Tilorone hydrochloride: a non-steroidal anti-inflammatory agent devoid of prostaglandin synthetase inhibitory activity

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Tilorone hydrochloride (2,7-bis(diethylaminoethoxy) fluoren-9-one hydrochloride) was first described as an orally active anti-viral agent (Kreuger & Mayer, 1970; Mayer & Kreuger, 1970). It was subsequently found to have the properties of stimulating humoral immune responses and inhibiting cell mediated immune responses and in the adjuvant arthritis test in the rat inhibited both the primary and secondary inflammatory responses (Megel, Raychaudhuri & others, 1974). Later work showed that in the rat it inhibited carrageenan-induced paw oedema, a carrageenan abscess model and the direct passive Arthus reaction provided that a priming dose was given 24 h before the inflammatory insult (Megel, Raychaudhuri & others, 1975).

The effect of tilorone on prostaglandin synthetase activity *in vitro* was measured using the guinea-pig lung enzyme and the *in vivo* actions on both the prostaglandin and leucocyte content of inflammatory exudates were measured in polyvinyl sponges implanted for 9 h in the rat (Walker, Smith & Ford-Hutchinson, 1976). No effect on the synthesis of prostaglandins *in vitro* was observed with tilorone at concentrations ranging from 10 to 100 μg ml^{-1}, but indomethacin produced a significant inhibition with concentrations as low as 0·1 μg ml^{-1} (43 % inhibition, 0·1 μg ml^{-1}, 83 % inhibition, 1 μg ml^{-1}). Tilorone exerted a pronounced effect on leucocyte emigration *in vivo* into the sponges when a 24 h priming dose was used (Table 1). In contrast no significant effects were observed on the prostaglandin content of the exudate at any of the doses studied. Conventional acidic non-steroidal anti-inflammatory drugs, such as aspirin, phenylbutazone and indomethacin, cause substantial inhibitory effects both on prostaglandin accumulation and leucocyte migration in this model (Walker & others, 1976) and in paw oedema and pleurisy in the rat (Di Rosa & Willoughby, 1971; Meacock & Kitchen, 1976). The only other compound known to exert its anti-inflammatory action in the sponge model by inhibiting leucocyte migration without affecting prostaglandin content, is a low molecular weight anti-inflammatory fraction isolated from normal human plasma (Smith & Ford-Hutchinson, 1975). It has been proposed that the human plasma fraction exerts its anti-leucotactic effect by a complement mediated mechanism (Walker, Smith & others, 1975). There is also evidence that tilorone may interact with the complement system possibly by interfering with the consumption of complement via either the classical or alternate pathways (Megel & others, 1975).

Table 1. *Effects of tilorone hydrochloride on prostaglandin content and leucocyte migration in 9 h sponge exudates in the rat.*

Dose of drug mg kg^{-1}	Total leucocyte counts $\times 10^3 \mu l^{-1}$	Inhib. %	Prostaglandin content ng ml^{-1}
Control	17.4 (4.5)	—	31 (12)
2 × 100 (4)	4.1 (1·5)*	76	19 (4)
2 × 67 (5)	3·3 (1·2)*	81	26 (3)
2 × 33 (9)	8·3 (4·7)*	53	31 (13)
2 × 15 (5)	14·0 (1·7)	20	nd
1 × 67 (5)	14·8 (4·2)	15	28 (6)

Results are expressed as means (with s.d.), number of rats in brackets. Tilorone hydrochloride was administered orally either at −1 h and — 24 h or at —1 h alone.
* $P < 0·01$ from results of corresponding control groups.

Tilorone hydrochloride may be representative of a class of anti-inflammatory agents with a novel mode of action. It would be of interest to test if it possesses analgesic and antipyretic properties, other actions of conventional non-steroidal anti-inflammatory drugs which have been postulated to be mediated by prostaglandin dependent mechanisms (Vane, 1973).

