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Speciation Studies of Adriamycin, Quelamycin and their Metal Complexes

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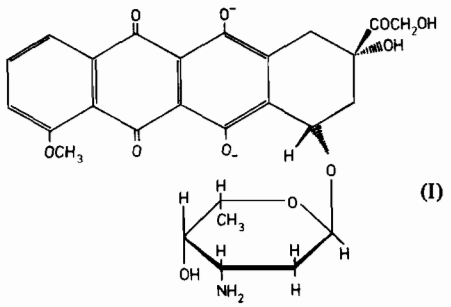
The formation constants for complexes formed between Adriamycin and some transition metals are reported. They are used to calculate the probable complex species concentrations present in gastro-intestinal fluids following Adriamycin and Quelamycin administration and to estimate the influence of these two agents upon the low-molecular-weight complexes normally present in blood plasma. Adriamycin forms a range of complexes in intestinal fluid and blood plasma, e.g. *CaAd and FeAdOH. The former suggests that Adriamycin may affect calcium metabolism whereas the latter suggests that co-administering ferric ions could interfere with this inhibition.*

Introduction

Adriamycin (I) is a cytotoxic agent having distinct antibiotic [1] and a wide range of anticancer properties [2–4]. It is produced by *Streptomyces peucetius var caesius* and was first reported in 1969 [5]. Unfortunately, the total dose of adriamycin is limited by cardiac toxicity which is said to arise from inhibition of Na–K-dependent adenosine triphosphatase [6]. The presence of calcium apparently alleviates these effects and so it has been suggested that chelation is involved [7]. This concept has led to the development of quelamycin [8–10], a 3:1 ferric:adriamycin



(I)

mixture. As this still exhibits some toxicity similar to iron toxicity [6], ratios of 2.9:1 ferric:adriamycin are now being screened [11].

It is very important to know which species of adriamycin and quelamycin exist *in vivo* and in solutions administered to humans. This quelamycin speciation cannot be determined by any known analytical technique and so the method of computer simulation that we pioneered some years ago was adopted [12–15]. Essentially, this assumes that solutions *in vivo* are close to equilibrium and that by determining each metal–ligand equilibrium constant *in vitro* by potentiometry, the equilibrium distribution of species in various biofluids can be computed. Only then is it possible to say whether or not a complex prevails at equilibrium *in vivo*.

It is important to make clear which areas of the overall picture we are modelling. Let us consider iron in blood: it exists in 5 forms [16]: (i) a very low concentration of aquated metal ions, (ii) a range of low molecular weight complexes, (iii) a circulating 'blotter' for ferric ions consisting of the protein transferrin, (iv) a reservoir form of iron – predominantly ferritin and (v) an inert form of iron such as haemoglobin or myoglobin. Forms (i)–(iii) are in labile equilibrium and are modelled as equilibrium systems. Form (iv), ferritin iron, is used as a larger store of iron when the transferrin blotter has become saturated. Similarly, this reservoir form can provide iron to the equilibrium system when the latter becomes depleted. However, both directions involve energetic processes. Form (v) does not have iron available to the equilibrium system until the haemoglobin *etc.* breaks down at the end of its lifetime (about 120 days). Quelamycin is a lyophilised monomeric form of ferric-adriamycin obtained by rapid freeze drying. *In vivo* it may be considered as form (iv) which slowly degrades to give adriamycin and ferric ions, both of which enter the equilibrium system of forms (i)–(iii) through which quelamycin probably produces its biological activity. Our models consider this equilibrium system.

The work described in this paper investigated the adriamycin–proton–ferric ion, and –cupric ion systems, characterizes them in terms of formation constants, estimates values for other pertinent adriamycin–metal formation constants, and computes concentrations of complexes present in solutions as administered in the clinic and in treated blood plasma.

Experimental

Solutions of adriamycin (molecular weight = 579.3, supplied by Dr. M. Gosálvez, University Autonoma, Madrid), copper oxide (Analar dissolved in HCl), ferric chloride hydrated (H and W AR), hydrochloric acid (ampoule BDH) and sodium hydroxide (ampoule BDH) were prepared in deionised, doubly-distilled water. The metal and acid contents of stock solutions were determined by gravimetry and titration against EDTA and Gran titrations as per our standard procedures [17].

