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Physicochemical characteristics of ferric adriamycin complexes

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Summary. *Ferric ions and adriamycin in solution interact to form complexes that can yield colloidal and flocculant mixtures. At high concentrations ($Fe^{3+} \geq 10^{-4}$ M, adriamycin $\geq 10^{-5}$ M) an absorption appears at 600 nm, indicating colloid formation, which is directly responsive to concentrations of the reactants. Evidence from dilution experiments by spectral analysis, ultracentrifugation, titration, and filtration indicate that phase transition that is sensitive to pH and time occurs with iron-adriamycin complexes to yield flocculated drug. We conclude that patients and animals treated wtih the iron-adriamycin preparations known as 'quelamycin' received flocculated iron-adriamycin, which accounts for the toxic and pharmacologic effects reported. It may be useful to utilize colloidal preparations of reactive or irritating drugs to avert acute toxic effects and to produce slower release of active drug.*

Introduction

The quinone polycyclic anthracycline antibiotics interact with multivalent cations such as calcium, magnesium, aluminium, and iron. Calendi et al. [4] studied the reactions of cations with daunorubicin and demonstrated alterations in the anthracycline absorption spectrum whereas Yesair et al. [13] described effects of cations on daunorubicin metabolism in vitro. Other types of quinone compounds have been investigated and used as model compounds for their interaction with cationic agents [12].

The clinical use of iron-anthracycline antibiotic combinations is controversial but has been pursued by Gosalvez as a means of alleviating the cardiotoxicity and other toxicities of anthracycline antibiotics [3]. Despite the claims concerning 'quelamycin' [6], recent analytical studies have indicated that ferric chloride and adriamycin actually form complexes unlike that proposed by Gosalvez [8, 9]. Because of our pharmacology data [5, 10], we felt that the iron-adriamycin preparations were subject to physicochemical alterations not considered by other investigators. We have investigated this problem and describe our results in this and other reports [5].

Materials and methods

Adriamycin (A) was provided by the Natural Products Branch, DCT, NCI; and ferric chloride ($FeCl_3 \cdot 6\ H_2O$) was obtained from Fisher Scientific Company (Silver Spring, MD). Solutions were made freshly in 18 megohm deionized water.

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Ultrafiltration. Solutions of A (5 m*M*) were made; and $FeCl_3$ was added from a concentrated solution to give molar ratios of 1 : 1 and 3 : 1 ($FeCl_3$: A). The solutions were kept at room temperature for varying periods of time and then 10 µl was filtered on a Millipore HA filter (0.45 µm, 47 mm diameter) in an all-glass filter apparatus attached to a vacuum flask. The filter was washed with 2 ml water and then placed in 2 ml acid alcohol (75% ethanol, 0.1 *M* HCl) to extract drug complex [1]. After 10 min, the acid alcohol extract was transferred to a fluorescence cuvette and the fluorescence was measured with an Aminco SPF-125 spectrofluorometer (excitation 470 nm, emission 585 nm).

Sedimentation. Solutions of A (5 m*M*) were mixed with sufficient $FeCl_3$ solution to give molar ratios of 1 : 2, 1 : 1, 2 : 1, and 5 : 1 ($FeCl_3$: A). The pH of the mixtures was adjusted with NaOH to between 6.0 and 7.0. Each preparation was lyophilized and reconstituted to the original volume in water. The reconstituted lyophilizates were centrifuged in a Beckman Airfuge for 30 min at 100,000 *g*. The supernatant solutions were saved, and the sedimented material was resuspended in water (150 µl) and centrifuged again in the Airfuge. The supernatant solutions were saved, and the sediments were dissolved in 150 µl/0.1 *M* HCl. The three samples (supernatant 1, supernatant 2, and final sediment) were analyzed for A (by fluorescence) and ferric ion (by 1,10 phenanthroline assay) [7]. We found that A interferes with the ferric determination by 1,10-phenanthroline, but A is easily removed selectively from the assay solutions by initial adsorption to XAD-2 resin beads. Since the XAD-2 beads bind A but not ferric ion, a single mixing of about 100 mg XAD-2 beads in the aqueous assay solution followed by sedimentation was sufficient to remove A. The supernatant solutions were then assayed for ferric ion.

