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THE MECHANISM OF BIOACTIVATION AND ANTIGEN FORMATION OF AMODIAQUINE IN THE RAT

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Abstract—A glutathione conjugate of amodiaquine has been isolated and characterized from rat bile after administration of [^{14}C]amodiaquine (50 μmol/kg, 5.0 μCi/rat) to anaesthetized male Wistar rats. Thioether conjugates of amodiaquine in rat bile accounted for a total of 12% of the dose, 5 hr after administration of the drug. In addition, 1% of the dose remained in the liver covalently bound to tissue proteins after 5 hr. These findings provide direct evidence that a chemically reactive metabolite, amodiaquine quinoneimine, has been formed from the drug *in vivo*. A second major metabolite, desethylamodiaquine, accounting for 14% of the given dose, was present in the liver after 5 hr. Enzyme inhibition studies with ketoconazole-pretreated rats showed that both amodiaquine quinoneimine and desethylamodiaquine formation can be catalysed by cytochrome P450. The demonstration that amodiaquine readily and extensively forms a metabolite *in vivo*, with strong reactivity towards protein and non-protein thiol groups, may help to explain the idiosyncratic toxicity observed in man.

Amodiaquine, a 4-aminoquinoline antimalarial drug, is effective against chloroquine resistant strains of *Plasmodium falciparum* [1]. However, the drug was withdrawn from prophylactic use because of its association with hepatotoxicity [2] and agranulocytosis [3], and is now only used for the treatment of chloroquine-resistant infections.

Neither the precise mechanism nor the causative agent (parent drug or metabolite) of these adverse reactions has been identified, although both direct cytotoxic [2] and indirect immunological [4] mechanisms have been proposed. Consistent with the latter proposal are studies which have demonstrated the immunogenicity of amodiaquine in man and in the rat [5, 6]. These studies have shown a high incidence of antiamodiaquine antibodies in patients suffering adverse reactions to amodiaquine. However, amodiaquine has too low a molecular weight to be immunogenic *per se*, but when covalently linked to a macromolecular carrier could act as an antigen. Such covalent binding must be preceded by activation of the amodiaquine molecule to a reactive species which can then undergo conjugation with protein. Previous *in vitro* studies have demonstrated that amodiaquine is readily oxidized by a number of chemical and biochemical agents to a reactive species, amodiaquine quinoneimine [7]. Conjugation with thiol-containing compounds was observed, which is indicative of quinoneimine formation, since the mechanism of conjugation is Michael-like addition and requires a 1,4-unsaturated system which is present in the quinoneimine but not in amodiaquine.

Paracetamol, which like amodiaquine has a 4-aminophenol moiety, has also been associated with hepatotoxicity. Paracetamol undergoes an analogous *in vitro* oxidation to its quinoneimine. Microsomal incubations with the drug led to the formation of the same conjugate as that formed from chemical reaction of the synthetic quinoneimine with cysteine [8], a thiol-containing amino acid. Bioactivation of paracetamol in biological systems is known to be cytochrome P450-mediated [9]. Therefore, amodiaquine may also undergo an analogous P450-mediated oxidation to its quinoneimine *in vivo*. Formation of this reactive species *in vivo* could explain the observed immunogenicity of amodiaquine.

The unstable nature of electrophilic metabolites prevents their direct isolation from biological samples, since further rapid biotransformations including conjugation, reduction and hydrolysis may occur. Therefore, we have investigated the metabolism of amodiaquine in the rat, with particular reference to thioether conjugate excretion and irreversible binding of the drug to tissue protein.

The rat was selected as the animal model because amodiaquine has been shown to be immunogenic in this species [6].

MATERIALS AND METHODS

Reagents

Amodiaquine was a gift from Parke-Davis (Ann Arbor, MI, U.S.A.). Radiolabelled amodiaquine (sp. act. 9.4 μCi/μmol, >95% pure by TLC) was a gift from Amersham International (Amersham, U.K.). Glutathione, γ-glutamyl transpeptidase, 4-chloro-1-naphthol, urethane, polyethylene glycol 200 and ketoconazole were obtained from the Sigma Chemical Co. (Poole, U.K.). Tetrabutylammonium-hexafluorophosphate (electrochemical grade) was obtained from Fluka (Buchs, Switzerland). Solvent filters (0.45 μm pore size) were obtained from Millipore (Bedford, MA, U.S.A.). SEP-PAK C_{18}

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cartridges were obtained from Waters (Milford, MA, U.S.A.). NCS tissue solubilizer was obtained from Amersham. Glycine, sodium dodecyl sulphate (SDS), Tris, casein, Triton X-100 and ammonium persulphate were obtained from BDH (Liverpool, U.K.). Acrylamide, *N,N'*-bisacrylamide and molecular weight markers were from Biorad (Richmond, CA, U.S.A.). Horseradish peroxidase (HPO†)-labelled goat anti-rabbit IgG (Fc fragment) was from Tissue Culture Services (Bucks, U.K.). Nitrocellulose membrane (mean pore size 0.45 μm) was obtained from Schleicher and Schnell (Dassell, Germany). All other reagents were of analytical grade.

Mass spectrometry was carried out using a VG-Tritech TS250 mass spectrometer (VG Analytical Ltd, Manchester, U.K.).

400 MHz proton NMR was carried out on a Bruker WMX 400, using deuterated dimethyl sulphoxide as a solvent.

HPLC analysis was carried out using an SP8800 ternary HPLC pump with a Spectra 100 variable wavelength detector (Spectra-Physics, San Jose, CA, U.S.A.) linked to an on-line Radiomatic A250 Flo-one/β radioactive flow detector (Canberra-Packard, Pangbourne, Berks, U.K.).

Cyclic voltammetry was carried out using an EG + G Model 173 potentiostat/galvanostat (Princeton Applied Research) interfaced via an EG + G Model 276 interface to an Apple II computer, which utilized EG + G PARC Electrochemistry Version 2.0 software.