All titrations used a digital voltmeter (Radiometer PHM64) having a combined microelectrode (Russell pH, Catalogue No. CMAW757). Due to the scarcity and expense of adriamycin, a specially prepared thermostatted glass microtitration vessel (Imperial Cancer Research Fund) holding up to 5 cm^3, and an Agla micrometer syringe were employed. The equipment was calibrated in terms of proton concentrations by using solutions of known acid and alkali concentrations as standards and by careful acid–base titrations. Blood plasma conditions of 37 °C and I = 150 mmol dm^{-3} sodium chloride were maintained throughout this study.

For the protonation titrations a range of ligand concentrations (seven titrations spanning the adriamycin concentration range from 1–10 mmol dm^{-3}) were performed and the results plotted as $\bar{Z}$ *versus* $-\log[H^+]$ using our ZPLOT program [17]. $\bar{Z}$ is the average number of protons per ligand. A typical series of plots is shown in Fig. 1. Clearly, the maximum number of sites available for protonation is three over the normal working range of pH. This data was then used in the SCOGS [18] and MINIQUAD [19] programs to obtain the protonation constants listed in Table I. Bearing in mind the very small quantities being used, these show a remarkably high degree of precision.

The iron(III) and copper(II) titrations were performed for a range of metal:ligand ratios (4:1–1:3.3) by adding sodium hydroxide from an Agla syringe. These results were plotted as $\bar{Z}$ *versus* $-\log[$free

TABLE I. Log Formation Constants for Adriamycin^{2-} $-H^+$, $-Fe^{3+}$ and $-Cu^{2+}$ Systems at 37 °C, I = 150 mmol dm^{-3} as determined in this study.

Species	Log β
AdH^-	11.210 ± 0.04
AdH_2^0	19.296 ± 0.06
AdH_3^+	21.434 ± 0.08
$FeAd^+$	17.985 ± 0.07
$FeAd_2^-$	29.034 ± 0.36
$FeAd_3^{3-}$	33.413 ± 0.47
$FeAd(OH)^0$	14.693 ± 0.14
$CuAd^0$	12.72 ± 0.16
$CuAd_2^{2-}$	19.27 ± 0.18
$CuAd_3^{4-}$	23.21 ± 0.18
$CuAd(OH)^-$	5.74 ± 0.48

The constants in this and Table II are used in the ECCLES models of blood plasma.

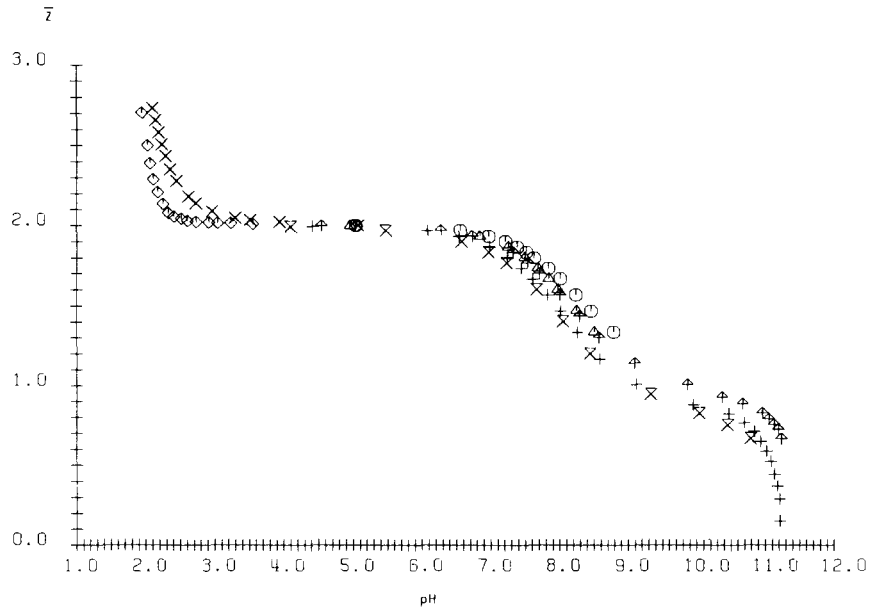


Fig. 1. Plots of $\bar{Z}$ *versus* pH for Ad^{2-} protonation at 37 °C, I = 150 mmol dm^{-3}. The different symbols refer to different ligand concentrations.