Spectral methods and light scattering. For all spectral measurements a Cary 14 or Cary 118 spectrophotometer with tungsten light source was used. Light scattering measurements were made with the Cary 118 Spectrophotometer from 350 nm to 450 nm, the spectral region where $FeCl_3$ and A absorption are minimal. Aqueous solutions of $FeCl_3$ and A were mixed together prior to absorbance measurements. Concentrations of A ranged from 10^{-3} *M* to 10^{-6} *M*, and $FeCl_3$ concentrations ranged from 10^{-4} *M* to 10^{-8} *M*.

Results

In our initial experiments with iron-adriamycin complexes we noted, as was described originally by Calendi et al. [4], that

ferric chloride dramatically altered the color of the adriamycin solutions when the two were mixed. The adriamycin solution changes from orange-red to purple-black, depending on reactant concentrations. We titrated adriamycin solutions with ferric chloride, theorizing that at the appropriate stoichiometric ratio of ferric ions to adriamycin we would see precise spectral alterations. At iron : adriamycin ratios of 1 : 10 and concentrations of iron as low as 10^{-8} *M* we saw no changes in the adriamycin absorption spectrum. We obtained incongruous spectral changes; and it appeared that several factors influenced this reaction. Neither (1 : 1) nor (3 : 1) $FeCl_3$: A mixtures produced the kind of spectral changes expected for the formation of a distinct complex. However, as we increased the ferric chloride concentration to 10^{-3} *M*, a new absorption shoulder appeared at about 600 nm (Fig. 1). It also appeared that a phase transition was occurring at a high (> 10^{-4} *M*) concentration of the $FeCl_3$ and that the absorption at 600 nm was related to complex formation.

To increase our understanding of these spectral changes, we obtained absorption spectra of equal quantities of reactants at differing molarities. By manipulating the light path distance, it is possible to observe the effects of concentration without changing the number of molecules intercepting the light beam. The light path length and concentration of $FeCl_3$: A mixture were varied inversely, and the resulting spectra were dependent on the concentration of the solutes (Fig. 2). The absorption spectrum associated with the dilute 10^{-5} *M* ($FeCl_3$: A × 10 cm) is identical with that of control adriamycin in aqueous solutions, and there is no indication of a 'complex absorption'. As the concentration of $FeCl_3$: A (3 : 1) mixture increased, the complex absorption at 600 nm increased. The color of the solution changed from orange (10^{-5} *M* A and 3 × 10^{-5} *M* $FeCl_3$) to purple-black (10^{-3} *M* A and 3 × 10^{-3} *M* $FeCl_3$). The spectral changes associated with the increase in reactant concentration and the visible color change show a decrease of the A chromophore absorption at 470 nm even though the amount of A in the solutions is unchanged. As the