Synthesis of amodiaquine quinoneimine

Amodiaquine quinoneimine was synthesized by a modification of the method of Dahlin and Nelson [10] for the synthesis of *N*-acetyl-*p*-benzoquinoneimine. Amodiaquine (0.6 g, 1.29 mmol) in chloroform (135 mL) over anhydrous Na_2SO_4 (0.3 g) with added silver oxide (1.5 g, 12.11 mmol) was stirred at room temperature under nitrogen for 30 min. The mixture was filtered and the filtrate evaporated *in vacuo*, to yield a tar. The material was seen to be pure by HPLC (10–25% CH_3CN in $NH_4H_2PO_4$ 10 mM/C_7S 5 mM, over 30 min, C_{18} 10μ column, flow rate 1.5 mL/min).

[^{14}C]Amodiaquine quinoneimine was synthesized from a mixture of [^{14}C]amodiaquine and unlabelled amodiaquine (molar ratio 1:25) using the above method.

Synthesis of glutathione adducts of amodiaquine

Amodiaquine quinoneimine (50 mg, 0.14 mmol), in chloroform (3 mL), was added to glutathione (75 mg, 0.24 mmol) in water (2 mL). The biphasic reaction mixture was shaken at room temperature for 30 min. The aqueous phase was removed, and the three regioisomeric products of the reaction were separated and isolated from the aqueous phase using the HPLC system described above. Fractions were collected for each peak (R_T 16, 18 and 20 min). Acetonitrile was evaporated from each fraction under a stream of nitrogen, and the aqueous residue passed through SEP-PAK C_{18} cartridges. The material was washed with water (*ca.* 20 mL) before being eluted from the cartridge with methanol (1.5 mL). All three glutathione adducts were characterized by fast ion bombardment (positive mode) (FIB+) mass spectrometry, and the least polar product was characterized by 400 MHz NMR.

Synthesis of a cysteine conjugate of amodiaquine

The cysteine conjugate of amodiaquine was synthesized by enzymic hydrolysis of the least polar glutathione adduct. The glutathione adduct (15 mg, 22.73 mmol) was dissolved in sodium phosphate buffer (0.1 M, pH 7.4, 3 mL) and Tris buffer (0.125 M, pH 9.0, 3 mL), and γ-glutamyl transpeptidase was added (24 U). The mixture was left overnight at 37°, resulting in the formation of a heavy yellow precipitate. The precipitate was removed by filtration, washed with water (2 × 3 mL) and dissolved in methanol (5 mL). The solution was filtered to remove a white solid, and the filtrate evaporated to dryness using nitrogen. The resulting yellow solid was characterized by FIB+ mass spectrometry.

Administration of [^{14}C]amodiaquine to male Wistar rats

Male Wistar rats (200–260 g) were anaesthetized with urethane (7% w/v in 0.9% saline, 20 mL/kg), and bile duct cannulae were inserted. Amodiaquine (50 μmol/kg, 5 μCi/rat, in polyethylene glycol (PEG) 200 saline 1:1, v/v) was injected via the hepatic portal vein, and bile fractions were collected every 30 min up to 5 hr after dosing. After 5 hr, the rats were killed by exsanguination and the major organs were removed. Bile was stored at −20°, and the organs at −80°.

Tissue distribution of [^{14}C]amodiaquine in the rat

A 25% homogenate (w/v) was prepared from each organ, in sodium phosphate buffer (0.1 M, pH 7.4). Aliquots were taken (2 × 200 μL), tissue solubilizer added (0.5 mL), and the mixtures left overnight at 50°. The solutions were cooled to 5°, decolourized with hydrogen peroxide (200 μL), neutralized with glacial acetic acid (60 μL) and assayed for radioactivity in 16 mL scintillant.

Irreversible binding of [^{14}C]amodiaquine to liver tissue

Aliquots (2 × 0.5 mL) were taken from 25% liver homogenates. Methanol (5 mL) was added to each aliquot, and the mixtures were left overnight at 5° to precipitate proteins. Exhaustive methanol extractions were carried out (4 × 5 mL), and the protein was washed in 70% methanol before being dissolved in sodium hydroxide (1.0 M, 2 mL). Aliquots were taken (2 × 0.5 mL), neutralized with glacial acetic acid (70 μL) and assayed for radioactivity in 16 mL scintillant. The samples were assayed for protein loss [11]. Supernatants from the precipitation and first extraction were analysed by HPLC (10–20% CH_3CN in NH_4OCOCH_3, 0.1 M, pH 3.8; over 18 min; C_{18} 10μ column; flow rate 1.5 mL/min).

† Abbreviations: HPO, horseradish peroxidase; PEG, polyethylene glycol; FIB+, fast ion bombardment (positive mode); EI+, electron impact (positive mode); i.p.v., intrahepatic portal vein.

Analysis and characterization of metabolites of [^{14}C]amodiaquine

Aliquots were taken from all bile samples (2 × 10 μL) and assayed for radioactivity in 4 mL scintillant. Individual and pooled bile fractions were analysed by HPLC (10–25% CH_3CN in $NH_4H_2PO_4$, 10 mM, pH 4.6, over 30 min, C_{18} 10μ column, flow rate 1.5 mL/min).

Biliary metabolite. Bile from the 30–60-min collection period (50 μL) was incubated with γ-glutamyl transpeptidase (1 U) in Tris buffer (0.125 M, pH 8.0, 350 μL) and magnesium chloride (0.1 M, 100 μL), at 37° for 90 min. The resulting mixture was analysed using the same HPLC system as that used for bile analysis.

The major biliary metabolite was isolated from bile using a semi-preparative HPLC column (CH_3CN in $NH_4H_2PO_4$, 10 mM, pH 4.6; 10–20% over 20 min then 20–35% over 10 min; C 18 25–40μ column; 250 × 8 mm; flow rate 2.5 mL/min). The eluent was processed as for isolation of the glutathione adducts, and then the material was purified further using an analytical column (conditions as for bile analysis). The metabolite was characterized by FIB+ mass spectrometry.