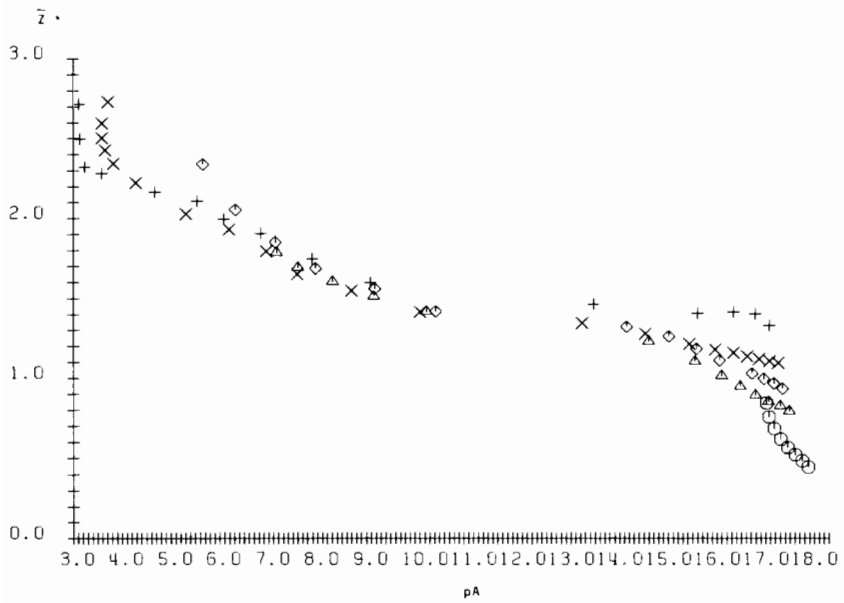


Fig. 2. Plots of $\bar{Z}$ *versus* pH for Ad^{2-}–Fe^{3+} complexing at 37 °C. I = 150 mmol dm^{-3}. The different symbols refer to different ligand concentrations and ligand to metal ratios.

adriamycin] using the ZPLOT program. A typical series of plots is shown in Fig. 2. It is noteworthy that up to three adriamycin ligands can be accommodated per metal ion. Octahedral coordination around the metal ion implies that under these circumstances the adriamycin is acting as a bidentate ligand.

The possibility that quelamycin, when dissolved into solution, has three ferric ions complexed to one adriamycin was checked by doing a large number of titrations in which the total metal concentration exceeded that of the total ligand. These iron titrations were fraught with drifting emfs as might be expected from the slow formation of polymeric metal hydroxy complexes. Nevertheless, some readings stabilised and gave meaningful points on the plots. This data was analysed using least squares formation constant programs such as SCOGS and MINIQUAD. Firstly, the mono, bis and tris complex constants were obtained and then metal–adriamycin–hydroxy constants. Extensive searches for complexes such as Fe_2adriamycin and Fe_3adriamycin (the latter is sometimes called Quelamycin in the literature but the term ought really to be reserved for the lyophilized solid) failed. This suggests that adriamycin–Fe^{3+} complexes containing three ferric ions do not exist at equilibrium in aqueous solution. The formation constants are listed in Table I.

The precision of the formation constants shown in Table I is considerably poorer than one would normally expect from our potentiometric approach. This arises from (i) the very small quantities used, (ii) the introduction of a *combination* microelectrode, and (iii) a small amount of ligand hydrolysis. Nevertheless, the constants determined are sufficiently reliable to permit them to be incorporated into the simulations and their uncertainty does not affect the conclusions of this paper.

Spectral Investigations

The standard Job's plot approach was applied at specified pH values (3.0 and 7.0), the pH being monitored using the electrodes and potentiometer used in the potentiometric part of this paper and the pH was adjusted using microadditions of sodium hydroxide or hydrochloric acid from Agla syringes [22]. The results were collected on a uv–visible spectrophotometer (Perkin Elmer PE402) at 37 °C.