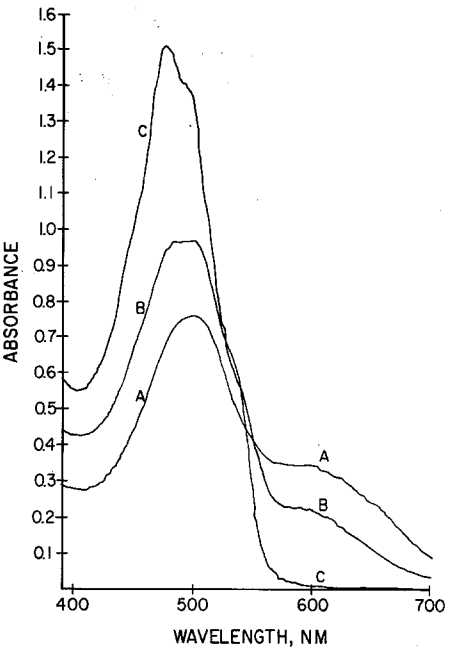

Fig. 2. Absorption spectra of equal quantities of adriamycin ferric chloride at differing molarities. The Cary 14 double-beam spectrophotometer was used with the tungsten light source. A stock solution of $FeCl_3$: A (3 : 1) was made in 18 megohm water. The concentration of A was 10^{-3} *M* and the concentration of $FeCl_3$ was 3 × 10^{-3} *M*. A spectrum (390–700 nm) of this solution was taken in a 1-mm path length cuvette (*Trace A*). This solution was diluted 1 : 10 (concentration of A was then 10^{-4} *M* and that of $FeCl_3$ was 3 × 10^{-4} *M*), and a spectrum was taken in a 1-cm path length cuvette (*Trace B*). The solution was diluted again 1 : 10 (concentration of A was 10^{-5} *M* and that of $FeCl_3$ was 3 × 10^{-5} *M*), and a spectrum was taken in a 10-cm path length cuvette (*Trace C*). All spectra were taken with the appropriate concentration of $FeCl_3$ in the reference cell of the same length as the experimental cell

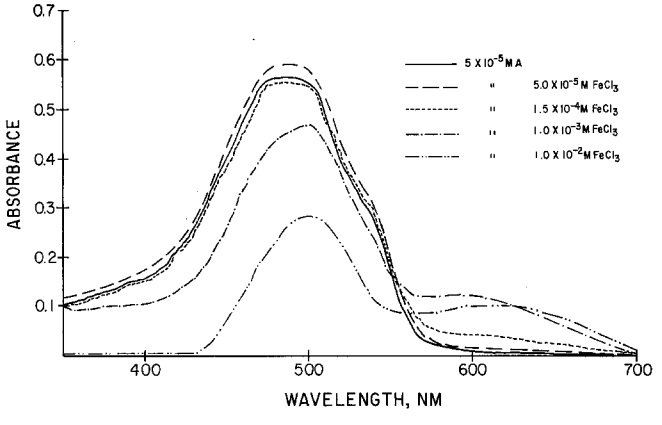

Fig. 1. Absorption spectral changes during adriamycin ferric chloride titration. The Cary 14 double-beam spectrophotometer was used with the tungsten light source. One-centimeter path length cuvettes were used. Stock solutions (2×) of A and $FeCl_3$ were prepared in 18 megohm water and mixed together to give the required concentrations. Each spectrum (350–700 nm) was recorded with the appropriate concentration of $FeCl_3$ in the reference beam

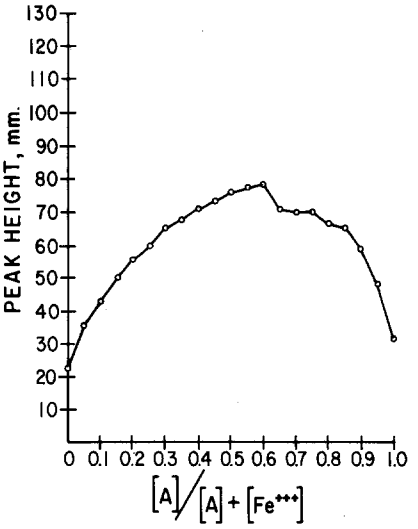

Fig. 3. Spectral changes during continuous variation method. The Cary 118 double-beam spectrophotometer was used with the tungsten light source. Solutions of A (5 × 10^{-4} *M*) and $FeCl_3$ (5 × 10^{-4} *M*) were made in 18 megohm water and were mixed to achieve the desired ratios. The spectrum of each mixture was recorded between 580 nm and 650 nm. The maximum absorbance was at nm. Peak heights at 600 nm were measured for each mixture to determine maximal complexation. All spectra were taken with the appropriate concentration of $FeCl_3$ in the reference cell

A chromophore absorption decreases, a new absorption appears at 600 nm.

We investigated the spectral changes further by the continuous variation method (12), titrating A versus $FeCl_3$ solutions (Fig. 3). Maximal absorption occurs at the A/A + Fe^{3+} ratio of 0.6 which corresponds to an $FeCl_3$: A ratio of 2 : 3. It is also evident that the absorbance of complex increases more rapidly with increasing relative amounts of iron than with increasing relative amounts of adriamycin. This observation indicates that iron serves as a multidentate ligand. There is no evidence for a distinct (3 : 1) $FeCl_3$: A stoichiometry.

