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Method for Analysis, and Distribution Profile, of Covalently-linked Ferritin–daunorubicin Conjugate in the Blood of Trypanosome-infected Mice

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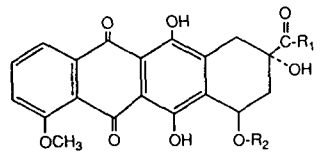
Abstract—Daunorubicin is a highly potent trypanocide in-vitro but is inactive in-vivo. When daunorubicin is conjugated to bovine serum albumin or horse spleen ferritin using Schiffs base linkages, the complex is trypanocidal in-vitro and in-vivo. We have developed novel analytical methods, using HPLC with fluorimetric detection, for the quantitation of daunorubicin and doxorubicin in biological samples, either as unconjugated drug, or when covalently linked to macromolecules or particles. Ferritin–daunorubicin conjugate (25 mg kg^{-1}) was administered intraperitoneally to mice infected with monomorphic *Trypanosoma brucei rhodesiense*; peak plasma levels occurred after 1·5 h, and were 5 times higher than those resulting from administration of an equivalent amount of unconjugated daunorubicin. Plasma levels then declined rapidly ($t_{\frac{1}{2}}$ for 1–6 h period was 0·58 and 0·86 h respectively for conjugated and unconjugated daunorubicin). However, higher plasma levels were seen 24 h after treatment, suggesting the distribution profile of daunorubicin when conjugated to ferritin is multiphasic with resultant high levels of daunorubicin obtained for a prolonged time period.

Daunorubicin (Fig. 1), a member of the anthracycline group of antitumour antibiotics, is used clinically in the treatment of the acute leukaemias (Buechner 1990). We previously showed that daunorubicin is also a highly potent trypanocide in-vitro against monomorphic *Trypanosoma brucei rhodesiense*, but is inactive when administered in high doses to rodents infected with the same trypanosome strain (Williamson & Scott-Finnigan 1978). Further studies showed that drug plasma levels following administration of daunorubicin to trypanosome-infected rodents, were above those necessary for an in-vitro trypanocidal effect (Brown et al 1982).

Many research groups including ours have investigated the use of macromolecules in an attempt to target anthracyclines to certain rapidly-dividing cells. Daunorubicin and the structurally-related and clinically-important doxorubicin (Fig. 1) have been specifically targeted via chemical linkage to immunoglobulins including monoclonal antibodies (reviewed by Blair & Ghose 1983), and also non-specifically targeted using macromolecules and particles including DNA, proteins, polysaccharides, synthetic polymers and liposomes (Poznansky & Cleland 1980; Williamson et al 1981; Couvreur et al 1982; Yokoyama et al 1990). These covalently or non-covalently linked drug particle or macromolecule complexes (reviewed by Langer 1990) were synthesized in attempts to increase plasma half-life of drug, and to enable lysosomotropic drug targeting (Trouet et al 1972).

We previously showed that when daunorubicin was covalently linked as Schiffs bases using glutaraldehyde as a cross-linking agent, to macromolecules such as horse spleen ferritin and bovine serum albumin (both known to be endocytosed by trypanosomes) the daunorubicin complex is trypanocidal both in-vitro and in-vivo (Williamson et al 1981). To characterize the in-vivo plasma level profiles it was necessary to determine both the conjugated drug and released drug after administration of these agents in-vivo. There is no satisfactory method reported for the quantitation of daunorubicin and doxorubicin in samples containing drug–macromolecular complexes; hence we set out to develop novel analytical methods which enable quantitative determination of ferritin–daunorubicin conjugate and non-conjugated daunorubicin in biological samples from ferritin–daunorubicin treated animals, and use these to determine the distribution of ferritin-daunorubicin complex in blood components and trypanosomes from mice infected with monomorphic *T. b. rhodesiense*.



Anthracyclinone — Daunosamine

Compound	R_1	R_2
Daunorubicin	CH_3	Daunosamine
Doxorubicin	CH_2OH	Daunosamine
Daunomycinone	CH_3	H
Adriamycinone	CH_2OH	H

FIG. 1. Structures of daunorubicin, doxorubicin and respective aglycones.