The data treatment followed the approach outlined in reference 22 and the free ligand concentrations were computed from the adriamycin pK values listed in Table I. The most reliable data (in terms of lack of cloudiness, drifting of optical density readings and ease of adjusting pH) were obtained at pH = 3.0 and produced formation constants of $\beta_1 = 3.19 \times 10^{16}$ mol^{-1} dm^3 and $\beta_2 = 4.166 \times 10^{32}$ mol^{-2} dm^6. Kinetically these solutions were quite stable (no further drift after 6–8 minutes) and could be kept and remeasured more than 24 hours later with identical results. However, the pH = 7.0 solutions hydrolysed and gave drifting spectral readings after about one hour.

The $\log \beta_1 = 16.50$ and $\log \beta_2 = 32.62$ values are within two orders of magnitude of the potentiometrically determined constants listed in Table I. Part of this discrepancy arises from the complexity

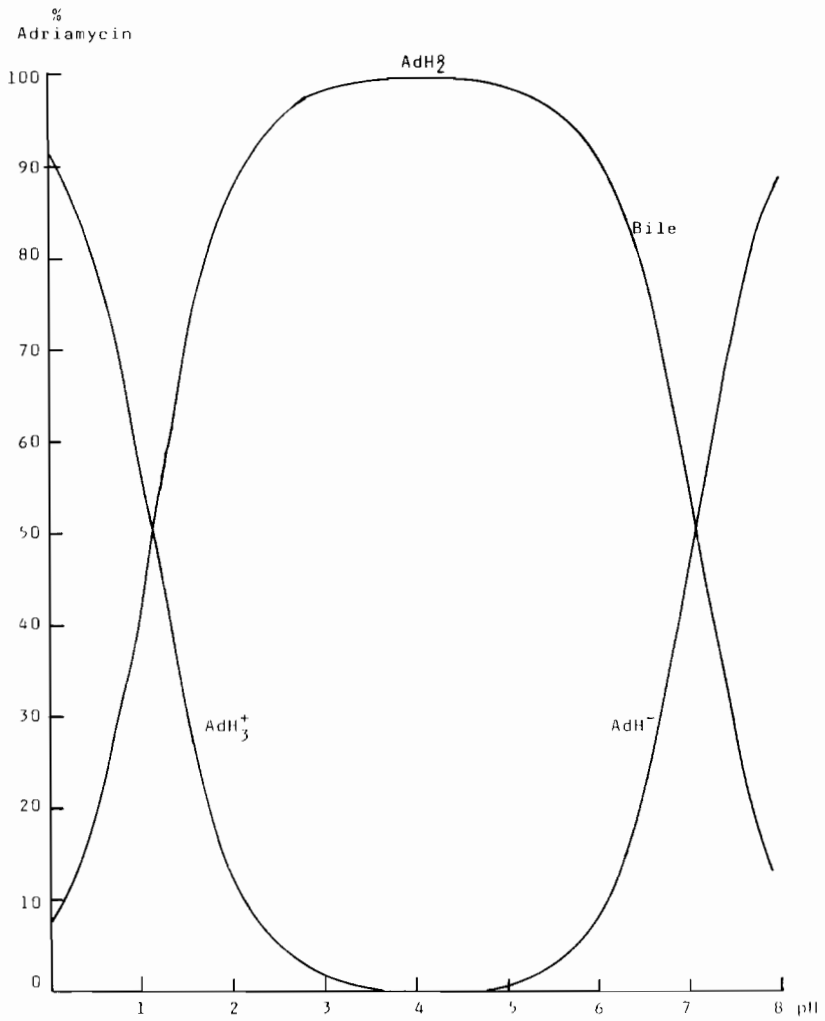


Fig. 3. The pH distribution of adriamycin between its various protonated species.

of the system exceeding the capabilities of the Job's plot approach and because of the small quantities used.

