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Anti-warfarin antibody preparation and its characterization for radioimmunoassay

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Anti-warfarin antiserum was prepared in rabbits by immunization with a synthesized warfarin antigen, 4′-azo-warfarin human serum albumin, which possesses two enantiomorphic haptenic sites of warfarin on the molecule. The antiserum recognized both *R*- and *S*-warfarin to the same degree, 50% cross reactivities of racemic warfarin, respectively. One of the warfarin metabolites, racemic warfarin alcohol, showed 1% cross reactivity, and *R*- or *S*-warfarin alcohol have half the reactivity of the racemic alcohol. Rabbit plasma warfarin levels were determined by radioimmunoassay using this antiserum and racemic [^{14}C]warfarin and by fluorometric assay after isolation by thin layer chromatography. After a single administration of warfarin (2 mg kg^{-1} orally or 500 μg kg^{-1} i.v.), the plasma levels determined by both assay methods showed a good correlation ($r = 0{\cdot}97$, $P < 0{\cdot}001$, $Y = 1{\cdot}04–0{\cdot}09$). The results show that the radioimmunoassay can determine total plasma warfarin without interference of plasma metabolite. The applicability and limitation of the radioimmunoassay for pharmacokinetic study are discussed.

Earlier methods for determination of warfarin concentrations in plasma are laborious, since they were based on native fluorescence (Lewis et al 1970), phosphorescence (Bridges 1974) or absorption (Fishwick & Taylor 1967) of the drug and therefore, required its extraction and separation from other drugs in the plasma.

We attempted to establish a radioimmunoassay for the direct determination of plasma warfarin avoiding extraction and separation. During the course of our study, Cook et al (1979) reported an excellent radioimmunoassay method for warfarin which can determine stereospecifically blood warfarin as racemic mixture.

The warfarin used clinically is a racemic mixture composed equally of two optical enantiomers, the (*R*) (+)-warfarin (*R*-warfarin) and the (*S*)(−)-warfarin (*S*-warfarin). *S*-warfarin has a hypoprothrombic effect four to five times greater than *R*-warfarin in both rat (Eble et al 1966) and man (O'Reilly 1974). Also the stereospecificity of the warfarin metabolism (Pohl et al 1976) and some drug interactions of warfarin (Lewis et al 1974) have been demonstrated.

We have obtained anti-warfarin rabbit antiserum, which recognizes both *R*- and *S*-warfarin to the same degree, by immunization of rabbits with racemic warfarin antigen synthesized using as hapten acenocoumarin, a nitro derivative of warfarin. With this antiserum, direct radioimmunoassay of warfarin in rabbit plasma after a single administration of racemic warfarin was made and the results compared with those from fluorometric determination combined with thin layer chromatography.

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MATERIALS AND METHODS

Radioactive warfarin, 3-α-acetonyl [α-^{14}C]benzyl-4-hydroxycoumarin (specific activity 49 mCi mmol^{-1} was purchased from The Radiochemical Centre Amersham (Buckinghamshire, HP79LL, U.K.). Warfarin was purchased from Sigma Chemical Company (St Louis, Mo., U.S.A.). Potassium warfarin was kindly provided by Eisai Co, Ltd (Tokyo, Japan). Acenocoumarin, 3-(α-acetonyl-*p*-nitrobenzyl)-4-hydroxy coumarin, was supplied as Sintrom tablet from Ciba-Geigy Ltd (Basel, Switzerland), and was extracted and recrystallized. The chemical purity was confirmed by melting point and elemental analysis. This compound is racemic of *R*- and *S*-4′-nitrowarfarin.

R- and *S*-Warfarin isomers were prepared by resolution of racemic warfarin according to West et al (1961) with slight modifications.

Racemic, *R*- and *S*- warfarin alcohols were synthesized from each corresponding warfarin i.e. racemic, *R*- and *S*-warfarin, as follows; 1 g of warfarin was dissolved in 10 ml of warmed 0·01 M NaOH and was slowly added with 0·1 g of $NaBH_4$ and stirred for 1 h at 0 °C.