Materials and Methods

The anthracyclines daunorubicin hydrochloride and doxorubicin hydrochloride used in this work were a generous gift from Professor F. Arcamone, Farmitalia Carlo Erba, Milan, Italy.

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All glassware was silanized with Repelcote (BDH) before use. Drug solutions were freshly prepared in double-distilled water, stored at 4°C, and were protected from light.

Determination of free and total daunorubicin in biological samples containing ferritin–daunorubicin complex

The reverse phase HPLC system with fluorimetric detection used for determination of daunorubicin in plasma and biological samples was as described previously (Brown et al 1981). Stock aqueous solutions of daunorubicin hydrochloride, and the internal standard doxorubicin hydrochloride (100 μg mL^{-1}) were prepared. Human citrated plasma was mixed with daunorubicin stock solution to produce plasma drug concentrations in the range 0·01 to 5·0 μg mL^{-1}. Doxorubicin hydrochloride internal standard was added to samples to produce concentrations in the range 0·02 to 2·0 μg mL^{-1} according to the concentration of daunorubicin used.

To determine free daunorubicin in samples containing ferritin–daunorubicin, a modification of the extraction method described by Bolanowska & Gessner (1982) was used. A sample of a saturated solution of $NaHCO_3$ in distilled water was added to plasma (100–150 μL). Solid NaCl was slowly added to the mixture until saturation occurred. Ethyl acetate (1·0 mL) was added to each plasma sample solution, followed by mixing (20 s) and centrifugation (2200 *g*, 5 min). Daunorubicin and doxorubicin were then back-extracted as follows: 0·1 M H_3PO_4 (120–250 μL) was added to a sample of the ethyl acetate layer (0·9 mL), then mixed and centrifuged as before; the ethyl acetate was discarded. Hexane (0·5 mL) was added to the aqueous solution and mixed to remove residual organic phase then centrifuged as before. A sample of the lower aqueous layer (20–200 μL) was injected directly onto the HPLC column. To determine total daunorubicin in samples containing ferritin-daunorubicin, 100 μL of sample was mixed with 2 M HNO_3 (50 μL). The solution was stoppered, mixed, and refluxed at 100°C for 3 min, to release the aglycone. After rapid cooling to 20°C, a solution containing 200 μL CH_3CN-phosphate buffer (pH 7·0, 0·1 M) (4:1) was added and the solution mixed (20 s) and centrifuged (2200 *g*, 10 min). A sample of the supernatant was filtered, then injected onto the HPLC column.

Radioimmunoassay of horse-spleen ferritin in biological samples using ^{125}I as radioactive label

The following reagents were used: phosphate buffered saline (PBS) (0·15 M NaCl, 0·02 M phosphate, pH 7·3); bovine serum albumin (BSA) (Sigma, Grade V); rabbit non-immune serum (NIS) diluted 1:5 with 1% BSA in PBS; rabbit anti-horse-spleen ferritin (RaF) antiserum diluted with NIS; and goat anti-rabbit IgG (GaR) antiserum used undiluted.

Radioiodination of horse-spleen ferritin. To ferritin solution (10 μL, 1 mg mL^{-1}) was added carrier-free sodium iodide with ^{125}I (1·0 mCi, 10 μL). Chloramine T (400 μg mL^{-1}) in PBS (50 μL) was added and the reaction mixture incubated at ambient temperature for 1 min. Sodium metabisulphite (1 mg mL^{-1}) in PBS (0·5 mL) and KI (20 mg mL^{-1}) in PBS (0·5 mL) were added to terminate the reaction, and the reaction mixture was transferred to a Sephadex G50 column (Pharmacia, 18 cm × 12 mm) eluting with 1% BSA in PBS.

Identification of fractions containing ^{125}I ferritin. Heparinized rabbit blood (10 mL) was centrifuged (1000 *g*, 10 min) and the supernatant aspirated and retained. The serum was diluted 1:5 with 1% BSA in PBS to give NIS.

Lyophilized anti-horse-spleen ferritin antiserum (Nordic) was reconstituted with PBS (1·0 mL) and diluted with NIS by serial dilution to give RaF 1:1000 in NIS.

