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.

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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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organic supernatant was pipetted into a clean tube and the extraction procedure repeated with a second 5 ml volume of ether. The pooled ether extract was washed with 2 ml borate buffer pH 9·5 after which 8 ml of the organic layer was shaken for 2 min with 4 ml phosphate borate buffer pH 7·85 to remove metabolites (when present). To 7 ml of the organic layer 4 ml of 0·1 M HCl was added. After it had been shaken for 2 min, the organic supernatant was removed by aspiration. To 3 ml of the acid layer was added 3 ml of 0·2 M sodium hydroxide and 3 ml phosphate borate buffer pH 7·85 and fluorescence was measured with a Perkin-Elmer model 204 spectrofluorimeter at excitation and emission wavelengths of 331 and 386 nm respectively using 1 cm cuvettes.

The material extracted from the above procedure was identified by thin layer chromatography. The pooled ether extract was evaporated to small volume (0·1 ml) and 0·1 ml methanol added. 2 $\mu$l was then spotted on a t.l.c. plate and developed in strong ammonia–methanol (1·5:100) for 30 min (equilibration time was 1 h). Location was by Dragendoff spray. The ether extracts of standard solutions of chloroquine in plasma, red cell and urine showed only one spot which was identical to the spot occupied by chloroquine added to 0·1 M HCl and passed through the same extraction process. The $R_F$ values for all the spots were the same.

Before a final decision on the pH at which fluorescence was to be read, the effect of pH on fluorescence was determined. The results showed that maximum fluorescence occurred at pH 9·9 to 10·2 and agree with those of Schulman & Young (1974) but differ from those of McChesney et al (1962, 1966) and Rubin et al (1965). The fluorescence spectra obtained at pH 9·9 and at pH 11·8 (used by Rubin et al 1965), with excitation wavelength set at 331 or 335 nm, were similar although fluorescence intensity was less at pH 11·8. Maximum excitation occurred at an emission wavelength of 386 nm but there was also a minor peak at 330–335 nm which was presumably Rayleigh scattered light. A standard curve was plotted for 0, 5, 10, 20, 50 and 100 ng ml$^{-1}$ chloroquine in HCl and gave a straight line. A similar curve was obtained for chloroquine standards in plasma, red cells and urine. The coefficient of variation of 5 determinations of a single sample of 5 ng ml$^{-1}$ was 20·7% and that of 100 ng ml$^{-1}$ was 2·2%. The mean coefficient of variation over the whole range of standard samples was 10·2 s.d. 3·3%. An assay of acceptable accuracy can thus be expected down to a concentration of 5 ng ml$^{-1}$.

Because comparison of limits of detection obtained in different studies are limited by the fact that different fluorimeters may well have different sensitivities, we compared the limit of detection and the percentage recovery by the above method with those using the method of Rubin et al (1965). Our results showed that percentage recovery was better with the modified method at concentrations between 0·1 and 1 $\mu$g ml$^{-1}$ and that at 10 ng ml$^{-1}$ and below, the method of Rubin et al had poor reproducibility. Diethyl ether appears to be the most suitable solvent because, having a lower boiling point than heptane and methylene dichloride, the samples used in the final fluorimetric assay are less easily contaminated by light-scattering droplets or the deposition of a solvent film on the inside of the cuvette. Double extraction, which is easy with ether since it forms the upper layer, improves the extraction ratio and efficiency (from 85–97%) and minimizes pipetting errors. Ether also has the advantage that it does not readily form emulsions with plasma.

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