While working with $FeCl_3$: A mixtures, we noted that a black precipitate formed after several minutes. This was especially evident at high (m*M*) concentrations of $FeCl_3$: A which were the same concentrations used to prepare clinical $FeCl_3$: A (quelamycin). The quantity of precipitate also increased at higher pH values. Whereas $FeCl_3$: A (3 : 1) solution has a pH value of 2, when this solution was alkalinized with NaOH to pH 6 or 7 for biological compatability the amount of precipitate increased greatly.

We attempted to isolate the precipitate by ultrafiltration for chemical analysis and fluorescence quantification of adriamycin content. $FeCl_3$: A mixtures were placed on cellulose nitrate filters (0.45 μm), yielding a black residue, the filters were rinsed with distilled water, and the remaining material on the filter was extracted and assayed for adriamycin. The results indicated that only a small quantity of $FeCl_3$: A remained on the filter (less then 2%). We concluded, since the residue readily dissolved when washed with water, the $FeCl_3$: A complex was readily dissociable.

Since the filtration method was unsatisfactory, we tried sedimentation for separation and isolation. Without pH adjustment, 100,000 *g* centrifugal force did not sediment the black precipitate from a 3 : 1 mixture of $FeCl_3$: A. With pH adjustment of the $FeCl_3$: A (3 : 1) solution to pH 6 or 7, the precipitate sedimented at 100,000 *g*. Again, efforts to wash the precipitate with water caused the material to dissolve readily. The centrifugal forces that we found necessary to sediment these mixtures indicated a material with colloidal properties.

Light-scattering experiments were conducted to determine the parameters of colloid formation in the $FeCl_3$: A mixtures. However, even at concentrations of A (10^{-6} *M*) and $FeCl_3$ (10^{-8} *M*) where colloid formation was not visibly evident, we saw evidence of light scattering. Since other anions in the reaction medium (e.g. hydroxyl ion, carbonate ion) may be participating in reactions involving Fe^{3+} or A, these may be interacting to contribute to the light-scattering changes we see at very low concentrations of A and $FeCl_3$.

Discussion

From our data and from data produced by Dr May and his collaborators [8], we believe that the anthracycline-cation complexation process is not a simple reaction. The iron-adriamycin complexation does take place, but it is not a complex characterized by a 3 Fe to 1A stoichiometry. Our analysis suggests multiple interactions where ferric ion complexes with not only adriamycin but with other anionic species in the medium, all of which vary according to the pH and the ionic strengths of the medium. In time, and under the correct conditions, complexes grow until phase transition, colloid formation, and flocculation become visibly evident. In that regard it is useful to consider the electrolytic equlibria involved.

Chemistry of aqueous solutions of ferric ion

Complexation studies in aqueous solution involving ferric ions are confounded by the numerous forms in which iron may be present. In general, a ferric ion complex will be octahedral (Fig. 4) [11].

If the source of ferric ion is the salt, ferric chloride hexahydrate, a strong electrolyte whose empirical formula may be written as $Fe(H_2O)_4Cl_2{}^+Cl^- \cdot 2\,H_2O$, the following equilibria will be established subsequent to dissolution of the salt:

Yellow *Colorless*

$$Fe(H_2O)_4Cl_2^+ + 2\,H_2O \rightleftharpoons Fe(H_2O)_6^{+3} + 2\,Cl^- \qquad \text{(Eq. 1)}$$

The hydrated ferric chloride ion, $Fe\,(H_2O)_4Cl_2{}^+$, confers a characteristic yellow color to the solution while the hydrated ferric ion, $Fe(H_2O)_6^{+3}$, is colorless. Dilution of the solution favors the right-hand side of Eq. (1), and is associated with a loss of color. If the $Fe(H_2O)_6^{+3}$ were stable, this solution would be neutral. However, the aquocomplex $Fe(H_2O)_6^{+3}$ is acidic (our solutions are approximately pH 2) [2]:

$$Fe(H_2O)_6^{+3} \rightleftharpoons Fe(H_2O)_5OH^{+2} + H^+ \qquad \text{(Eq. 2)}$$

The hydrolysis present in Eq. (2) is responsible for the acidic nature of the ferric salt. Also, the hydroxy complex, $Fe(H_2O)_5OH^{+5}$, is responsible for the yellow-brown color so characteristic of ferric solutions.