A sample (50 μL) of each fraction from the radioiodination of horse-spleen ferritin was first diluted tenfold with 1% BSA in PBS. To a sample (100 μL) of diluted fraction in a 4 mL polystyrene tube (LKB) was added RaF (1:1000) and to a second sample of the same diluted fraction (100 μL) was added NIS (100 μL); these were mixed and incubated at 37°C for 1 h. Rabbit IgG was precipitated as follows: GaR antiserum (RIA) was added undiluted (50 μL) to each sample, followed by incubation for 15 min at room temperature (21°C). Polyethylene glycol 12·5% in PBS (500 μL) was added to each tube and after briefly mixing, each tube was centrifuged (1000 *g*, 15 min) and the supernatant aspirated. The precipitates were counted for gamma emission using a NaI detector and an Ekco M5024B scaler/timer. Fractions with highest RaF to NIS count ratio were pooled and adjusted with 1% BSA in PBS to 3·5 μCi/10 mL. This solution was used as ferritin tracer. The RaF dilution which showed optimal activity was 1:15 000 dilution.

Radioimmunoassay of horse-spleen ferritin. A standard solution (100 μL) of ferritin (10, 25, 100, 200, 400 or 1000 ng mL^{-1}) in 1% BSA in PBS was transferred to a 4 mL tube, and 1% BSA in PBS (100 μL) was transferred to a pair of tubes denoted 'non-specific binding, NSB'. Ferritin tracer (100 μL) was added to all standard and sample tubes, plus two extra tubes denoted 'total counts'. To the pair of NSB tubes was added NIS (100 μL), and all tubes were incubated at 37°C for 1 h. All tubes except 'total counts' were then precipitated and counted.

Isolation of blood fractions from trypanosome-infected mice treated with ferritin–daunorubicin conjugate

Ferritin-daunorubicin conjugate (25 mg kg^{-1}) was administered intraperitoneally to specific-pathogen-free Parkes mice infected two days previously with a monomorphic strain of *T. b. rhodesiense* (Yorke et al 1929) and blood components and trypanosomes isolated as previously described (Brown et al 1982).

Results

HPLC of total daunorubicin in samples containing daunorubicin–ferritin conjugate by conversion to daunomycinone aglycone

Daunorubicin was quantitatively converted to the aglycone daunomycinone by hydrolysis of the glycosidic linkage with the aminosugar daunosamine (Fig. 1). This conversion was achieved with greater than 95% efficiency using daunorubicin concentrations up to 5 μg mL^{-1} (n = 5). A linear relationship was found between daunorubicin concentration in the range 0·05 to 0·25 μg mL^{-1} and the resultant peak height for daunomycinone (r = 0·996, n = 5). Hence total daunorubicin can be quantitated in samples containing either free or covalently bound daunorubicin by this method, since either form of drug will liberate daunomycinone

aglycone on acid hydrolysis. Doxorubicin was similarly converted to adriamycinone aglycone (Fig. 1) and quantitated with greater than 95% efficiency (n = 5) in the range 0·05 to 0·25 $\mu g\ mL^{-1}$ using this hydrolysis procedure.

HPLC of free daunorubicin in samples containing daunorubicin–ferritin conjugate

The assay method for the determination of unconjugated daunorubicin in samples containing conjugated daunorubicin was a modification of that described by Bolanowska & Gessner (1982). This method extracts daunorubicin in free base form into ethyl acetate, then back extracts daunorubicin into aqueous phase after acidification and protonation of the daunosamine amino group, followed by HPLC with fluorimetric analysis of the extract. Drug extraction efficiencies similar to those described by Bolanowska & Gessner (1982) were found in the range 0·05 to 0·25 $\mu g\ mL^{-1}$ (> 95%, n = 5).

Radioimmunoassay of horse-spleen ferritin

The results of the radioimmunoassay of solutions containing horse-spleen ferritin are shown in Fig. 2. These data indicate a sigmoidal relationship between % binding of ferritin to anti-horse-spleen ferritin antibody and log[ferritin]. To linearize these data points logit transforms were used where

$$\text{logit } y = \frac{\text{counts min}^{-1} \text{ in sample} - \text{NSB counts min}^{-1}}{\text{counts min}^{-1} \text{ in blank} - \text{NSB counts min}^{-1}}$$

The results were replotted using logit transform vs log ferritin concentration and these are shown in Fig. 2 (inset). These data indicate a linear relationship between logit transform and log[ferritin], and these plots were used as calibration curves for horse-spleen ferritin.

