggregation numbers with larger hydrophobic moieties as provided by the additional phenyl rings.

Table 3
pK_a values[a]

Solvent	RA[b]	MTTO[c]	TTNPB[d]	TTNN[e]
150 mM NaCl	6.05 ± 0.06	7.18 ± 0.07	6.49 ± 0.08	6.54 ± 0.18
5 mM NaCl	6.49 ± 0.04	7.90 ± 0.06	7.22 ± 0.03	7.29 ± 0.14
Model compound pK_a	4.75	4.75	4.19	4.17

[a] Mean $\pm$ S.D. with $n = 3$.
[b] Concentration was 1.83 μM.
[c] Concentration was 1.41 μM.
[d] Concentration was 1.55 μM.
[e] Concentration was 1.05 μM.

A hypsochromic shift was observed with an increase in pH for each retinoid. This shift was larger than the difference in the λ_{max} observed between aqueous and ethanol solutions. The difference between the aqueous and ethanol solution may be a result of the loss of hydrogen bonding among the retinoids which provides an extended conjugated system. The basis for this lies in the observation that in non-polar solvents, retinoic acid is self-associated through hydrogen bonds, and the addition of hydrogen bonding solvents was shown to disrupt the hydrogen bonding between the retinoids and result in a hypsochromic shift (Chihara and Waddell, 1980; Takemura et al., 1980). In water, the retinoids are also believed to be self-associated largely as a result of the hydrophobic effect, but hydrogen bonding is evidently also an important aspect of the association. With the addition of ethanol, the aggregate is disrupted which breaks the intermolecular hydrogen bonding, and thus a large hypsochromic shift is observed.

As with the difference between water and ethanol, the hypsochromic shift associated with ionization may also be a result of the loss of intermolecular hydrogen bonding. That is, replacing the proton of the carboxylic acid with a sodium ion interferes with the conjugation arising from the hydrogen bonding of the retinoids. However, the effect of aggregation on the spectra shift of RA present at low pH must also be considered. Furthermore, the ionization of the carboxyl group leaves an isolated negative charge which diminishes the electron attracting tendency of the carboxyl group. Thus, replacement of the proton with a sodium ion appears to be largely responsible for the hypsochromic shift observed with increasing pH, although the more subtle effects of the packing environment remain unknown.

In analyzing the values of the λ_{max} for these compounds, the free electron model is useful (Wade Jr., 1995). In this model, an increase in conjugation is predicted to provide better stabilization of the excited state and thereby result in a bathochromic shift. From the λ_{max} values listed in Table 2, it is evident that the addition of sterically restricting benzene rings did not lead to greater conjugation. Nevertheless, an examination of the preferred molecular conformation is particularly revealing (Suzuki, 1959, 1960; Lewin and Carroll, 1981; Strickland et al., 1983).

The X-ray crystal structure of retinoic acid indicates that the molecule is largely planar except for the minor torsional strains brought about by the methyl groups, particularly at the C6–C7–C8 (Stam, 1972). In contrast, the crystal structure of TTNPB places the restricting rings near the polar head group perpendicular to the plane of the remaining molecule (cf Fig. 1 Lewin and Carroll, 1981). In this conformation, there would be very little resonance stabilization of the excited state which is consistent with the lower maximum wavelength (Suzuki, 1959, 1960). MTTO and TTNN also have benzene rings that would be more favorably placed perpendicular to the conjugated polyene chain which relieves the steric repulsion between the methyl groups and protons (Suzuki, 1959, 1960). By this argument, the rank order of the values of the λ_{max} are consistent with the free electron theory. That is, retinoic acid is a

Table 4
Fluorescent intensity and polarization values of TTNN[a]

Saline solvent (mM)	Intensity $\times 10^{-4}$		Polarization	
	Low pH	High pH	Low pH	High pH
5	4.39 ± 0.02	39.2 ± 0.04	0.194 ± 0.009	0.011 ± 0.007
150	4.68 ± 0.02	31.5 ± 0.03	0.198 ± 0.004	0.023 ± 0.010

[a] Mean $\pm$ S.D. with $n = 3$.

linear polyene of five double bonds, MTTO is a polyene consisting of four double bonds, TTNPB has styrene as its chromophore, and TTNN has naphthoic acid as its chromophore.

Prior to measuring the apparent acid dissociation constants, the stability of the retinoids to irradiation by ultraviolet light in aqueous solution was determined by monitoring the absorbance as a function of time at low and high pH. While a decrease in absorbance was evident after four hours, the change in absorbance could be considered negligible ($< 1\%$) in the analysis of the ionization behavior.