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Fluorimetric determination of cephalexin

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Previous methods for the quantitative measurement of cephalexin include ultraviolet spectrophotometry (Marrelli, 1972; Kirschbaum, 1974) and hydroxamate colour formation (Mays, Bangert & others, 1975). Because these methods are of low sensitivity, microbiological assays have generally been used for the determination of cephalexin at the low concentrations, encountered in biological fluids. On alkaline hydrolysis the β-lactam ring of cephalexin opens and the corresponding cephalosporoate is formed which on heating yields a fluorescent derivative (Cohen, Funke & Puar, 1973; Indelicato, Norvilas & others, 1974; Yamana, Tsuji & others, 1974). This has been made the basis of a fluorimetric assay procedure.

Cephalexin monohydrate of reference standard quality (Lilly) was used for fluorimetric determinations. All other chemicals used were of analytical grade.

Citrate buffer pH 5·0: 42·0 g citric acid was dissolved in 204·0 ml of 2 N NaOH and the mixture was diluted to 1 litre with double distilled water.

Formaldehyde (10·0 ml) was mixed with 990·0 ml citrate buffer pH 5·0 to make 1% formaldehyde solution in the buffer.

Quinine solution (1·0 μg ml^{-1}) was prepared in 0·1 N sulphuric acid as a standard fluorescence solution.

Method in aqueous solution. 1·0 ml aqueous solution containing cephalexin was pipetted into a test tube containing 0·5 ml N NaOH; 10 min later 0·5 ml N HCl was added followed by 3·0 ml of pH 5·0 citric acid-sodium hydroxide buffer containing 1% formaldehyde. A fluorescent product was obtained by heating for 30 min at 100° the tubes being covered with loosely fitting polythene balls to minimize evaporation. After the tubes had been cooled at 20° for 10 min, the fluorescence intensity was measured using a Baird Atomic Fluoripoint Spectrofluorimeter with a Xenon lamp at 420 nm (excitation 345 nm).

Method for plasma. To 1·0 ml plasma, 0·8 ml 20% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 3000 rev min^{-1} (10 min). 1·0 ml of supernatant was pipetted into a test tube containing 0·5 ml 2 N NaOH and after 10 min 0·5 ml 2 N HCl was added, followed by 3·0 ml citric acid-sodium hydroxide buffer (pH 5·0) containing 1% formaldehyde. A fluorescent product was obtained from this solution and its intensity measured under the conditions described for the aqueous solution above. A blank value was determined by treating cephalexin free plasma in the same way.

The excitation and emission spectra of the fluorescent product are shown in Fig. 1. Profiles of the product formed from analytical cephalexin and from cephalexin obtained from plasma are identical.

Variation of fluorescence with pH suggested that measurements should be made at pH 5. At this value the fluorescent derivative is formed after between 30 and 50 min incubation at 100°. The necessity to incorporate 1% formaldehyde in the buffer solution is shown by a drop in fluorescent yield under the assay conditions when the formaldehyde is omitted (from 120 to 65 units).

A linear dependence of fluorescence intensity on cephalexin concentration is obtained between zero and 20 μg ml^{-1} in the aqueous solution and in the pooled human plasma (regresium equation is $y = 8{\cdot}7 + 22{\cdot}4\alpha$).

The precise chemical identity of the fluorescent alkali degradation product is uncertain, but there is evidence to suggest that it may be the diketopiperazine derivative. Intramolecular nucleophilic attack of the α-amino group on the side chain of the cephalexin has been suggested as a decomposition mechanism for the formation of the fluorescent derivative (Cohen & others, 1973; Indelicato & others, 1974; Yamana & others, 1974). Addition of formaldehyde catalyses the reaction by reducing the basicity of the amino group (Jusko, 1971). Alkaline hydrolysis of ampicillin followed by heating at 100° in pH 5·0 buffer containing 1% formaldehyde results in a fluorescent derivative which possesses identical excita-

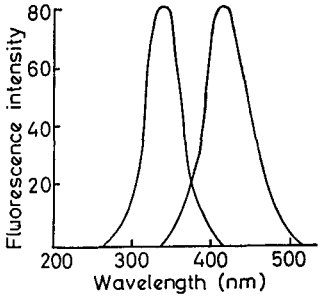

FIG. 1. Spectrophotofluorimetric excitation (345 nm) and emission (420 nm) spectra for the fluorescence product of cephalexin.

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