Liver metabolite. Liver homogenates (25%) were combined and aliquots taken (8 × 4 mL). Acetonitrile (10 mL) was added to each, and the mixtures were left at 5° overnight. The precipitated protein was washed with methanol (10 mL) and the supernatant combined with the acetonitrile from the precipitation stage. The solvents were evaporated to dryness under nitrogen, and water (2 mL) was added to each tube. The aqueous solutions were extracted with diethyl ether (5 mL) and the organic phase was removed and shaken with hydrochloric acid (0.1 M, 2 mL). The acid extracts were combined and neutralized by the dropwise addition of sodium hydroxide (0.75 M). The neutral solution was further extracted with diethyl ether (2 × 10 mL) which was then evaporated to dryness under nitrogen. Methanol (200 μL) was added to the residue, and the metabolite was isolated by HPLC (10–20% CH_3CN in NH_4OCOCH_3, 0.1 M, pH 3.8; over 18 min; C_{18} 10μ column; flow rate 1.5 mL/min), and the eluents processed as described for the isolation of the glutathione adducts. The metabolite was characterized by FIB+ mass spectrometry.

Pretreatment of rats with ketoconazole

Ketoconazole (100 mg) was dissolved in HCl (1.0 M, 800 μL) and NaOH (0.5 M, 3 × 50 μL) was added whilst shaking vigorously, resulting in a solution of pH 3–4. Water (3.05 mL) was added and the solution passed through a 0.45 μm filter to yield a 25 mg/mL solution.

Male Wistar rats (N = 6, 225–280 g) were administered ketoconazole (50 mg/kg, i.p.) in the above solution. The rats were anaesthetized, and [^{14}C]amodiaquine was administered as described above, exactly 1 hr after ketoconazole pretreatment.

Control rats (N = 4, 245–295 g) received a mixture of HCl (1.0 M)/NaOH (0.5 M), pH 3–4, diluted 1 in 4 (2.0 mL/kg, i.p.), in place of the ketoconazole solution.

Total biliary and hepatic radioactivity, analysis of biliary and hepatic radioactivity, and irreversible binding to liver tissue protein were determined as described above.

Detection of amodiaquine antigens in rat liver after chronic administration

Two groups of male Wistar rats (250–300 g, N = 4) were administered either amodiaquine (269 μmol/kg) in PEG 200 (1 mL/kg) or PEG 200 alone i.m. for four consecutive days. The animals were killed by cervical dislocation, and the livers excised into ice-cold sodium phosphate buffer (pH 7.4, 67 mM). The livers were homogenized (25% in sodium phosphate buffer), filtered through two thicknesses of muslin and stored at −20° until analysed by immunoblotting.

For immunoblotting analysis, homogenate samples were loaded at 100 μg protein/track and SDS–PAGE electrophoresis ("Mighty Small", Hoefer, U.K.) was carried out in a 7% polyacrylamide gel under reducing conditions (6 mg/mL dithiothreitol) at 30 mA/gel, as described by Laemmli [12]. Protein was transferred from the gels to nitrocellulose membrane at 150 mA for 2 hr. The blots were stained for protein with 1% (w/v) amido black or probed with rabbit anti-amodiaquine antiserum, prepared as described previously [6] at a dilution of 1/100, for 3 hr at 20°. After washing, the blots were exposed to HPO-conjugated goat anti-rabbit IgG antibody (1/500 dilution) overnight at 20°. Bound HPO-conjugated antibody was visualized with freshly prepared substrate (4-chloronaphthol/H_2O_2) as described by Kenna *et al.* [13] for 20 min, and the reaction terminated by rinsing the blots with water.

Determination of oxidation potentials by cyclic voltammetry

A 0.1 M solution of tetrabutylammonium-hexafluorophosphate in dimethyl-formamide was passed down a column of activated alumina. The test compound was then dissolved in this solution to give an approximate concentration of 5.0 mM. The test solution was placed in a glass cell, and nitrogen was gently bubbled through for 5 min. Three electrodes were placed in the solution; a platinum counter, a platinum working and a silver reference electrode. A range of potentials were applied across the solution, from 0 to 2000 mV at a rate of 100 mV/sec, while the current through the solution was being measured. Measurements were carried out at 20°. A plot of current versus applied potential was obtained, and the oxidation potentials were determined from the current maxima on these plots [14].

Statistics

All values are given as the mean ± the SD of the mean. All statistical analyses were carried out using an unpaired Student's *t*-test, following analysis with the F-test to ensure homogeneity of variance. Differences were deemed significant at the 5% level.