As first noted by Noy (1992a), the value of the pK_a of retinoic acid is much higher than expected. From the well-known dissociation behavior of carboxylic acids, the expected pK_a would be between 4 and 5. The explanation provided for the discrepancy was that the retinoic acid self-associates in aqueous solution (Noy, 1992a,b). The shift in the pK_a was postulated to be a result of the partial shielding of the carboxyl group from the bulk aqueous phase by the less polar environment of the aggregate (Noy, 1992a). Our results for RA are in complete agreement. Moreover, the results from the remaining compounds suggest that self-association is a general phenomenon of all retinoids. These results also corroborate an earlier study where self-association of non-ionizable retinoids was suggested (Li et al., 1966).

While self-association has been established, several features of the process deserve comment. First, the concentrations at which the experiments were performed were less than 2 μM. This is a relatively low concentration for self-association. However, the retinoids have in excess of 26 carbon atoms with the carboxylic acid moiety as the sole polar functional group. Another aspect of the experimental data is the remarkably good fit of the retinoids to the relatively simple, Henderson–Hasselbalch relationship. Implicit in the fit are the assumptions that a single moiety undergoes ionization determined by a unique ionization constant and this ionization constant is independent of pH. In addition, the absorptivities of the ionized and non-ionized species at the chosen wavelengths are independent of the pH. However, these assumptions are not expected to be valid (Fernandez and Fromherz, 1977).

Specifically, as the pH is increased near the pK_a, the retinoids must necessarily become ionized. With ionization, the retinoid will create a negative charge at the surface of the aggregate (Fernandez and Fromherz, 1977; Drummond et al., 1986, 1989). The presence of a negative charge will create a proton activity gradient from the bulk to the surface of the aggregate. This increase in the proton activity at the surface of aggregate will reduce the likelihood of the ionization of the remaining retinoid molecules in the aggregate. The overall effect is that the titration curve does not have a transition which is as sharp as predicted by the Henderson–Hasselbalch relation. In fact, the curve should be broadened, and the observed midpoint or effective pK_a be higher than what would be observed in the absence of self-association. Nevertheless, there was good fit of the data to Eq. (1) for all the retinoids which suggests a more complicated situation.

To further assess the nature of the aggregation, the three factors contributing to the $pK_{a_{obs}}$ are considered in the following equation (Fernandez and Fromherz, 1977; Drummond et al., 1986, 1989):

$$pK_{a_{obs}} = pK_{a_{aq}} + f(\varepsilon) - F\Psi/2.3RT \quad (3)$$

The first term is the $pK_{a_{aq}}$ which is the negative logarithm of the ionization constant of the retinoid present in an infinitely dilute aqueous solution. The $pK_{a_{aq}}$ is dependent only on the chemical structure and its ability to stabilize the negative charge in the molecule in an aqueous solution at a specified temperature and pressure. The second contribution, f(ε), is dependent on the polarity or effective dielectric constant of the environment of the carboxylic acid group. The third term is the electrostatic contribution which accounts for the surface charge alluded to above.

To estimate the $pK_{a_{aq}}$, the $pK_{a_{obs}}$ was measured at lower concentrations. However, as Noy found with retinoic acid (Noy, 1992a), the ionization constant of the retinoids can not be measured in a sufficiently dilute aqueous solution where aggregation does not occur. While these results prevent a direct analysis of the ionization, they do suggest that the nature of the aggregate changes with concentration, which is consistent with a polydispersed distribution. The planar portions of the retinoids may lead to a stacked arrangement in solution as has been found with dyes which also form polydispersed distributions of aggregates (Mukerjee, 1974).

Since the $pK_{a_{aq}}$ of the retinoids could not be experimentally obtained, the values of the pK_a of model compounds were used for comparison. Retinoic acid was postulated to have a planar polyene chain which forms a conjugated system. The values of the pK_a for 2-butenoic acid and 2,4-hexadienoic acid are 4.70 and 4.76 in aqueous solution at 25°C. These are similar to acetic acid and suggest there is only a small inductive effect from the long chains. MTTO will be similar, since its carboxylic acid is also attached to a polyene chain. For TTNPB, the carboxylic acid is attached to a benzene ring. The choice for a model compound becomes benzoic acid which has a pK_a of 4.19. TTNN has its carboxylic acid attached to a naphthylene ring, and the pK_a of 2-naphthoic acid is 4.17. With these choices, the difference between the $pK_{a_{obs}}$ and the $pK_{a_{aq}}$ can be attributed to the effect of electrostatic surface charge and polarity.

To further explore the electrostatic contribution, the pK_a was measured in a solution with a sodium chloride concentration of 5 mM. Comparing the values of the pK_a in 5 and 150 mM NaCl, an increase in the pK_a with a decrease in ionic strength was observed. The direction of the change is consistent with a decrease in the shielding of the surface charge of the aggregate. The change is much larger than that expected based on the Debye–Huckel equation for an isolated carboxylic acid in aqueous solution. However, increasing the salt concentration may also have modified the interaction of water with the hydrophobic groups of the retinoids. This may have caused an alteration in the structure of the aggregate which accounts for the change in the observed pK_a.