Discussion

The adriamycin anion, $Ad^{=}$, has three pK values at 11.2, 8.1 and 2.1. It is not possible to use equilibrium data for assigning such numbers to specific protonation sites but the two higher figures presumably refer to the $-O^-$ groups on the naphthacene rings at positions 6 and 11. It is reasonable to assume that when the ligand complexes metal ions these two donor sites will be involved and the sugar amino moiety would then provide an optional binding site. However, it must be stressed that our work gives stoichiometric information only, not structural details.

It is usual to administer adriamycin intravenously and quelamycin either intravenously or orally in enteric-coated pills and so the COMPLOT [23, 24] and ECCLES [12] programs were used to calculate the species distributions at equilibrium in both types of solutions (Figs. 3 and 4). The solution injected intravenously at pH = 7–8 contains a mixture of AdH^- and $AdH_2^{\pm}$. Both complexes are expected to dissociate and give other complexes in plasma (as discussed later). Assuming that the components of digestive juices and stomach contents do not interact with quelamycin, in the stomach at pH = 1–2 the charged species AdH_3^+ predominates, there being only a small portion of neutral stomach-wall transmittable species present (*i.e.* $AdH_2^{\pm}$ — up to 30% of the total adriamycin) and then in the small intestine all the adriamycin is present as a neutral species $FeAd(OH)^{\pm}$.

Thus, the addition of iron(III) to adriamycin in the ratio of Ad:Fe = 1:3 appreciably affects the bio-

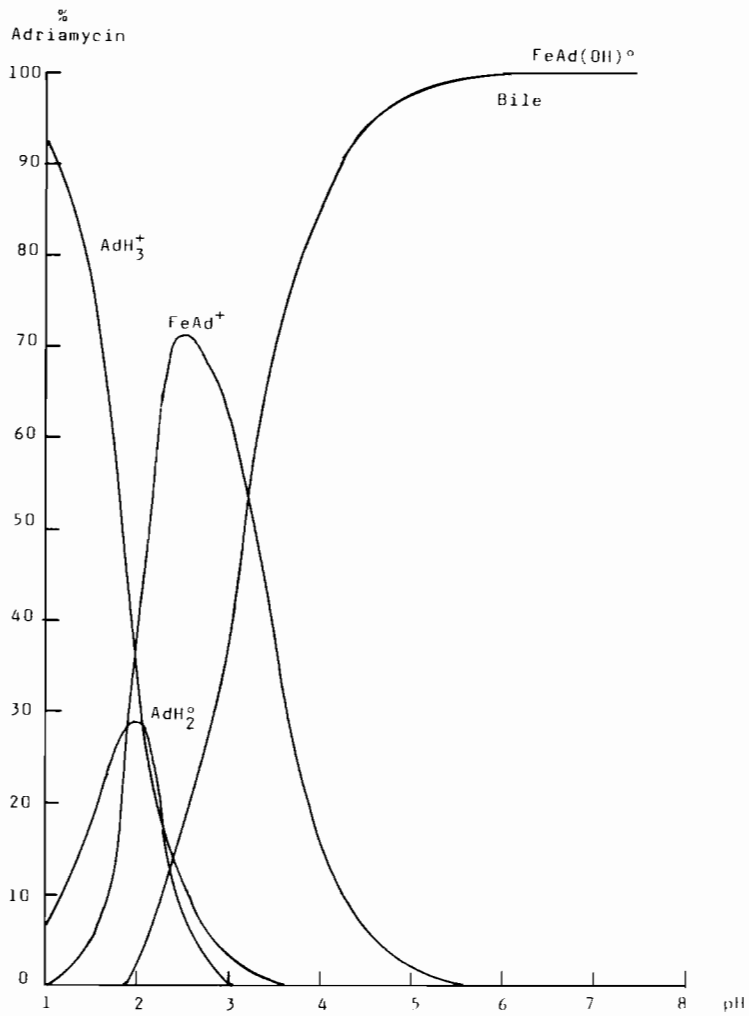


Fig. 4. Speciation plots over a range of pH for the adriamyciniron system plotted as percentage of ligand.

availabilities of the adriamycin and of the Fe(III) in the small intestine (up to 35% of the iron is present as FeAd(OH)° – Fig. 5).