The reaction mixture was acidified with 0·1 M HCl and the resulting precipitate was washed with water and dried in a vacuum. The dried powder was dissolved in a small amount of acetone and applied

on preparative thin layer chromatography plates and developed in a solvent, methanol–benzene (33:77). Under 254 nm light the warfarin band was detected, scraped and extracted with acetone. After evaporation of the acetone, the warfarin was recrystallized from isopropyl alcohol. Racemic warfarin alcohol contains two alcohol isomers, (*R,R*)-, (*R,S*)-; and similarly, the *S*-warfarin alcohol contains (*S,S*)-, (*S,R*)-warfarin alcohols. The chemical structure and purity of each warfarin alcohol were confirmed by melting point, elemental analysis and $[\alpha]_D^{24}$ value, and were verified with the reported values (Trager 1970). Dicoumarol and vitamin Ks were obtained commercially from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Human serum albumin (HSA) was purchased as fraction V powder from Armer Pharmaceutical Co Ltd (Tower phenix, Ariz., 85077, U.S.A.).

Preparation of immunogen

4′-Aminowarfarin, 3-(α-acetonyl-*p*-aminobenzyl)-4-hydroxy-coumarin, was prepared by the catalytic reduction method of Butler (1956) with a slight modification. The chemical structure of the crude crystal (70% yield) was confirmed by i.r. spectrum and immediately used for immunogen synthesis. Warfarin antigen was synthesized by the coupling of crude 4′-aminowarfarin with HSA via diazo bond by a method similar to that described previously (Satoh et al 1973) (Fig. 1).

Sodium nitrite, 9·7 mg in 10 ml of water, was added to 4′-amino-warfarin, 350 mg dissolved in 50 ml of an aqueous solution containing 0·1 M HCl and 7 mM potassium bromide, for 20 min with continuous stirring and the solution was kept in an ice bath for 1 h. During the addition, the pH was kept between 9·0 to 9·5 with 0·1 M NaOH. The reaction mixture was further stirred for overnight at 0 °C and then dialysed against water.

Thus, warfarin antigen obtained as 4′-azo-warfarin HSA was lyophylized and stored at −20 °C.

Immunization and test of antiserum

A solution in 0·9% NaCl of 0·5% 4′-azowarfarin HSA was emulsified with an equal volume of Freund's complete adjuvant. Male albino rabbits, 3 kg, were injected intramuscularly in both thighs and subcutaneously in eight different sites at the back. The total dose administered per rabbit was 3 mg of 4′-azowarfarin HSA. Booster injections were repeated monthly. Blood was obtained monthly from the vein of the ear one week after the booster injection, and after coagulation, the serum was separated by centrifugation. All sera thus obtained were tested for binding capacity with [^{14}C]warfarin, which was determined as follows: [^{14}C]warfarin, about 10 000 counts min^{-1} in 0·1 ml, 0·1 ml of one-tenth diluted test serum and 0·3 ml of 0·1 M Tris-HCl buffer (pH 7·6) were mixed and allowed to stand for 45 min at room temperature (20 °C). After the addition of 0·5 ml of saturated ammonium sulphate solution in Tris-HCl buffer to the mixture, the precipitate was collected by centrifugation (3000 rev min^{-1} for 15 min). The precipitate which contains [^{14}C]warfarin bound with antibody was washed twice with 0·5 ml of 50% saturated ammonium sulphate solution. The wall of the test tube was wiped with tissue every time after the decantation of supernatant and the precipitates finally obtained were dissolved in 1 ml of distilled water and transferred to a counting vial containing 10 ml of scintillator. Normal serum, which lacked binding activity to warfarin, was used as a blank and its count was subtracted as a background.

The scintillator was toluene-Triton emulsion phosphor. 2,5-Diphenyl-oxazole (DPO) (4 g) and 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene

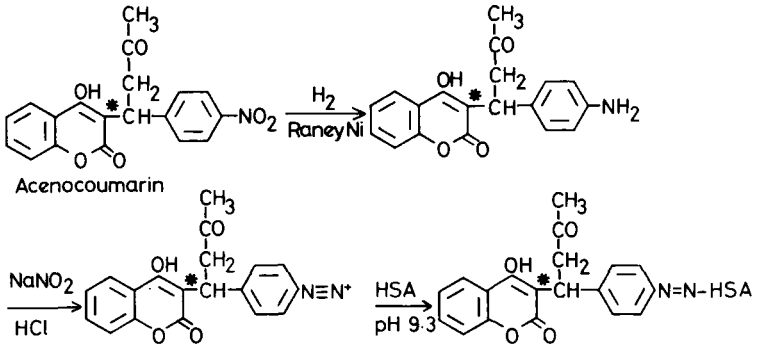

FIG. 1. Synthetic pathway to immunogen for racemic warfarin.