RESULTS

Glutathione adducts of amodiaquine

A mixture of three regioisomers was obtained

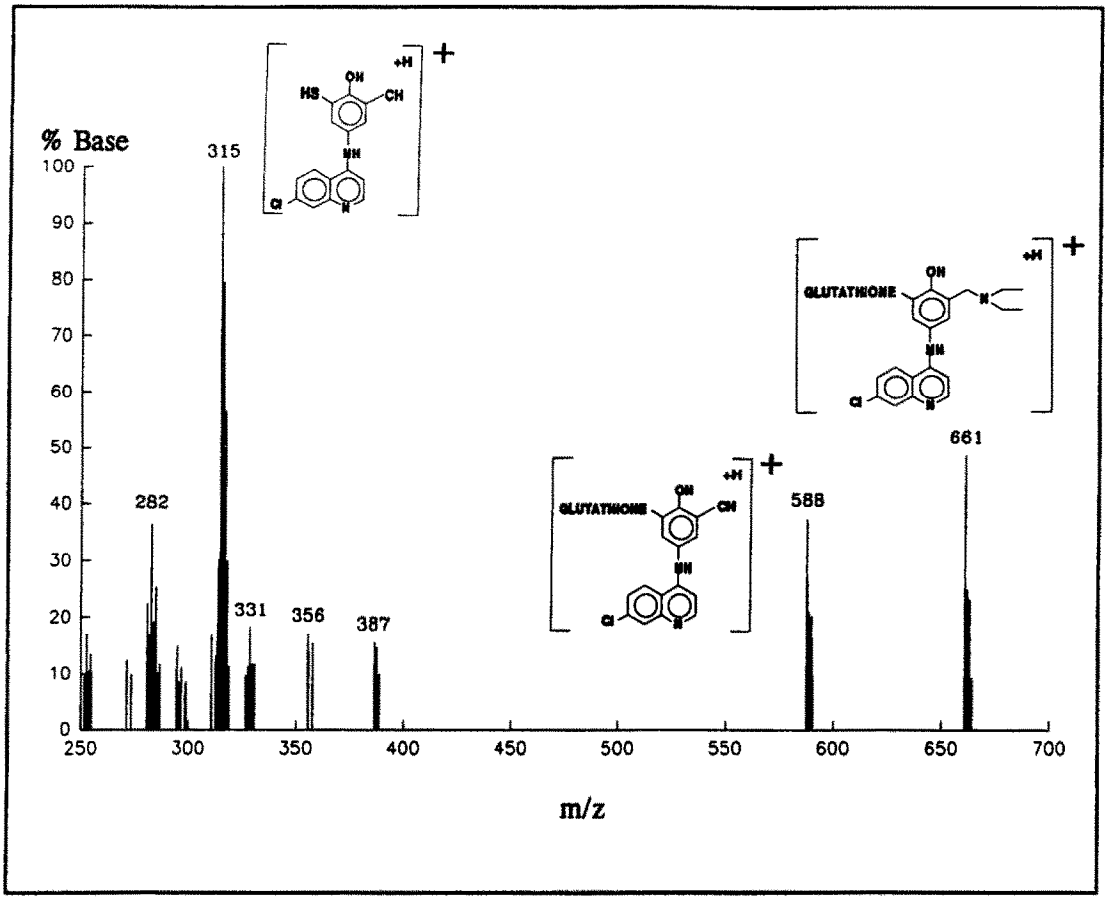
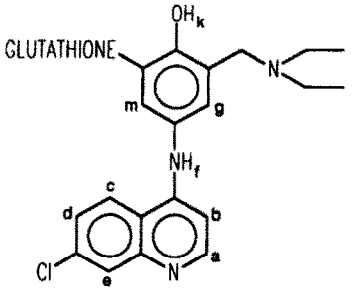


Fig. 1. FIB+ mass spectrum of the major synthetic isomer of the amodiaquine–glutathione adducts. The figure shows the characteristic ions at 661 $[M + 1]^+$, 588 and 315, displayed with the relevant fragment structures.



Proton	Chemical Shift (δ)	Integration	Multiplicity	Coupling Constant (H_z)
a	8.39	1	d	5.2
b	6.66	1	d	5.2
c	8.54	1	d	9.2
d	7.52	1	dd	9.2, 2.0
e	7.87	1	d	2.0
f	9.18	1	s	
g	6.97	1	s	
k	8.66	1	s	
m	7.17	1	s	

Fig. 2. The position of addition of glutathione on the amodiaquine molecule for the major synthetic isomer of the amodiaquine–glutathione adducts, and 400 MHz ^{1}H-NMR data of the aromatic region of the spectrum. Lower case letters denote each aromatic proton, and the position of addition is derived from the chemical shifts and peak splitting pattern associated with each aromatic proton (s = singlet, d = doublet, dd = doublet of doublets).

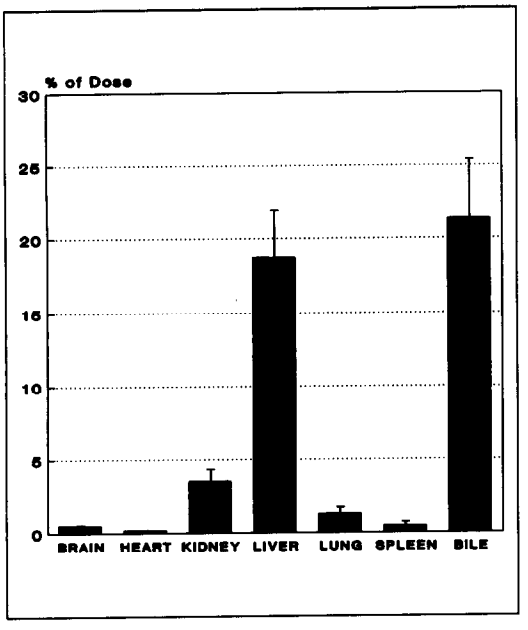


Fig. 3. Distribution of radioactivity (shown as % of dose) in the rat, 5 hr after administration of [^{14}C]amodiaquine (50 μmol/kg, 5 μCi, i.p.v.).

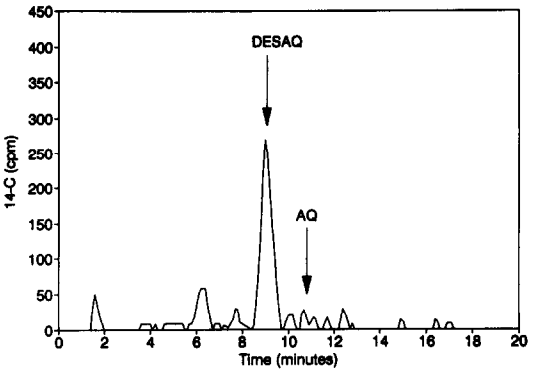


Fig. 4. Radiometric HPLC profile of extractable radioactivity from rat liver homogenates, 5 hr after administration of [^{14}C]amodiaquine (50 μmol/kg, 5 μCi, i.p.v.), showing one major radiolabelled peak. Arrows indicate the retention times of authentic amodiaquine and desethylamodiaquine (using NH_4OCOCH_3 buffer, pH 3.8/CH_3CN eluents).