The ECCLES program can be used to calculate the concentrations of complexes present at equilibrium in multicomponent fluids such as blood plasma [12–15]. This requires a knowledge of all the metal–ligand formation constants for the systems present. The proton, cupric ion, and ferric ion–adriamycin complexes can be introduced into this model using data from Table I. Ideally, we ought to have the constants for the other metals in plasma complexing with adriamycin but, with limitations on both the time and the amount of ligand available, the constants in Table II were used. These are estimates based upon those actually measured in this study and quoted in Table I. The blood plasma models revealed that for low adriamycin concentrations 71% of the iron(III) is present as Fe(citrate)-$(OH)_2^{2-}$ and 29% as Fe(citrate)$(OH)^-$, both being charged species not open to membrane permeability. Figures 5 and 6 show that as the drug concentration is increased, a neutral species, FeAd(OH)°, is increasingly preferred from the naturally occurring low molecular weight citrate complexes of iron and from transferrin. Up to 90% of the low molecular weight iron(III) can be made membrane diffusable in this fashion. Although this situation may not be realised under equilibrium conditions in plasma it may be approached in localised concentrations, *e.g.* in cells.

Adriamycin may thus be expected to complex both calcium and magnesium ions in blood plasma. The distribution of the ligand is AdH_2 > AdH > CaAd > MgAd > FeAdOH. This sequence arises since, although the Ca and Mg constants are estimated to be much lower than those for ferric ions, the alkaline earth ions are present at much greater concentrations

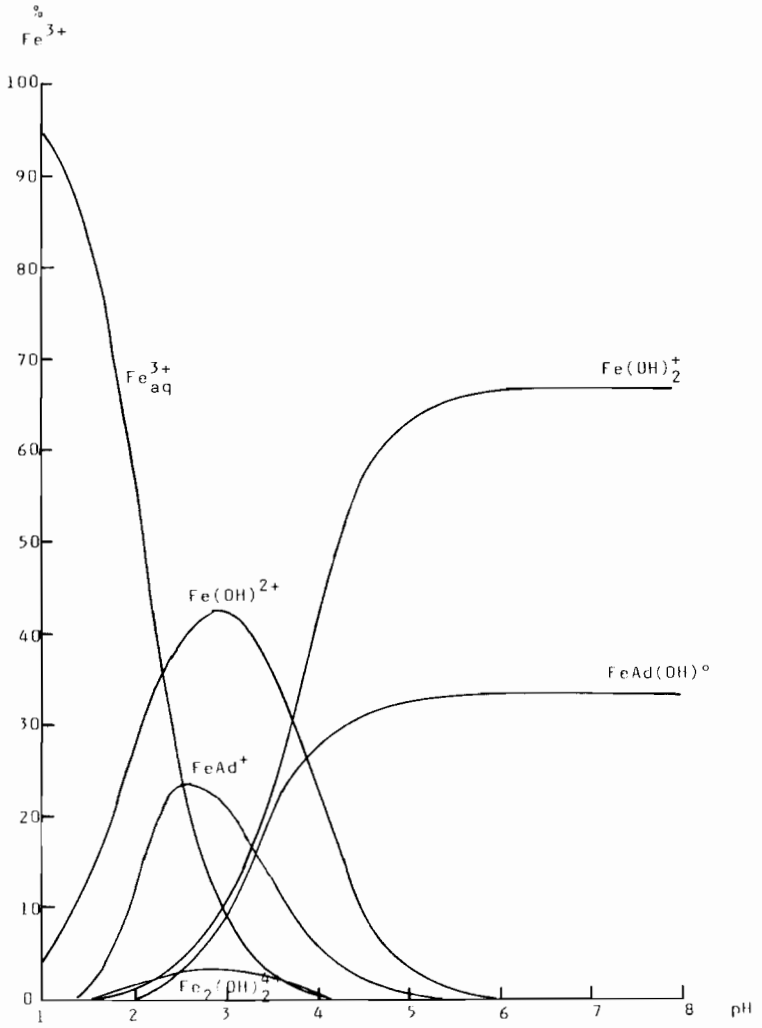


Fig. 5. Speciation plots over a range of pH for the adriamycin–iron system plotted as percentage of Fe^{3+}.

than the latter. Although we detected species such as CaAd and MgAd and these might be linked with the inhibition of Na–K ADP-ase – as mentioned in the introduction, the evidence ought to be based on laboratory determined constants, not estimated values. Certainly, our studies do not rule out the possibility of enzyme inhibition being the source of the cardiac toxicity. Clearly, more work is desirable in this area.