Determination of free and conjugated daunorubicin, and ferritin, in biological samples from trypanosome-infected mice dosed intraperitoneally with ferritin–daunorubicin conjugate

Ferritin–daunorubicin conjugate (25 mg daunorubicin kg^{-1}) was administered intraperitoneally to specific pathogen-free Parkes mice heavily infected to ++ on a subjective scale (> 10^6 trypanosomes mL^{-1} tail blood) with *T. b. rhodesiense*.

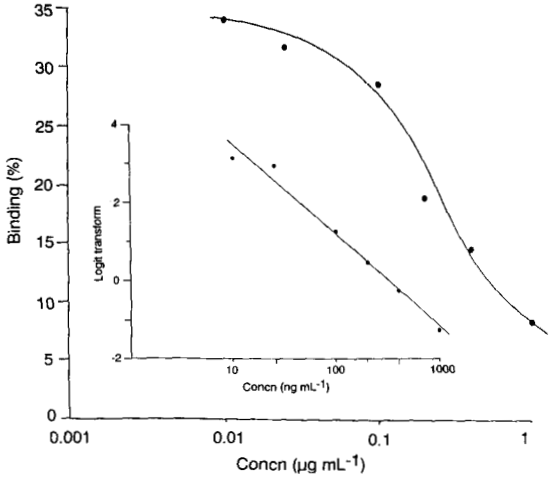

FIG. 2. Plot of % binding of horse spleen ferritin to rabbit anti-horse spleen ferritin antibody against log ferritin concentration. Inset: plot of logit transform against log ferritin concentration.

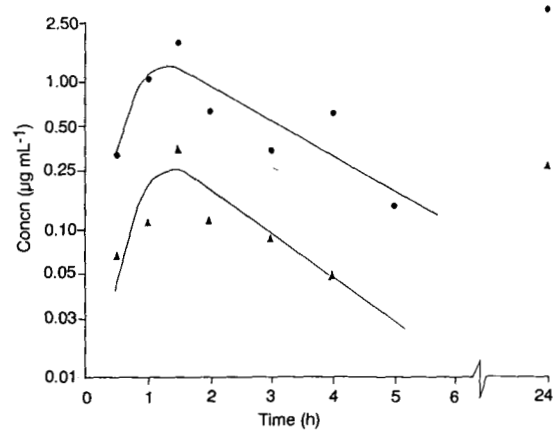

FIG. 3. Distribution of daunorubicin either unconjugated (▲) or conjugated (●) to ferritin in plasma of mice infected with *T. b. rhodesiense* and dosed with daunorubicin (25 mg kg^{-1}, i.p.)–ferritin conjugate.

At each time point two mice were killed and the trypanosome-infected blood was citrated and removed, and separated into blood components and trypanosomes. These fractions were then analysed for free and conjugated daunorubicin, and horse-spleen ferritin by methods previously described (Brown et al 1981). The results of the daunorubicin determinations in plasma are shown in Fig. 3. Peak plasma levels for both conjugated daunorubicin (1·8 $\mu g\ mL^{-1}$) and released daunorubicin (0·35 $\mu g\ mL^{-1}$) occurred after 1·5 h, then declined sharply, with $t_{\frac{1}{2}}$ values of 0·58 and 0·86 h, respectively, calculated for the 1–6 h period. However, conjugated and unconjugated plasma daunorubicin levels were higher than those found at the 1·5 h peak, 24 h after administration of ferritin–daunorubicin (3·0 $\mu g\ mL^{-1}$), indicating a sustained and multiphasic release of daunorubicin. These data were compared with similar data for trypanosome-infected rodents dosed intraperitoneally with an equivalent dose of daunorubicin (Brown et al 1982) which showed that the initial peak concentration of free drug (0·35 $\mu g\ mL^{-1}$) also occurred after 1·5 h, and showed a rebound increase 6 h after treatment, with drug levels of only 0·05 μg