Actually, a dihydroxy complex can form and the entire acid base equilibria are [2]:

$$Fe(H_2O)_6^{+3} \rightleftharpoons Fe(H_2O)_5OH^{+2} + H^+ \qquad pK_1 = 2.2 \quad \text{(Eq. 3)}$$

$$Fe(H_2O)_5OH^{+2} \rightleftharpoons Fe(H_2O)_4(OH)_2^+ + H^+ \quad pK_2 = 3.3 \quad \text{(Eq. 4)}$$

Another characteristic of the hydroxy complex is its propensity toward olation: splitting out water between hydroxo groups to form a polynuclear complex [2]. This is exemplified by the formation of ferric oxide:

$$Fe(H_2O)_5OH^{+2} + OH^- \rightarrow Fe(H_2O)_4(OH)_2^+ + H_2O \qquad \text{(Eq. 5)}$$

$$Fe(H_2O)_4(OH)_2^+ + OH^- \rightarrow Fe(H_2O)_3(OH)_3 + H_2O \qquad \text{(Eq. 6)}$$

ferric hydroxide

$$2\,Fe(H_2O)_3(OH)_3 \rightarrow Fe_2O_3(H_2O_2)\downarrow + 7\,H_2O \qquad \text{(Eq. 7)}$$

hydrated ferric oxide (black)

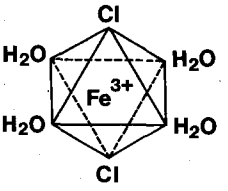

Fig. 4. Hydrated ferric chloride

Dilute solutions and low pH favor the colorless $Fe(H_2O)_6^{+3}$ ion. The presence of a yellow color in dilute solution indicates the presence of $Fe(H_2O)_5OH^{+2}$ or $Fe(H_2O)_4(OH)_2^{+}$. Since the pK_1 in Eq. (4) is approximately 2, significant amounts of hydroxy compounds may be present in quite acidic solutions.

Adriamycin equilibria

Adriamycin is amphoteric with 3 pKa values [8]:

$$AH_3^+ \overset{K_1}{\rightleftharpoons} AH_2^0 + H^+ \qquad pK_1 = 2.1$$

$$AH_2^0 \overset{K_2}{\rightleftharpoons} AH^- + H^+ \qquad pK = 8.1$$

$$AH^- \overset{K_3}{\rightleftharpoons} A^= + H^+ \qquad pK_3 = 11.2$$

At acidic pH, the species present which are capable of complexing with iron are AH_3^+, AH_2^0, and AH^-.

Complexation

Resolution of the stoichiometry of an iron-adriamycin complex and the effect of pH upon that complex clearly depends upon a recognition of the participating species.

Between pH 3 and 7, the following species predominate in dilute solution:

Iron species	*adriamycin*
$Fe(H_2O)_5OH^{2+}$	AH_2^0
$Fe(H_2O)_4OH_2^+$	AH^-

Based upon the species present, it is unreasonable to postulate an iron : adriamycin complex in a ratio of 3 : 1 as proposed by Gosalvez [6]. The protonated amino group repels the positively charged iron. A ratio of 1 : 1 is more likely, according to our data here and our biological experiments [5]. Also several hydrolytic processes associated with aquated ferric ion lead to a complicated *time-dependent* set of chemical species. This is particularly true as the pH is increased.

Perhaps the simplest postulate for the ion-adriamycin interactions we observe is an initial complex formation of one iron molecule to one adriamycin molecule (Fig. 5). These Fe : A complexes may then interact with one another to form polymers which are colloidal in nature. After continued polymerization, flocculation and precipitation occur. The exact polymeric species is difficult to determine, but the physical chemical change is easily seen.