Other outstanding questions which might be answered by further investigations along these lines include the adriamycin:iron ratio dependence of

TABLE II. Log Formation Constants for Adriamycinate^{2-}–metal ion^{2+} Systems Estimated from the Chemical Literature and from a Knowledge of the Values Reported in Table I.

log β (Ferrous–Ad)^0	= 6.5	log β (Ferrous–Ad_2)$^{2-}$	= 10.5
log β (Calcium–Ad)^0	= 3.0	log β (Calcium–Ad_2)$^{2-}$	= 4.0
log β (Magnesium–Ad)^0	= 3.0	log β (Magnesium–Ad_2)$^{2-}$	= 4.0
log β (Manganese–Ad)^0	= 7.0	log β (Manganese–Ad_2)$^{2-}$	= 9.5
log β (Zinc–Ad)^0	= 8.1	log β (Zinc–Ad_2)$^{2-}$	= 12.0
log β (Lead–Ad)^0	= 7.0	log β (Lead–Ad_2)$^{2-}$	= 9.5

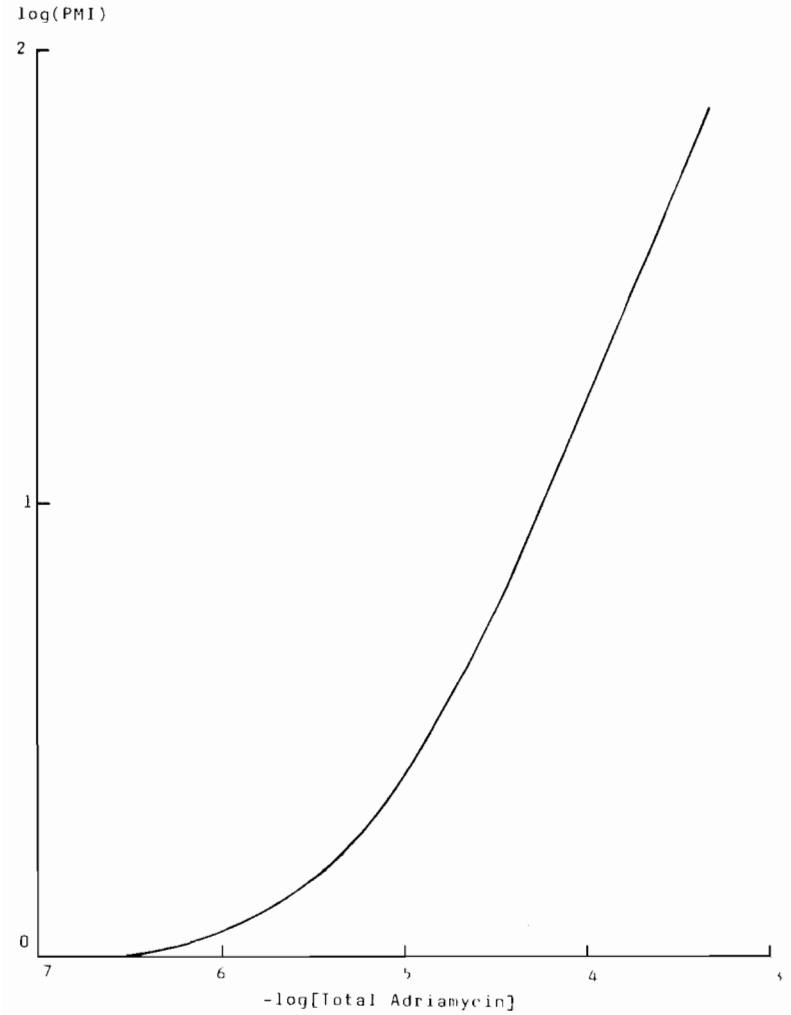


Fig. 6. Log (P.M.I.) plot for adriamycin *versus* total drug plasma concentration. (For a comparison with discussion of other iron(III) mobilizing ligands see refs. 15 and 16.)

benefits and side effects (*i.e.* dose optimisation), topping up with ferrous complexes, models of the complexes present inside cells, the kinetic dependence of the reactions investigated; finally the important $FeAd(OH)^{\circ}$ species merits further studies.