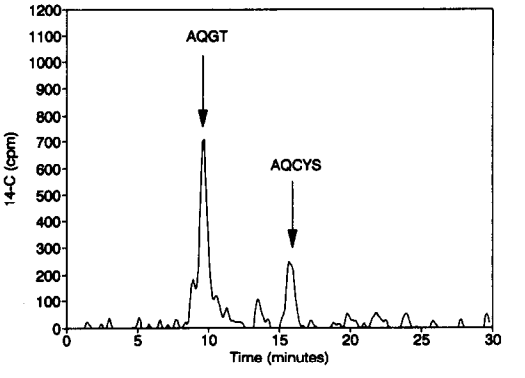


Fig. 5. Radiometric HPLC profile of pooled rat bile samples, collected during the 5 hr after administration of [^{14}C]amodiaquine (50 μmol/kg, 5 μCi, i.p.v.). Arrows indicate the retention times of the major synthetic glutathione adduct of amodiaquine (AQGT) and the cystein adduct derived from this glutathione adduct (AQCYS) (using $NH_4H_2PO_4$ buffer, pH 4.6/CH_3CN eluents).

in the ratio 2:3:10. FIB+ gave unambiguous characterization of all three isomers as glutathione adducts of amodiaquine [m/z 661 $(M+1)^+$, 588, 315] (Fig. 1).

^{1}H NMR (400 MHz) of the major isomer identified the position of conjugation in this isomer as C5′ (*ortho* to OH, Fig. 2).

Cysteine adduct of amodiaquine

Enzyme degradation of the major glutathione adduct yielded a single product. FIB+ mass spectrometry gave unambiguous characterization of this product as a cysteine conjugate of AQ [m/z 472 $(M+1)^+$, 402, 315].

Distribution of radioactivity after administration of [^{14}C]amodiaquine to male rats

Two major sites of accumulation of radioactivity were observed, the liver (18.76 ± 3.24) and bile (22.67 ± 4.45). All other organs each accounted for less than 3.5% of dose (lung 1.27 ± 0.44, kidney 3.47 ± 0.87, heart 0.20 ± 0.02, spleen 0.45 ± 0.26, brain 0.52 ± 0.04, Fig. 3). Overall, approximately 50% of the dose was recovered in the organs and bile 5 hr after dosing.

Irreversible binding to liver tissue

After correction for protein loss during extraction, the proportion of radioactivity which had become irreversibly bound to liver tissue protein was 1.10 ± 0.11% dose. Extractable radioactivity was predominantly one single product (Fig. 4) (R_T 9 min, 14.35 ± 2.53% dose) which co-chromatographed by HPLC with authentic desethylamodiaquine. A second unidentified peak (R_T 6.5 min) was also observed.

Biliary metabolite of amodiaquine

Radiometric HPLC analysis of bile samples from both amodiaquine- and amodiaquine quinoneimine dosed rats (Fig. 5) showed one major peak (R_T 10 min, 9.34 ± 1.97% dose) which co-chromatographed with the least polar synthetic glutathione adduct of amodiaquine. This metabolite underwent complete degradation by γ-glutamyl transpeptidase to a product which co-chromatographed with synthetic cysteine adduct of amodiaquine. FIB+ mass spectrometry gave unambiguous characterization of the metabolite as a glutathione adduct of amodiaquine

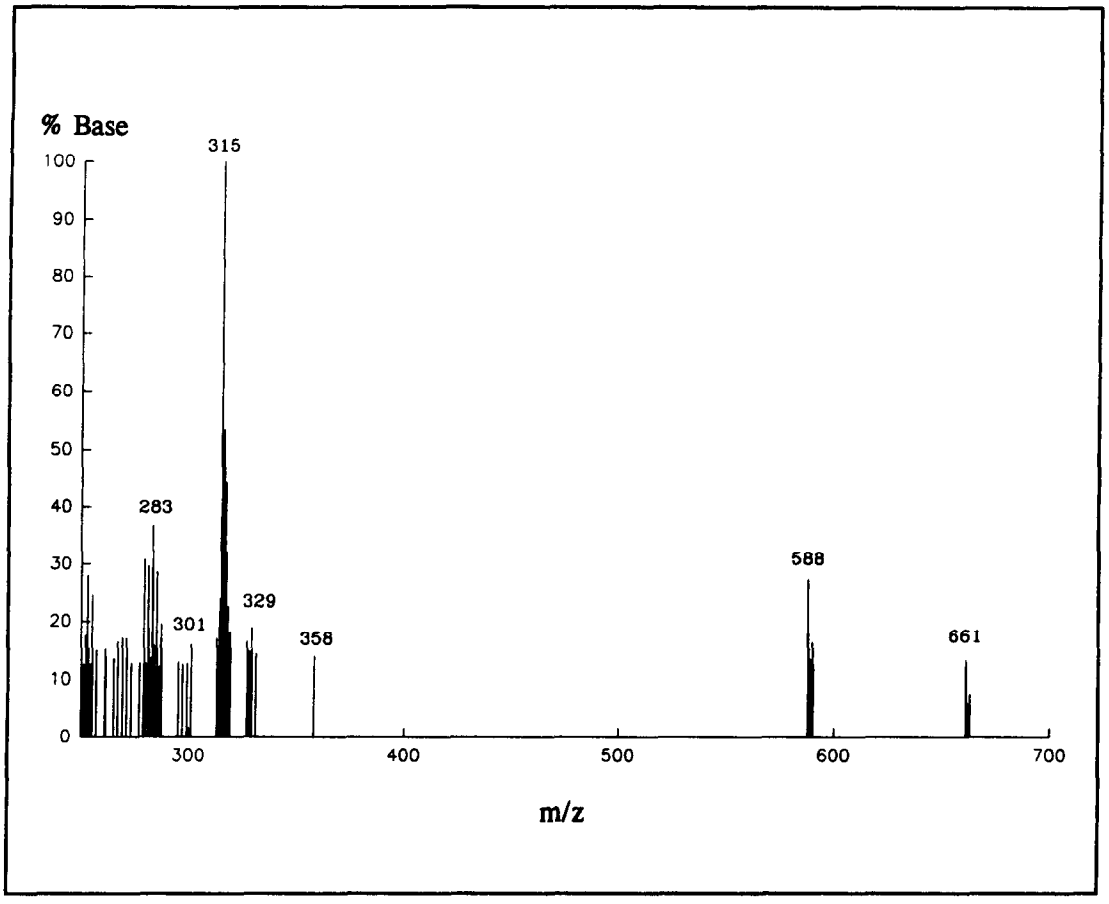


Fig. 6. FIB+ mass spectrum of the major biliary metabolite of amodiaquine. The figure shows characteristic ions at 661 $[M + 1]^+$, 588 and 315. These ion peaks are characteristic of a glutathione adduct of amodiaquine (see Fig. 1).