To differentiate the electrostatic shielding from a change in the hydrophobic interactions, the influence of the pH and ionic strength on the polarization was determined. Polarization provides a measure of the average rotation of a molecule during the lifetime of the excited state of fluorescence. With the assumptions that there was no change in the lifetime and the rate of rotation is dependent on the size of the aggregate, the polarization values may be used to infer a change in the size of the aggregate.

The polarization of TTNN at low pH was higher than that at high pH. This observation supports the hypothesis that the size of the retinoid aggregate decreases as the pH is increased. At high pH, the estimated size of the aggregate at high ionic strength was about 50% larger than that at low ionic strength. The additional salt would screen the negative charges and thereby allow the formation of a larger aggregate. The polarization of the aggregate at low pH was insensitive to ionic strength. The absence of a change in the polarization at low pH with an increase in salt concentration suggests that the electrostatic shielding is the major cause of the pK_a change and there is little effect of the salt on the aggregate size.

In a closer examination of the change in the pK_a with salt, the arotinoids differ from retinoic acid. For retinoic acid, the increase in the pK_a with ionic strength was 0.44 whereas for the re-

maining retinoids, the increase was between 0.72 and 0.75. This suggests that arotinoids form aggregates which are distinct from retinoic acid. Since the salt concentration largely swamps out the charge effect, the difference in the observed pK_a's may indicate that the three arotinoids form aggregates with a larger surface charge than retinoic acid. The larger surface charge could be a result of a difference in the geometry of packing or simply be a result of the formation of aggregates with a larger aggregation number as suggested by the solubility measurements.

The effective polarity was assessed with the values of the $pK_{a_{aq}}$ of the model compounds. The difference between the values of the $pK_{a_{obs}}$ in 150 mM NaCl and the values of pK_a of model compounds are 1.25 for retinoic acid and 2.43, 2.3, and 2.37 for MTTO, TTNPB, and TTNN, respectively. The substantially smaller value observed for retinoic acid in comparison to the three derivatives adds further support to the suggestion that RA exhibits distinct aggregation behavior. Moreover, the similarity of the values for the three derivatives as found above may also indicate that they form similar types of aggregates. The larger difference between the measured pK_a and model compound pK_a would correspond to a less polar environment for the three derivatives in addition to a larger surface charge. This also represents evidence that the arotinoids form larger aggregates than retinoic acid.

Finally, the good fit of the data to the Henderson–Hasselbalch equation observed even at the low ionic strength deserves comment. The absence of any electrostatic broadening in the titration curve is probably due to the dependence of the aggregation number on pH. That is, as the retinoid molecules become ionized, they leave the aggregate, and there is little or no contribution to the surface charge.

In summary, the similarities among retinoic acid and the three derivatives are that they all undergo self-association in aqueous solution with the primarily driving force of the hydrophobic effect modulated by intermolecular hydrogen bonding. The aggregation affects the ionization behavior, and although the Henderson–Hasselbach relationship is followed, this is most likely a consequence of a decrease in the aggregation number with ionization. The observed pK_a for these compounds are more than two units higher than expected and are sensitive to concentration and salt concentration.

The differences between retinoic acid and the arotinoids are evident in the solid state properties where retinoic acid may exist as a triclinic and monoclinic state. This, in turn, influences the observed amount of retinoic acid in solution. In addition, the nature of the aggregation for retinoic acid appears distinct from that of MTTO, TTNPB, and TTNN possibly due to the formation of smaller aggregates. The formation of aggregates in aqueous solution complicates the experimental investigation into the intestinal absorption of the retinoids especially for retinoic acid which displays additional time dependent properties (Regazzi et al., 1997).

Acknowledgements

Financial support was provided by PHS RO1 CA55493 and by University of Minnesota ISWOP. We acknowledge Professor Zimmerman whose interest in the intestinal absorption of retinoids led to this work. We are also grateful for the use of her equipment, guidance in the HPLC analysis of the retinoids and review of this manuscript.

References

Albert, A., Serjeant, E.P., 1971. In: The Determination of Ionization constants. Chapman and Hall, London, pp 44–60.

Canter, C.R., Schimmel, P.R., 1980. Biophysical Chemistry. Part II: Techniques for the study of biological structure and function. W.H. Freeman, San Francisco, chapters 7–8.

Chihara, K., Waddell, W.H., 1980. Electronic and vibrational spectral investigation of the molecular association of the all-trans isomers of retinal, retinol, and retinoic acid. J. Am. Chem. Soc. 102, 2963–2968.