(dimethyl-POPOP) (100 mg) were dissolved in 1000 ml of toluene and to this solution was added 500 ml of Triton X-100. The radioactivity was counted in Aloka Liquid Scintillation Counter (Japan Radioactive & Medical Electronics Inc.).

Radioimmunoassay procedure

The radioimmunoassay is based on competitive binding of unlabelled and labelled warfarin to anti-warfarin antibody. In this experiment, 0·1 M Tris-HCl buffer (pH 7·6) was used for the dilution of antisera, sample plasma and preparation of cold ligands. Incubation mixture: 0·1 ml of [^{14}C]warfarin solution about 4000 counts min^{-1} was added to a 10 mm × 8 mm siliconized brown glass tube containing 0·1 ml of one-tenth diluted antiserum and 0·1 ml or 0·05 ml of cold ligand solution. Tris-HCl buffer was added to make a final volume of 0·5 ml. The mixture was allowed to stand overnight at 4 °C. The subsequent procedure was the same as described in the determination of antibody binding capacity with [^{14}C]warfarin.

Animal study

An animal study was made to examine the effect of warfarin metabolites, or any intrinsic substance which may exist in plasma, on the binding of [^{14}C]warfarin to the antibodies.

After 40 h fasting, two male rabbits were given orally potassium warfarin, 2 mg kg^{-1} (as warfarin). In another experiment, potassium warfarin (500 μg kg^{-1} as warfarin) was intravenously injected into two male rabbits. Blood samples were collected from the vein of the ear at 0·5, 1, 2, 3, 6, 9 and 12 h after the drug administration. Blood was drawn into a syringe containing 0·2 ml of 0·1 M sodium oxalate. After centrifugation, the plasma was used for radioimmunoassay in one-tenth dilution and 1 ml of the original plasma was used for fluorometric assay by the method of Bachman & Burkman (1975), in which the warfarin spot after thin layer chromatography is eluted with 2 ml × 2 acetone. Three ml of the elute was taken to dryness under vacuum and the residue dissolved. The fluorescence was read in 1 ml of *NN*-dimethylformamide at excitation and emission wavelength of 330 and 408 nm respectively, with a Hitachi Fluorescence Spectrophotometer MPF-2A.

RESULTS

Antibody production

Two rabbits were immunized and their antibody production was checked as described under Materials and Methods. The production of anti-warfarin antibodies followed the pattern shown in Fig. 2. Two rabbits immunized with synthesized warfarin antigen demonstrated a primary antibody response to [^{14}C]warfarin.

The antiserum obtained in rabbit No. 1 one week after three booster injections bound 50% of added [^{14}C]warfarin (4000 counts min^{-1}) in ten-fold dilution.

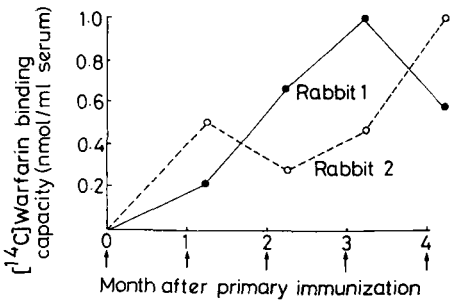

FIG. 2. Binding of [^{14}C]warfarin by sera of two rabbits during the course of the immune response to warfarin.

Characterization of antibodies

For characterization of the antibodies, the radioimmunoassay was performed with various ligands using antiserum obtained in rabbit No. 1 one week after three booster injections. An inhibition curve of the binding of [^{14}C]warfarin to the antibodies by racemic warfarin is shown in Fig. 3. Cross reactivities of various ligands were represented from the reciprocal of the number of moles required for 50% inhibition of binding, as shown in Table 1(a) and (b).

In the Table 1(a), the coumarin anticoagulant, dicoumarol, had 0·05% cross reactivity and vitamin Ks showed no detectable affinity to the antibodies.

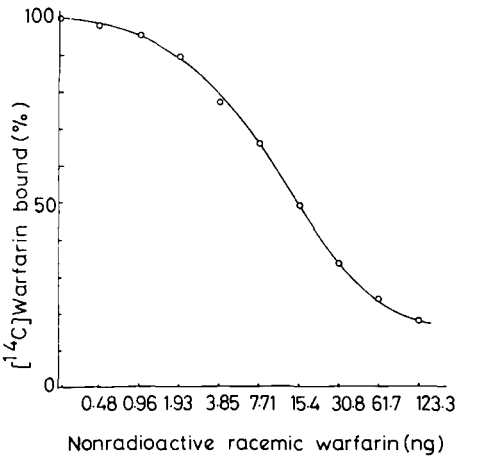

FIG. 3. An inhibition curve of the binding of [^{14}C]warfarin to the antibodies by racemic warfarin.

