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The anticoagulant activity of some selected warfarin analogues

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The anticoagulant activities of 6-, 7-, 8-, 4′-hydroxy, 6-chloro- and 6-bromowarfarin were determined in rabbits after intraperitoneal administration of 16·2 μmol kg^{-1} over 96 h. Substitution on the 4-hydroxycoumarin moiety resulted in reduction of the anticoagulant activity. 6-Chlorowarfarin was more potent than 6-bromowarfarin suggesting that the molecular size of 4-hydroxycoumarin moiety may be crucial for biological activity.

Warfarin is metabolized in-vivo in man to three oxidized derivatives, 4′-hydroxy-, 6-hydroxy- and 7-hydroxywarfarin and three reduced derivatives, the diastereoisomers of warfarin alcohol (designated 'alcohol 1' and 'alcohol 2') and dehydrowarfarin (Lewis & Trager 1970; Fasco et al 1978). In-vitro metabolic studies have shown that 8-hydroxywarfarin is a minor metabolite of warfarin when liver microsomes are uninduced, but that induction of the liver microsomes by polycyclic aromatic hydrocarbons causes about a 16-fold increase in the level of that metabolite (Fasco & Cashin 1980). The anticoagulant activity of the metabolites and other derivatives of warfarin has been of interest. It was reported that none of the metabolites except 4′-hydroxywarfarin possess significant anticoagulant activity (Barker et al 1970). However, Fasco & Cashin (1980) suggested that they may contribute to the anticoagulant activity of the parent drug. We have therefore examined the anticoagulant activity of some selected metabolites of warfarin.

Warfarin and its monohydroxylated analogues except 5′-hydroxywarfarin exist in solution in ring–chain tautomers (Scheme I) (Obaseki 1982). Barker et al (1970) postulated that the competitive inhibition of vitamin K by the coumarin anticoagulants was due to the production of a tautomer with characteristics resembling the synthetic water-soluble vitamin K, 2-methyl-1,4-naphthoquinone at pH 7 to 8. It was argued that the addition of any group on the benzene ring of the coumarin moiety could destroy the anticoagulant activity of the compound. This postulation was tested by investigating the potency of warfarin analogues with similar substituents (chlorine and bromine) on the same benzenoid carbon atom, carbon-6.

Materials and methods

Compounds. (±)-Warfarin (Sigma Chemical Co., St Louis, MO, USA) was recrystallized from 70% aqueous acetone; 6-hydroxy-, 8-hydroxy-, 6-bromo- and 6-chlorowarfarin were synthesized and chemically characterized as described by Obaseki et al (1985). 4′-Hydroxy- and 7-hydroxywarfarin were synthesized using the method of Hermodson et al (1971). 147·2 μmol of the test compound was dissolved in 0·1 M KOH (5 mL) and diluted to 12·5 mL with isotonic phosphate buffer (pH 7·3).

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Animals and treatments. Male Dutch white rabbits (1·3 to 1·6 kg), from a colony bred and maintained in the College of Medicine of the University of Lagos Animal House, were acclimatized to 12 h on–off light cycle for 7 days. They were permitted free access to food and water. 16·2 μmol kg^{-1} of the test compound was injected intraperitoneally to a group of four rabbits. Different groups of four rabbits were used for each of the analogues.

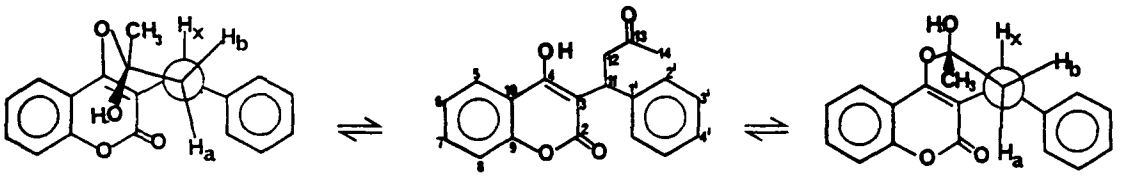
Anticoagulation studies. Blood (~1·0 mL) withdrawn from the ear vein of each rabbit into a plastic tube was mixed in a proportion of 9 : 1 with 3·2% sodium citrate in 0·7% NaCl. The blood was centrifuged at 2000 rev min^{-1} for 20 min at 4 °C. 'Prothrombin complex activity' was determined by the one-stage prothrombin time method of Quick (1966) utilizing human thromboplastin (Thromborel S, Hoechst-Behring, W. Germany) at 37 °C.