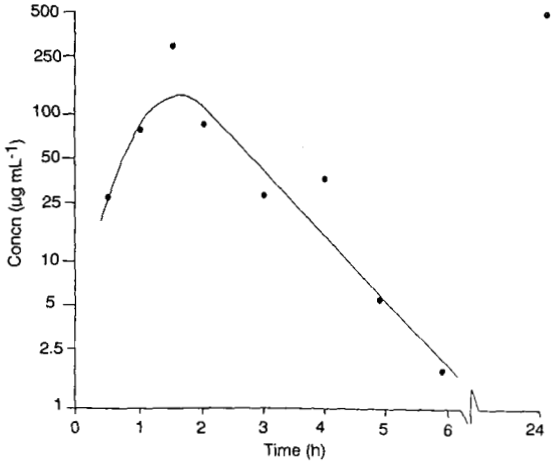

FIG. 4. Ferritin levels in plasma of mice infected with *T. b. rhodesiense* following daunorubicin (25 mg kg^{-1}, i.p.) conjugated to ferritin.

mL^{-1} 24 h after treatment. The results of the radioimmunoassay of the horse spleen ferritin component of ferritin–daunorubicin conjugate are shown in Fig. 4. These data show that plasma levels of ferritin were maximal in the 1–6 h region (264 $\mu g\ mL^{-1}$) after 1·5 h, then declined rapidly, similar to the data for conjugated daunorubicin, with higher ferritin levels observed 24 h after treatment (479 $\mu g\ mL^{-1}$), consistent with the daunorubicin determinations in these samples, and similarly indicating multiphasic ferritin–daunorubicin levels following treatment with this conjugate.

Discussion

These data show that an intraperitoneal dose of 25 mg kg^{-1} daunorubicin conjugated to ferritin administered to mice infected with monomorphic *T. b. rhodesiense* resulted in plasma daunorubicin levels over 5 times greater than those obtained following administration of an equivalent dose of unconjugated drug. Unconjugated daunorubicin is inactive when administered to rodents infected with monomorphic *T. b. rhodesiense*, hence either the peak plasma levels of unconjugated daunorubicin are insufficient for a trypanocidal effect following 25 mg kg^{-1} intraperitoneal injection, or sustained daunorubicin release from the ferritin–daunorubicin conjugate for >24 h, is an important factor in the trypanocidal activity of the daunorubicin conjugate. We have previously shown that plasma levels resulting from daunorubicin administration were in excess of levels which are trypanocidal in-vitro, suggesting that peak plasma daunorubicin levels may be less important in determining trypanocidal activity than the time over which trypanosomes are exposed to such daunorubicin concentrations (Brown et al 1982). The mode of action of anthracyclines may be mediated by inhibition of DNA replication via a DNA intercalative mechanism (Waldes & Center 1981). We have previously shown that daunorubicin is localized largely in the cell nucleus of trypanosomes (Golightly et al 1983), consistent with an intercalative mode of binding, since DNA intercalation results in the accumulation of drug in the nucleus. Cell cycle-specific inhibitors, including anthracyclines, may be dependent for activity on the changes in DNA conformation that occur during cell cycle progression (Schwartz & Kanter 1980). Hence trypanosomes may need to be exposed to adequate in-vivo daunorubicin concentrations for a sufficient time period in excess of that which can be achieved using free daunorubicin, but which is attained following administration of equivalent doses of daunorubicin conjugated to ferritin. Furthermore the intracellular distribution of daunorubicin conjugated to macromolecules such as ferritin, which are endocytosed by trypanosomes (Brown et al 1965), may also have a significant effect on the trypanocidal activity of daunorubicin. We previously showed that following cellular uptake, complexes of BSA conjugated to daunorubicin are located in the lysosome fraction of trypanosomes (Golightly et al 1986). Lysosomal vesicles contain hydrolytic enzymes capable of cleaving Schiffs base conjugates, and hence these conjugates may additionally provide a sustained intracellular source of free daunorubicin, which is available for interaction and subsequent interference with trypanosomal nuclear DNA.

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