Table 1. Cross reactivities of structure related compounds (a) and each enantiomer of warfarin and warfarin alcohols (b) against antiserum.

Ligands	Cross reactivities (%)
(a)	
Warfarin	100
Acenocoumarin	240
Dicoumarol	0.051
Vitamin K_1	<0·037
Vitamin K_3	—
Vitamin K_5	—
(b)	
(±)-Warfarin	100
R(+)-Warfarin	48·6
S(−)-Warfarin	47·2
(±)-Warfarin alcohol (*R-R, R-S, S-S, S-R*)	1·02
R-Warfarin alcohol (*R-R, R-S*)	0·45
S-Warfarin alcohol (*S-S, S-R*)	0·50

Acenocoumarin, the original substance of haptenic sites in immunogen, had a larger affinity than warfarin.

As shown in Table 1(b), the *R*-, *S*-warfarin showed fifty-fifty cross reactivity and racemic warfarin alcohols showed 1% of the cross reactivity of racemic warfarin.

The affinity of both *R*-, and *S*-warfarin alcohol is one-half that of the racemic warfarin alcohol.

Plasma levels of racemic warfarin

Plasma levels of warfarin administered as racemic warfarin were determined using a standard curve obtained using the antiserum of rabbit No. 1 as shown in Fig. 3 and were compared with those obtained by fluorometric assay (Fig. 4).

Plasma levels in rabbits showed a highly significant correlation between radioimmunoassay and fluorometric assay after thin layer chromatography ($r = 0{\cdot}97$, $P < 0{\cdot}001$, $Y = 1{\cdot}04X - 0{\cdot}09$, $n = 28$). Those results showed that direct radioimmunoassay of diluted plasma could determine the total plasma level of racemic warfarin virtually without interference from the plasma component and from metabolites of warfarin.

DISCUSSION

Although the titre was not so high, we obtained an antiserum with a binding affinity of fifty-fifty to each enantiomer, which is unusual since antisera prepared by immunization with an antigen that possesses two enantiomorphic haptenic sites, i.e. d and l, in most cases show an imbalance in binding affinity to each isomer (Maeda & Tsuji 1981).

Radioimmunoassay using antisera with no stereospecificity and commercially available racemic labelled warfarin cannot determine each isomer but are suitable for the total determination of warfarin. Therefore, we examined whether total warfarin in the plasma, where the *R*- and *S*-warfarin isomers are probably present in imbalanced amounts, can be determined by our assay and confirmed this by the fact that the plasma levels of warfarin determined by direct radioimmunoassay of plasma and those by fluorometric assay after isolation of warfarin from plasma were consistent.

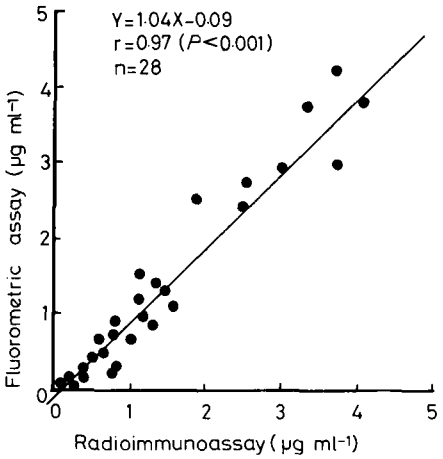

FIG. 4. Comparison of plasma warfarin levels in 28 plasma samples, as determined by direct radioimmunoassay of plasma and by fluorometry after separation by thin layer chromatography.

Although trace amounts of warfarin alcohol and hydroxywarfarin were detected in the rabbit plasma by thin layer chromatography (data not shown), they did not intefere in rabbit plasma radioimmunoassay. Many warfarin metabolites have been reported in various species (Fasco et al 1978 a, b, c) but we were not able to determine the cross reactivities of those metabolites except warfarin alcohol because they were not available.

Although our warfarin antiserum is not stereospecific, it could determine total warfarin in rabbit plasma after a single administration of racemic warfarin and is practically specific for warfarin versus its metabolites in plasma, considering the low cross reactivity of warfarin alcohols as shown in Table 1b. The sensitivity of the assay, 10 ng at 50% inhibition point, is considerably greater (100×) than fluorometry and has the advantage of simplicity.

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