Plasma warfarin, 6-chloro- and 6-bromowarfarin determination. Warfarin, 6-chloro- and 6-bromowarfarin concentrations in plasma were determined spectrofluorometrically (O'Reilly et al 1963). Linear calibration curves with correlation coefficients equal or greater than 0·97 in the concentration range of 0 to 50 μg mL^{-1}, were obtained. Average recoveries of warfarin, 6-chloro- and 6-bromowarfarin were 98·0, 99·0 and 97·5%, with coefficients of variation of 8·0, 6·5 and 7·0%, respectively, in series of replicate analysis. Average recoveries of the monohydroxylated warfarins were less than 60% with high coefficients of variation. Hence, they have not been reported.

Statistical analysis. All results are expressed as means ± standard deviation. Significance of the difference between means was calculated using Student's *t*-test. The significance level was $P < 0.05$.

Results and discussion

The mean time course of the anticoagulant activity of warfarin and selected warfarin analogues as estimated by the prothrombin complex activity (PCA) (Nagashima et al 1969) after a single intraperitoneal dose



Scheme I

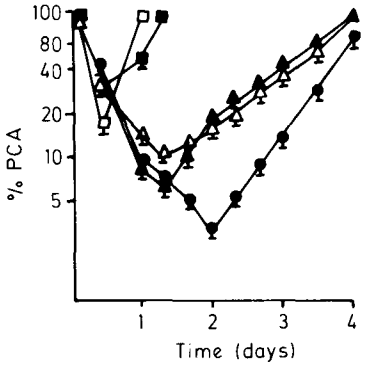


FIG. 1. Time course of mean percent of normal prothrombin complex activity (% PCA) after a single intraperitoneal dose (16·2 μmol kg^{-1}) in rabbits (n = 4 for each analogue): ● (±)-warfarin, ■ 7-hydroxywarfarin (typical of 6-hydroxy and 8-hydroxywarfarin), □ 4′-hydroxywarfarin, ▲ 6-chlorowarfarin, △ 6-bromowarfarin.

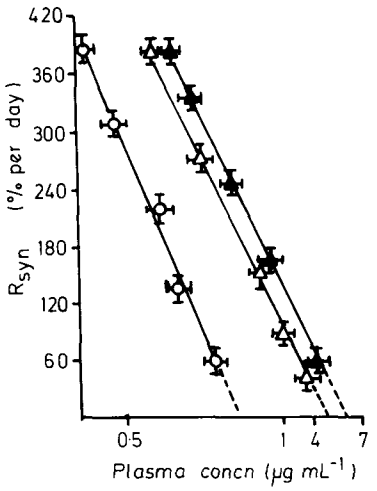


FIG. 2. Mean synthesis rate of prothrombin complex activity as a function of plasma concentration in rabbits (n = 4): ○ (±)-warfarin, △ 6-chlorowarfarin, ▲ 6-bromowarfarin.

(16·2 μmol kg^{-1}) is shown in Fig. 1. The maximum anticoagulant activities of warfarin, 6-chloro- and 6-bromowarfarin expressed as percent normal prothrombin activity were 3·1 ± 0·2, 6·0 ± 0·4, 10·2 ± 0·5% with corresponding plasma levels of 0·81 ± 0·04, 2·33 ± 0·12 and 3·05 ± 0·17 μg mL^{-1}, respectively. The areas under the curve of the plot of percent of normal prothrombin activity versus time for the substituted analogues studied were significantly less than that of warfarin ($P < 0·05$). It is obvious that substituents on the parent warfarin molecule reduced the anticoagulant activity.

Dose–response relationship is measurable in terms of 'prothrombin complex activity' versus plasma concentration. The 'real' effect of halogenation (X = Cl, Br) of the coumarin ring of warfarin was determined by the method of Nagashima et al (1969). This method is based on the fact that blood coagulability is a function of the rates of clotting factor synthesis (R_{syn}) and degradation (R_{deg}) in the body. Since coumarins inhibit the synthesis of clotting factors, R_{syn} is used as a measure of potency. R_{syn} values were calculated from the usually measured characteristic, prothrombin time. Fig. 2 shows the plot of R_{syn} versus log plasma concentration of warfarin, 6-chloro- and 6-bromowarfarin. Halogenation significantly reduces the 'real' anticoagulant activity; the plots are shifted to the right, the shift of the halogenated warfarins compared with warfarin may be explained in terms of decrease in 'fit' of the 4-hydroxycoumarin moiety to the active sites. Our observation agrees with the work of Overman et al (1944) which speculated that the minimal structural requirement for anticoagulant activity was an intact 4-hydroxycoumarin moiety with either a hydrogen or a carbon residue attached to C (3).