Drummond, C.J., Grieser, F., Healy, T.W., 1986. A single spectroscopic probe for the determination of both the interfacial solvent properties and electrostatic surface potential of model lipid membranes. Faraday Discuss. Chem. Soc. 1 (81), 95–106.

Drummond, C.J., Grieser, F., Healy, T.W., 1989. Acid–base equilibria in aqueous micellar solutions. J. Chem. Soc. Faraday Trans. 1 (85), 521–535.

Fernandez, M.S., Fromherz, P., 1977. Lipoid pH indicators as probes of electrical potential and polarity in micelles. J. Phys. Chem. 81, 1755–1761.

Grant, D.W.J., Higuchi, T., 1991. Solubility behavior of organic compounds. In: Saunders Jr., W.H., Weissberger, A. (Eds.), Techniques of Chemistry, vol 21. Wiley, New York, pp. 12–88.

Han, C.H., Zimmerman, C.L., Wiedmann, T.S., 1998. Spectral properties and ionization behavior of retinods. II. Bile salt solutions. Int. J. Pharm. 172, 229–240.

Lakowicz, J.R., 1986. Principles of Fluorescence Spectroscopy. Plenum Press, New York, chapter 8.

Lewin, A.H., Carroll, F.I., 1981. A novel conformation of retinoids. J. Am. Chem. Soc. 103, 6527–6529.

Li, C.Y., Zimmerman, C.L., Wiedmann, T.S., 1966. Solubilization of retinoids by bile salt/phospholipids aggregates. Pharm. Res. 13, 907–913.

Mukerjee, P., 1974. Micellar properties of drugs: micellar and nonmicellar patterns of self-association of hydropohobic solutes of different molecular structures-monomer fraction, availability, and misuses of micellar hypothesis. J. Pharm. Sci. 63, 972–981.

Noy, N., 1992a. The ionization behavior of retinoic acid in aqueous environment and bound to albumin. Biochem. Biophys. Acta 1106, 151–158.

Noy, N., 1992b. The ionization behavior of retinoic acid in lipid bilayers and in membranes. Biochem. Biophys. Acta 1106, 159–164.

Regazzi, M.B., Iacona, I., Gervasutti, C., Lazzarino, M., Toma, S., 1997. Clinical pharmacokinetics of tretinoin. Clin. Pharmacokinet. 32, 382–402.

Stam, C.H., MacGillavry, C.H., 1963. The crystal structure of the triclinic modification of vitamin-A acid. Acta Cryst. 16, 62–67.

Stam, C.H., 1972. The crystal structure of a monoclinic modification and the refinement of a triclinic modification of vitamin-A acid (Retinoic acid), $C_{20}H_{28}O_2$. Acta Cryst. B28, 2936–2945.

Strickland, S., Breitman, T.R., Frickel, F., Nurrenbach, A., Hadicke, E., Sporn, M.B., 1983. Structure–activity relationships of a new series of retinoidal benzoic acid derivatives as measured by induction of differentiation of murine F9 teratocarcinoma cells and human HL-60 promyelocytic leukemia cells. Cancer Res. 43, 5268–5272.

Suzuki, H., 1959. Relations between electronic absorption spectra and spatial configurations of conjugated systems, II: *O*-alkyl- and o,o′-dialkylbiphenyls. Bull. Chem. Soc. Japan 32, 1350–1356.

Suzuki, H., 1960. Relations between electronic absorption spectra and spatial configurations of conjugated systems, VII: a substituted and a,a′-disubstituted stilbenes. Bull. Chem. Soc. Japan 33, 396–405.

Szuts, E.Z., Harosi, F.I., 1991. Solublity of retinoids in water. Arch. Biochem. Biophys. 287, 297–304.

Takemura, T., Chihara, K., Becker, R.S., Das, P.K., Hug, G.L., 1980. Visual pigments: II, spectroscopy and photophysics of retinoic acids and all-*trans* methyl retinoate. J. Am. Chem. Soc. 102, 2604–2609.

Thongnopnua, P., Zimmerman, C.L., 1988. Simultaneous microassay for etretinate and its active metabolite etretin by reversed-phase high-performance liquid chromatogaphy. J. Chromatogr. 433, 345–351.

Wade Jr., L.G., 1995. Conjugated systems, orbital symmetry, and ultraviolet spectroscopy. In: Organic Chemistry, 3rd ed. Prentice-Hall, Englewood Cliffs, NJ, chapter 15.

Weigand, U.W., Busslinger, A.A., Chou, R.C., Jensen, B.K., 1993. In: Livrea, M.A., Packer, L. (Eds.), Retinoids: Progress in Research and clinical applications. Marcel Dekker, New York.