[m/z 661 $(M + 1)^+$, 588, 315] (Fig. 6). Co-chromatography with the least polar isomer shows that the position of conjugation of the metabolite is C5'.

The secondary peak (R_T 16 min, 2.53 ± 1.98% dose) observed in pooled bile samples has been characterized by co-chromatography as a cysteine conjugate of amodiaquine.

Liver metabolite

Electron impact (positive mode) (EI+) mass spectrometry gave unambiguous characterization of the liver metabolite as desethylamodiaquine [m/z 327 (M+), 282, 253, 218] (Fig. 7).

Ketoconazole inhibition

Pretreatment with ketoconazole resulted in a 2-fold decrease in biliary excretion of radioactivity from 22.30 ± 1.23% of dose in control rats to 11.31 ± 1.47% ($P < 0.001$) in pretreated rats (Fig. 8). A corresponding significant increase in accumulation of radioactivity in the livers was also observed, from 23.01 ± 2.78% of dose in control rats to 30.88 ± 4.00% in pretreated rats ($P < 0.01$). The hepatic ratio of amodiaquine to desethylamodiaquine increased from 0.17 ± 0.06: 1 in controls to 1.44 ± 0.32: 1 in pretreated animals, and the total amount of desethylamodiaquine in the liver decreased from 14.35 ± 2.53% of dose to 10.28 ± 2.41% ($P < 0.05$). A significant decrease in binding to liver tissue protein was observed from 1.10 ± 0.11% of dose in controls to 0.82 ± 0.11% in pretreated animals ($P < 0.005$).

Detection of amodiaquine antigens in rat liver

Immunoblots of liver homogenates from four individual rats treated with amodiaquine and one rat treated with injection vehicle alone are shown in Fig. 9. Under the conditions of exposure used here, no bands were evident in the control homogenates whereas clearly discernible binding of the anti-amodiaquine antibodies was observed in all four liver samples taken from rats treated chronically with amodiaquine. Several bands were seen in each amodiaquine-treated rat sample, the pattern being highly reproducible between rats. However, two high molecular weight proteins (apparent molecular

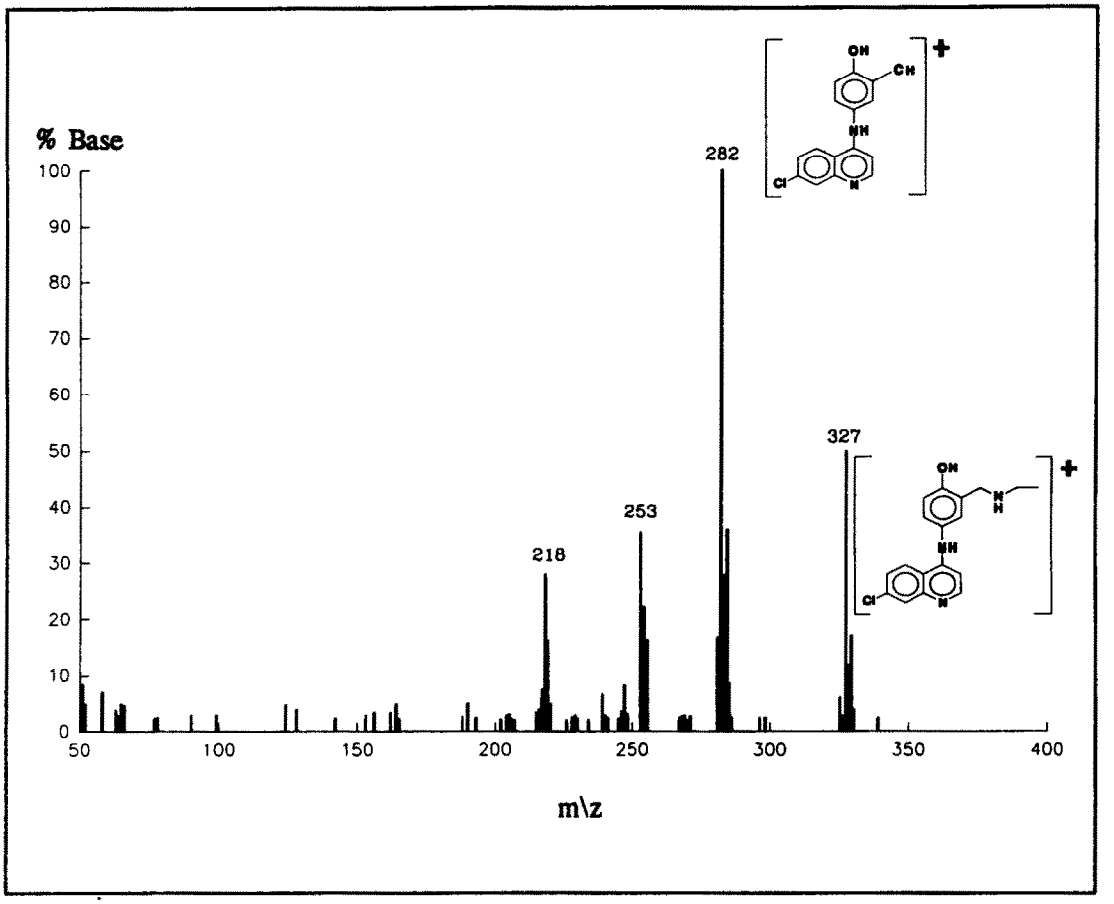


Fig. 7. Electron impact (positive mode) mass spectrum of the metabolite of amodiaquine present in the liver 5 hr after administration of [^{14}C]amodiaquine (50 μmol/kg, 5 μCi, i.p.v.). The figure shows characteristic ions at 327 (M^+), 282, 253, and 218, displayed with the relevant fragment structures. This spectrum is identical to that for synthetic desethylamodiaquine.