The monohydroxylated warfarins reduced the prothrombin activity more rapidly than warfarin whereas no such significant difference was observed between warfarin and the halogenated analogues as a group (Fig. 1). Warfarin is highly bound to serum ablumin; 6-, 7- or 8-hydroxywarfarin are less bound (O'Reilly 1971). Equimolar doses of warfarin and its analogues were used, therefore, the concentration of free monohydroxylated warfarins in the liver mav be higher initially than that of warfarin despite warfarin having a longer biological half life. This may account for the observed faster reduction of the prothrombin activity by the monohydroxylated analogues.

In conclusion, substitution of halogen or hydroxy moiety on the 4-hydroxycoumarin ring of warfarin results in significant loss of anticoagulant activity in rabbits. Despite 6- and 7-hydroxywarfarin plasma levels reaching considerable concentrations in experimental animals given warfarin, their contribution to anticoagulant activity is likely to be nominal.

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Influence of water deprivation on the disposition of paracetamol

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The effect of acute (96 h) water deprivation on the disposition of paracetamol (acetaminophen) has been examined in male Sprague-Dawley rats. Plasma and urinary concentrations of the drug and its two major metabolites, the glucuronide and sulphate, were determined by a sensitive and specific high performance liquid chromatographic assay. Following an intravenous dose of 100 mg kg^{-1} of paracetamol, no significant changes were found in the elimination rate constant (k), the mean residence time (MRT), total plasma clearance (Cl) and the apparent volume of distribution at steady-state (Vss). However, rats deprived of water for 96 h excreted a larger percentage of the administered dose as the glucuronide conjugate (15·3 vs 7·9%) and a smaller percentage as unchanged paracetamol (7·3 vs 20·7%) in the urine. In addition, there was a significant two-fold increase in the partial metabolic clearance to paracetamol glucuronide. Water deprivation also led to a significant reduction in the renal clearance of paracetamol accompanied by an increase in the renal clearance of the glucuronide.

It is known that humans do not always replace orally all the fluids lost in sweat and urine, a condition referred to as voluntary dehydration (Greenleaf & Sargent 1965). Most frequently, the system may be dehydrated as a result of (i) disease states such as polyurea, diarrhoea, oesophageal or pyloric obstructions, (ii) exposure to tropical climates and high or humid temperatures, (iii) accidental fluid loss as in the case of haemorrhage, and (iv) circumstantial water deprivation as in severe muscular exercise. Any type of water deprivation, either acute or chronic, whether developed or induced, subjects the system to a stress, leading to significant hormonal, enzymatic and physiological changes (Jones & Pickering 1969; Hatton 1971; Baetjer & Rubin 1976; Keil & Severs 1977).

Ahmad et al (1982) and Bakar & Niazi (1983) have reported that the pharmacokinetics of aspirin and chloramphenicol are altered in water-deprived rats. Specifically, it has been shown that the rate of microsomal oxidative reactions (phase I) using a model drug, antipyrine, can be altered substantially (Prasad et al 1985). However, there is a paucity of data on phase II enzymes. Of the major conjugation reactions in animals and man, glucuronide and sulphate formation are two of the most common routes of drug biotransformation for many drugs and are quantitatively the most important of the phase II conjugations.

The present study was undertaken to investigate the disposition kinetics of paracetamol (acetaminophen) in control (food and water freely available) and 96 h water-deprived (food freely available) rats. Paracetamol was chosen as a model drug for the study of phase II metabolic pathways because of its widespread use and clinical importance, low binding to plasma proteins and quantitative metabolism primarily to the glucuronide and sulphate conjugates without having to go through a phase I metabolism.

Methods

Male Sprague Dawley rats (Locke-Erickson Laboratories, Oak Park, IL), 225 to 300 g, were randomly assigned to a treatment (water-deprived) or control group and given free access to food. The treatment rats were deprived of water for 96 h before administration of the drug while the control group was allowed water. Body weights of all rats were recorded before and after the 96 h to assess the influence of water deprivation.

At the end of the 96 h the right jugular vein and carotid artery were catheterized with silastic and polyethylene tubing, respectively, under light ether anaesthesia. All animals were housed individually in plastic

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