Although we believe that the iron-adriamycin preparations that have been made do not possess a single entity structure, we show from our data that what was administered in the animal and clinical trials was not a distinct (3 : 1) iron-adriamycin complex solution; rather it was an iron-adriamycin colloidal complex. The original methodological description [6] was to combine adriamycin and ferric chloride at pH 7, to filter very rapidly any precipitate formed, and to lyophilize the remaining solution. The lyophilized material was reconstituted for patient or animal administration. We have shown that the process of mixing adriamycin and ferric ion produced colloid very rapidly and that the quantity of colloid increased with time and also increased as the concentration of ferric ion and adriamycin increased. Therefore, after the process of filtration and during lyophilization, the ferric ion and adriamycin continue to react. The lyophilizing and reconstituting procedures add time, increase the effective concentration of the substrates, and result in an increased production of colloid. Biological evidence for this is reported in our animal experiments [5].

Our belief is that all of the iron-adriamycin preparations that have been used in experimental animals and clinically are composed of iron-adriamycin colloid or flocculant. This

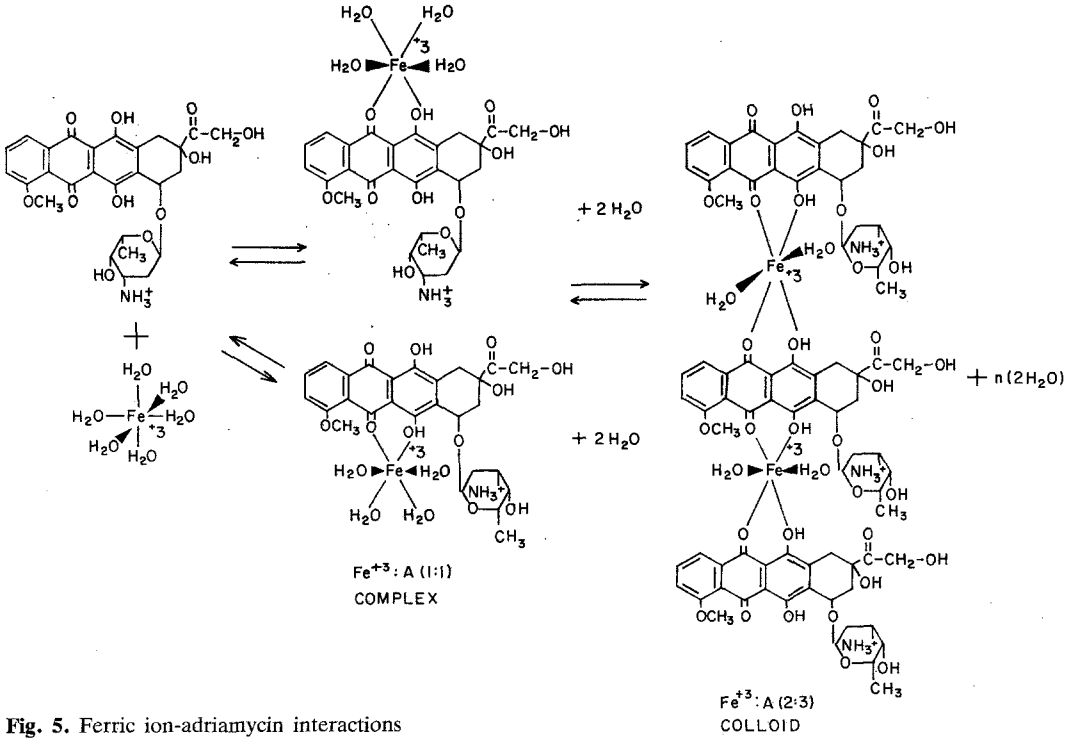

Fig. 5. Ferric ion-adriamycin interactions

physical modification produces significant biological modification in the pharmacokinetics of the adriamycin [5, 10]. When the iron-adriamycin colloid is administered to animals IP or IV it remains as a depot source of adriamycin until sufficient dilution occurs for reversible dissociation of the complex with liberation of adriamycin to interact with tissue components. When trapped as the colloid the adriamycin appears to be nonirritating and, depending on the size of the particles, may be trapped in the capillary beds [5].

A rational combination of iron and adriamycin appears to produce a complex which serves as a sustained release drug delivery system. It may be a useful pharmaceutical maneuver to modify very reactive or irritating drugs such as adriamycin by incorporating them into colloidal flocculents which produce a slower release of drug, as long as effective levels of drug are achieved. This maneuver may reduce the toxicity associated with high initial blood levels and with local irritation and necrosis.

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