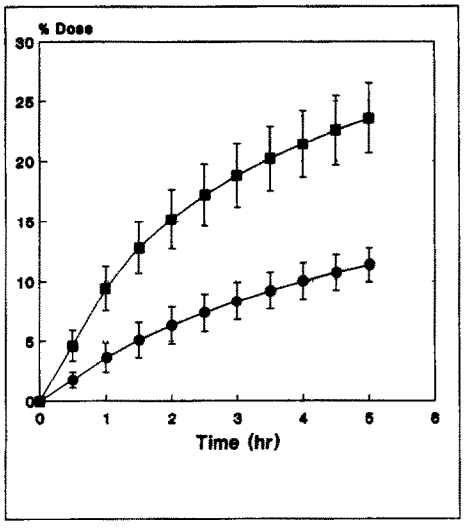


Fig. 8. Cumulative excretion of radioactivity (expressed as % of dose) into the bile of control (■) and ketoconazole-pretreated (●) rats, during the 5 hr after administration of [^{14}C]amodiaquine (50 μmol/kg, 5 μCi, i.p.v.).

masses 200 and 246) appeared to have the highest affinity for the anti-amodiaquine anti-serum.

Oxidation potentials by cyclic voltammetry

The oxidation potential for amodiaquine was 774 mV whereas that of desethylamodiaquine was 693 mV.

DISCUSSION

It has been suggested that the majority of radiolabelled material excreted from the rat after dosing with [^{14}C]amodiaquine is in the form of drug conjugates [15]. However, despite extensive studies, previous workers were unable to characterize such metabolites. This was primarily due to the refractory nature of the metabolite(s) to simple enzyme hydrolysis. By using more selective methods, such as acid hydrolysis and (more reliably) γ-glutamyl transpeptidase hydrolysis, we were able to determine the identity of the major biliary metabolite. The major biliary metabolite of amodiaquine has been unambiguously characterized by mass spectrometry, with supporting evidence from HPLC and enzyme hydrolysis, as a glutathione conjugate of the drug. The minor biliary metabolite has been tentatively

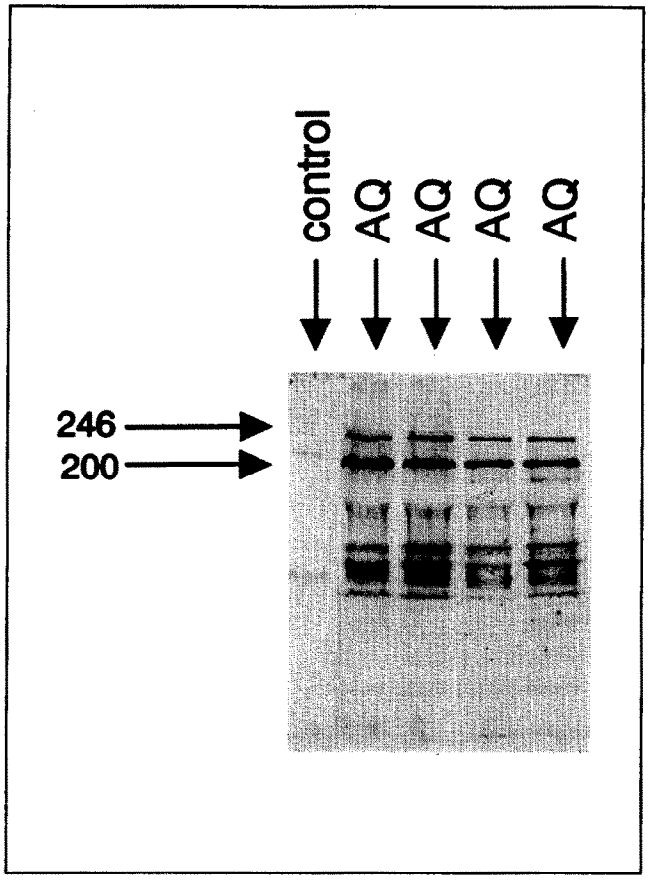


Fig. 9. Immunoblots of liver homogenates from four individual rats treated for four consecutive days with amodiaquine (AQ) (269 μmol/kg, i.m.), and a control rat treated with vehicle alone. Blots were probed with rabbit anti-amodiaquine antiserum (1/100 dilution) and HPO-labelled goat anti-rabbit IgG antibodies (1/500 dilution). Bands were exposed by incubating with 4-chloro-1-naphthol/H_2O_2. The two major bands in amodiaquine rats were calculated to have apparent molecular masses of 200 and 246.

characterized as the corresponding cysteine conjugate. The characterization of the metabolite as a glutathione adduct explains why attempted deconjugation using sulphatase and glucuronidase enzymes was unsuccessful.

There are only a few examples of xenobiotics which undergo such extensive conjugation with glutathione as observed with amodiaquine. These include the environmental pollutant, 1-nitropyrene [16], and drugs which conjugate directly to glutathione such as α-bromoisovalerylurea and ethacrynic acid [17, 18].

The amodiaquine molecule *per se* has no electrophilic site susceptible to attack by glutathione, and therefore must be bioactivated in order for conjugation to occur *in vivo*. Previous studies from our laboratory indicate a facile *in vitro* oxidation of amodiaquine to a highly electrophilic intermediate, amodiaquine quinoneimine [7]. Direct reaction of synthetic amodiaquine quinoneimine (the product of a chemical oxidation of amodiaquine) with glutathione affords a mixture of three geometric isomers. The isomer which co-chromatographed with the *in vivo* glutathione conjugate was the major product of the chemical reaction. Proton NMR studies carried out on this synthetic isomer have identified the position of conjugation as C5′ on the *p*-aminophenol moiety (Fig. 2). Favourable electronic effects [19], as well as decreased steric hinderance, may dictate preference for addition at this site *in vitro*.

