J. Pharm. Pharmacol. 1980, 32: 709

COMMUNICATIONS

Displacement of albumin-bound warfarin by anti-inflammatory agents in vitro

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Much work has been done on the examination of warfarin binding to, and displacement from, serum albumin binding sites especially by phenylbutazone (for example, Aggeler et al 1967; Schary et al 1975). Inhibition of the metabolism of the *S*-isomer of warfarin by phenylbutazone has also been suggested as an alternative mechanism for the increased anticoagulation (Lewis et al 1974; Schary et al 1975).

Azapropazone, which is structurally similar to phenylbutazone, has also been implicated in increased warfarin anticoagulation in the clinic (Green et al 1977; Powell-Jackson 1977). Since azapropazone is highly bound (approximately 95%) to human serum (Jones 1976) and since it displaces warfarin in vitro from plasma binding (McElnay & D'Arcy 1978) we have compared this displacement quantitatively with that given by a range of other non-steroidal anti-inflammatory agents that have been implicated in warfarin displacement interactions in man as the results might indicate the relative potential of these agents to interact with warfarin at albumin binding sites.

Materials and methods. The spectrofluorometric titration technique used was based on the method of Henry & Wosilait (1975) which depends on increased warfarin fluorescence when it is bound to albumin and a subsequent decrease in fluorescence when warfarin is displaced. An excitation wavelength of 320 nm and an emission wavelength of 390 nm was used.

Drugs and albumin solutions were made up in Tris buffer (75 mM, pH 7·4). Some of the anti-inflammatory agents examined were not readily soluble in buffer and were therefore dissolved in a few drops of 0·1 M NaOH and made up to volume with buffer, the pH being adjusted as required.

A sample of 3·5 ml of human serum albumin (0·5 mg ml^{-1}) plus anti-inflammatory drug (20 μl volume) was added to a cuvette; fluorescence emission was zeroed to negate native fluorescence of the protein as well as any enhancement or quenching of fluorescence by the anti-inflammatory drug. A 10 μl sample of warfarin (0·02 mg ml^{-1}) was then added to the albumin-anti-inflammatory drug mixture; the contents were mixed by inversion and the fluorescence emission again measured. This procedure of serial alternate additions and recordings was repeated 15 times.

The titration of warfarin was also carried out by this procedure but with either albumin or buffer alone in the cuvette; the former gave a control reading with which anti-inflammatory data could be compared, while the latter gave background fluorescence for warfarin. The latter background results were subtracted from all experimental results before titration curves were plotted. All titrations were in triplicate.

The maximum warfarin fluorescence in the presence of each anti-inflammatory agent was compared as a percentage of that of the control (warfarin-albumin alone). The percentage decrease in fluorescence value was then used as a measure of the agent's ability to displace warfarin from its albumin binding.

In the clinical setting, plasma concentrations attained during the treatment of inflammatory disease vary from one anti-inflammatory agent to another; therefore the drug concentrations tested were varied in the same ratio as those of the therapeutic plasma concentrations of the drugs (see Table 1). The actual solutions used were the normal therapeutic plasma concentrations multiplied by the factor 0·0175; this gave optimal cuvette concentrations for the interaction study.

Results. Warfarin fluorescence increased greatly when it was bound to human serum albumin (Fig. 1); the fluorescence was quenched when known displacing agents were added to the in vitro system. A typical example of such quenching is illustrated in Fig. 2 which shows a marked decrease in warfarin fluorescence at all points on its spectrofluorometric titration curve in the presence of azapropazone.

Comparative data for the other anti-inflammatory drugs tested have been presented in Table 2; paired *t*-test values for the triplicate end-point pairs (control versus test in each case) are shown.

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Table 1. Therapeutic plasma concentrations of anti-inflammatory drugs obtained during normal treatment. Where a range of plasma concentrations is quoted that used in the present experiments is given in parentheses.

Drug	Therapeutic concn (μg ml^{-1})	Drug solution used (μg ml^{-1})	Reference
Azapropazone	90	1·575	Leach 1976
Phenylbutazone	25·3–95·0 (75)	1·3125	Orme 1977
Naproxen	40–60 (50)	0·875	Personal communication (Syntex 1978)
Indomethacin	0·5–3 (2)	0·035	Alvan et al 1975
Mefenamic acid	2–8 (5)	0·0875	Personal communication (Parke-Davis 1978)
Ketoprofen	0·4–8 (6)	0·105	Personal communication (Bayer 1978)
Ibuprofen	30–40 (35)	0·6125	Slattery & Levy 1977

* selected therapeutic plasma concentration (in parentheses) multiplied by a factor of 0·0175 to give optimal cuvette concentration; 20 μl was added to the cuvette.

Azapropazone evoked the greatest degree of quenching of warfarin fluorescence (85·4%) which illustrated its displacement of warfarin from its albumin binding, followed by phenylbutazone (74·2%). Naproxen, indomethacin, mefenamic acid and ketoprofen produced a lesser degree of fluorescence quenching but at a statistically significant level ($P < 0.05$).

Unexpectedly, ibuprofen produced a slight (5·69%) but significant increase in fluorescence of warfarin; this effect was surprising since Slattery & Levy (1977) reported decreased warfarin binding to albumin in the presence of this drug.