When one examines the fate of adriamycin in blood plasma from the computer simulation, one finds that, in spite of the iron interactions observed, the vast majority of adriamycin stays as protonated forms (17.2% as AdH^{-} and 82.81% as AdH_2°). This suggests that the drug passively diffuses through membranes into cells as AdH_2° where it brings about its biological effects.

In conclusion, this work has shown that quelamycin Fe_3Ad^{7+} is not formed in blood plasma from adriamycin and that side-effects of adriamycin medication may be calcium chelation dependent. Administered iron could effectively compete for the attentions of adriamycin *in vivo* and reduce these side effects but too much iron is expected to be toxic.

Acknowledgements

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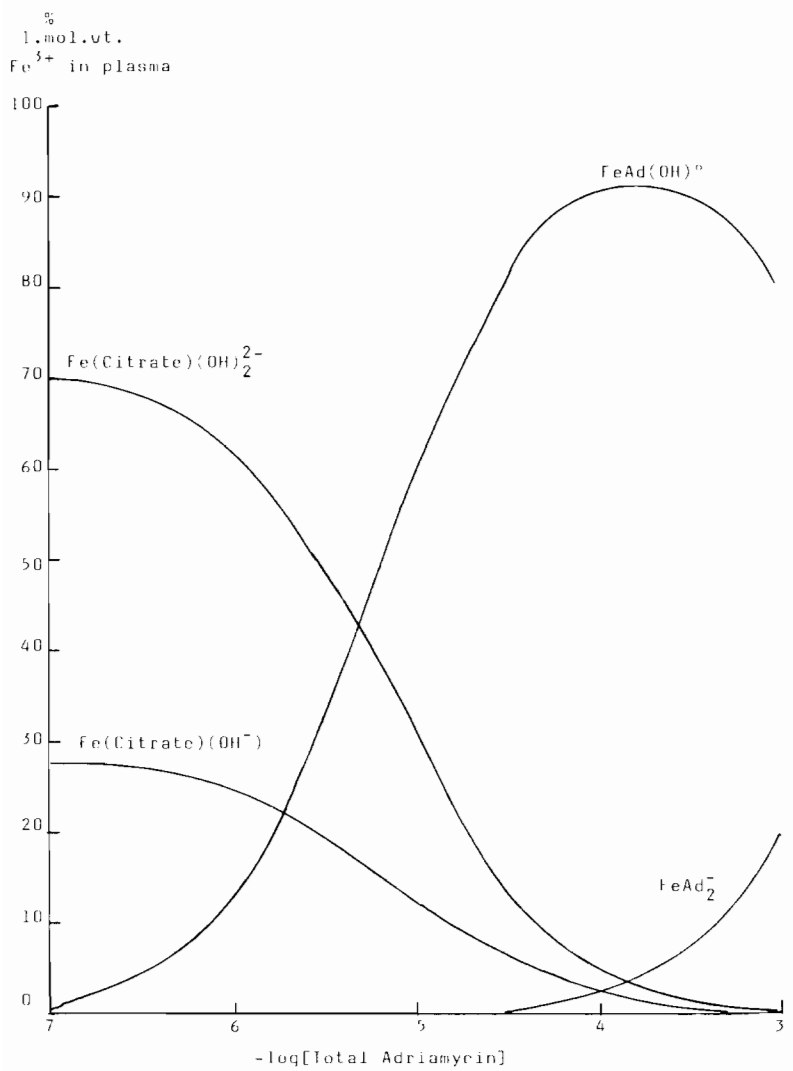

Fig. 7. Trends in the main low molecular weight complexes of iron(III) in blood plasma as adriamycin content is increased.

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