The detection and characterization of a glutathione conjugate of amodiaquine excreted into bile provides direct evidence that bioactivation of the drug to a chemically reactive intermediate has occurred *in vivo*. The formation of only one isomer *in vivo* suggests that the conjugation is enzyme-mediated, possibly by glutathione transferase, since the *in vivo* reaction is regioselective whereas the direct chemical reaction is not. Furthermore, conjugation of glutathione at the observed position indicates that the electrophilic metabolite formed from amodiaquine *in vivo* is amodiaquine quinoneimine.

To confirm amodiaquine quinoneimine formation *in vivo*, a study was carried out in which rats were administered synthetic radiolabelled amodiaquine

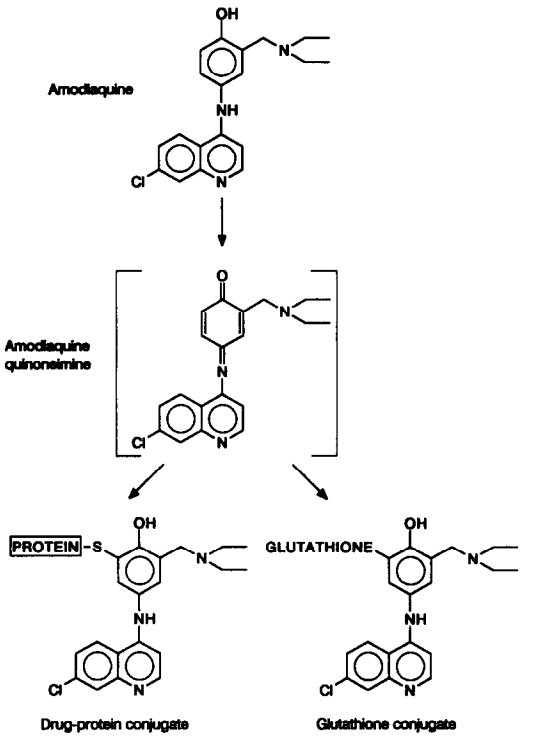


Fig. 10. Scheme to show the bioactivation, detoxication and antigen formation for amodiaquine *in vivo* in the rat.

quinoneimine. The biliary metabolite profile was identical to that seen in rats administered amodiaquine itself. This provides further evidence that the thioether conjugates detected in bile, after administration of amodiaquine, arise from amodiaquine quinoneimine.

The extensive bioactivation of amodiaquine to amodiaquine quinoneimine reflects the relatively low oxidation potential of the parent drug (774 mV) in comparison with drugs with similar chemical structures, e.g. paracetamol (1286 mV). The oxidation of paracetamol to its quinoneimine is catalysed by cytochrome P450 in humans (CYPIA2 and CYPIIE1 [9]) and in the rat [20], but is a minor pathway *in vivo* compared with conjugation pathways, such as glucuronidation and sulphation. These conjugation pathways appear to play no significant role in the metabolism of amodiaquine in the rat.

Enzyme inhibition studies using ketoconazole resulted in a significant decrease in the excretion of thioether metabolites in rat bile. Ketoconazole binds to cytochrome P450 with high affinity and is a potent inhibitor of P450-dependent drug oxidations in rat liver *in vitro* and *in vivo* [21, 22]. This suggests that the formation of thioether conjugates from amodiaquine in the rat is preceded by a cytochrome P450-mediated oxidation of the drug.

Distribution of radioactivity revealed two major sites of accumulation, the liver and bile. The radioactivity in bile has been identified as thioether conjugates of amodiaquine. There was no free amodiaquine in bile and very little in the liver. The major hepatic metabolite was unambiguously characterized by HPLC and mass spectrometry as desethylamodiaquine. The formation of the desethyl metabolite was also inhibited by ketoconazole, indicating that de-ethylation is catalysed by a P450 enzyme.

This metabolite has been demonstrated to possess 33–100% of the antimalarial activity of the parent drug [23]. The accumulation of desethylamodiaquine in the liver may have therapeutic and toxicological significance, since the life cycle of *P. falciparum* involves a hepatic phase. Interestingly, there was no evidence of the presence of a glutathione conjugate of desethylamodiaquine, despite the compound having a comparable oxidation potential to that of amodiaquine (693 mV and 774 mV, respectively).

We have demonstrated that amodiaquine becomes extensively metabolized to a chemically reactive intermediate, amodiaquine quinoneimine, in the rat, and that this metabolic activation can be cytochrome P450-mediated. Bioactivation of amodiaquine to its quinoneimine *in vivo* may have considerable toxicological significance. Chemically reactive metabolites such as this can bind to endogenous proteins, thus forming hapten conjugates. This can lead to the formation of anti-drug antibodies [24] and, indeed, chronic administration of amodiaquine to humans, rats and rabbits has resulted in the formation of antibodies recognizing amodiaquine–protein conjugates [6]. Direct evidence for the formation of protein conjugates from amodiaquine in the rat *in vivo* came from two complementary methods of analysis; irreversible binding to liver tissue after an acute dose, and immunoblotting of liver homogenates after chronic administration of radiolabelled amodiaquine. The combination of these two methods gives both quantitative and qualitative information on the formation of new antigenic determinants on liver proteins. One percent of a single dose of amodiaquine remained covalently bound in the liver, and such binding appeared to represent widespread modification of hepatic proteins, since Western blots of liver samples from rats after chronic amodiaquine treatment revealed several bands recognized by the amodiaquine antiserum. A similar pattern of multiple drug–protein conjugate formation was seen after administration of halothane to rats [13].

In conclusion, this study provides a metabolic rationale for the immunogenicity of amodiaquine in the rat (Fig. 10). This involves bioactivation of amodiaquine to amodiaquine quinoneimine, which binds spontaneously and covalently to thiol groups in endogenous proteins. If this metabolic pathway operates in humans, then induction or enhanced ability for this biotransformation, in particular individuals, may explain the observed toxic effects associated with amodiaquine in man.

The metabolism of amodiaquine in the rat provides a good model for metabolic activation leading to antigenicity of a drug.

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