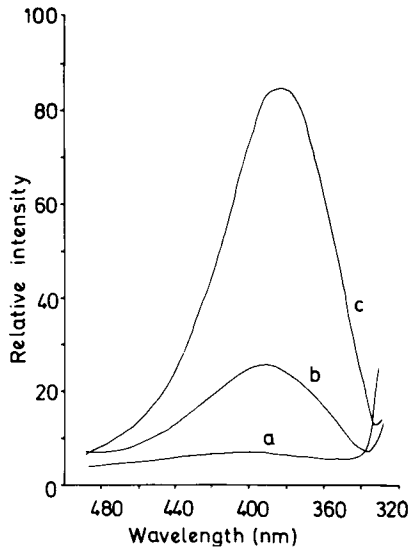

FIG. 1. Fluorescence emission scans of albumin (a), warfarin (b) and a warfarin-albumin complex (c).

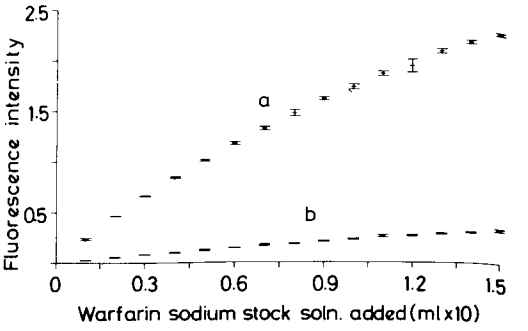

FIG. 2. Spectrofluorometric titration of warfarin alone in albumin (a) and while in combination with azapropazone (b). The plotted values have been corrected for background warfarin fluorescence and represent the mean ± s.e. of triplicate titration results

Discussion. From the present experimental evidence it is possible to predict that only azapropazone and phenylbutazone are likely to enter into clinically important displacement of warfarin from its albumin binding. Such a prediction agrees well with data on actual clinical interaction in which azapropazone (Powell-Jackson 1977) and phenylbutazone (Aggeler et al 1967) have given rise to increased anticoagulation. The other anti-inflammatory drugs tested in the present study do not seem to cause clinically significant interaction with warfarin. For example, Penner & Abbrecht (1975) demonstrated a lack of interaction between ibuprofen and warfarin in man, while Jain et al (1979) found that naproxen had little effect on warfarin anticoagulation. Vessell et al (1975) failed to show an interaction between warfarin and indomethacin in man; although Self et al (1978) described one case of warfarin potentiation by indomethacin, they commented that the interaction was obviously of low incidence. The lack of case reports would also suggest that ketoprofen and mefenamic acid do not evoke clinically important interactions with warfarin.

Sellers (1978), while considering the implications of drug displacement from albumin binding, suggested that increases in free and active drug concentrations were transient because of compensatory elimination and redistribution effects. He concluded that, as a sole mechanism of interaction, protein binding displacement would be clinically unimportant but that a combination of decreased drug clearance and plasma binding displacement could often result in serious interaction.

The fact that drugs capable of competing with each other for albumin binding sites might also compete for elimination and metabolic pathways has already been emphasized by Gillette (1973). It may be suggested therefore, since azapropazone and phenylbutazone give rise to increased warfarin anticoagulation in man and

Table 2. Fluorescence changes of warfarin while in combination with the anti-inflammatory agents (concentrations varied in the same ratio as normal therapeutic plasma concentrations). Data obtained using the spectrofluorometric quenching titration technique described in the text.

Drug	Fluorescence decrease %	t*	P	Order of displacing potency
Azapropazone	85·37	89·23	< 0·001	1
Phenylbutazone	74·22	57·60	< 0·001	2
Naproxen	14·89	6·15	< 0·05	3
Indomethacin	11·11	7·45	< 0·02	4
Mefenamic acid	10·76	7·06	< 0·02	5
Ketoprofen	6·47	11·64	< 0·01	6
Ibuprofen	− 5·69	5·88	< 0·05	fluorescence enhancement

* paired t-test value with 2 degrees of freedom (i.e. number of pairs minus one)

also to warfarin displacement in vitro, that protein binding displacement may also have a role in the prediction of drug involvement in the unrelated elimination interaction mechanism.

The results from a model capable of comparing the effects of the agents on warfarin metabolism together with binding results could lead to a more comprehensive prediction of warfarin—anti-inflammatory interactions.

February 29, 1980

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Improved fluorimetric assay of chloroquine in biological samples

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Chloroquine concentration in biological samples is now commonly determined using the fluorimetric method of McChesney et al (1962) as modified by Rubin et al (1965). The method consists of extraction of chloroquine into heptane or methylene dichloride from an alkali medium buffered with borate, pH 9·5. Chloroquine is re-extracted into 0·1 M HCl which is then mixed with an equal volume of 0·2 M alcoholic NaOH and fluorescence read at activation and emission wavelengths of 335 and 400 nm respectively. The accuracy and sensitivity of the Rubin et al (1965) method was criticized by Schulman & Young (1974) because the fluorescence of chloroquine depends on pH and the pH conditions specified by Rubin et al (1965) appear to provide an ideal condition only for the separation of chloroquine from its metabolites but not to provide maximum sensitivity in fluorimetric analysis. The Rubin et al (1965) method, even as modified by Schulman & Young (1974), often results in erratically high values for blanks and has a poor reproducibility below 10 ng ml^{-1}. By introducing minor modifications to the method of Rubin et al (1965) we have substantially increased the sensitivity and accuracy of the method.

Chloroquine was added from a stock solution (1 mg ml^{-1} in 0·1 M HCl) into 1 ml of plasma, red cell, urine or 0·1 M HCl in 15 ml round-bottomed Quickfit centrifuge tubes, made alkaline with 0·2 ml ammonia solution (S.G. 0·91) and extracted with 5 ml purified diethyl ether by shaking on a Gallenkamp electric shaker for 5 min. The aqueous and organic layers were separated by centrifugation at 2500 rev min^{-1} for 2 min. The

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