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Adsorption of tamoxifen and 2-methyl derivatives under cell culture conditions

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Summary

Adsorption of triphenylethylene compounds (tamoxifen, 4-hydroxytamoxifen and their 2-methyl derivatives) on glass and plastic surfaces was separately evaluated under specific conditions met during the preparation of solutions and their incubation. The composition of the culture medium (whether supplemented or not with serum), the material of the recipient (glass or plastic), the concentration of the drug and the substituents on the molecule dramatically affected the amount of active principle left in solution. When evaluated without cells, incubation of a less polar molecule (i.e., tamoxifen) at a low concentration (10^{-8} M) in a culture medium not supplemented with serum in a plastic dish left only 10% of the initial concentration after 1 h. Under the same conditions, the more polar 4-hydroxytamoxifen had a recovery rate of 20%. Advice is given on circumventing these problems which may lead to erroneous conclusions.

Introduction

The nonsteroidal antioestrogen tamoxifen (ICI, Nolvadex, *trans-(Z)*-1-[4-[2-(dimethylamino) ethoxy]phenyl]-1,2-diphenyl-1-butene) has been used in the treatment of both pre- and post-menopausal women with breast cancer (Lerner et al., 1976; Jordan et al., 1991; De Placido et al., 1990; Stewart, 1991). This triphenylethylene derivative is suggested to inhibit mammary cell growth not only by competing with oestradiol through binding to the oestrogen receptor and by interfering with growth factors activity (Clarke et al., 1990; Freiss et al., 1990) but also by blocking the activation of protein kinase C (Bignon et al., 1990) and calmodulin (Rowland et al., 1990; Bouhoute and Leclercq, 1992) and by binding to antioestrogen binding sites (Rochefort, 1987; Leo et al., 1991; Lopes et al., 1991). Tamoxifen has been extensively studied in clinical trials and in laboratory experiments on animals and cell cultures (Buckley and Goa, 1989; Sunderland and Osborne, 1991).

In vitro culture systems use synthetic culture media supplemented with animal sera containing

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endogenous steroids and factors modulating the effect of oestradiol. These compounds cannot be completely eliminated even by treatment with dextran-coated charcoal (DCC) (Briand and Lykkesfelt, 1984; Devleeschouwer et al., 1987). Therefore, to evaluate the effects of oestrogen agonists and antagonists on cellular parameters, some authors tend to decrease or suppress the percentage of serum added to the culture medium (Briand and Lykkesfelt, 1984; Freiss et al., 1990; Issandou et al., 1990; Gong et al., 1992). Following such modifications, the cells require a period of adaptation (Briand and Lykkesfelt, 1984). Another problem, already mentioned by some authors (Lien et al., 1989; Custodio et al., 1991) but not yet quantified, arises with the unspecific adsorption of tamoxifen on container surfaces.

The aim of the study was the determination of tamoxifen, 4-hydroxytamoxifen and their non-isomerisable 2-methyl derivatives left in solution after contact between the solution and the glass or plastic surfaces. The chosen conditions reproduced those occurring during the preparation of the solution (room temperature: 22°C) and its incubation with cell cultures (37°C). The 2-methylated compounds were synthesized by Foster et al. (1985). Their physicochemical properties and biological activities were evaluated by Jarman et al. (1986) and McCague et al. (1988). They were determined by high-performance liquid chromatography (HPLC) and electrochemical detection (ED) by Chamart et al. (1989).

Materials and Methods

[*N-methyl*-^{3}H]Tamoxifen (specific activity: 90 Ci/mmol) and 4-[^{3}H]hydroxytamoxifen (specific activity: 90 Ci/mmol) were purchased from Amersham International, Bucks, U.K. and kept in darkness at −20°C. Tamoxifen citrate, 2-methyltamoxifen hydrochloride and 2-methyl-4-hydroxytamoxifen were provided by Dr M. Jarman (Drug Development Section, Institute of Cancer Research, Sutton, U.K.) and kept in darkness at 4°C.

All solvents and reagents used were analytical grade. Bidistilled water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) (resistivity = 15 MΩ cm). The culture medium, with or without Hepes, was Earle's Minimum Essential Medium (MEM) (Gibco, Paisley, U.K.) supplemented with L-glutamine (585 mg/l), penicillin (100 000 U/l) and streptomycin (100 mg/l). The foetal calf serum (FCS) (Gibco) was inactivated by heating (1 h at 56°C) and depleted of steroids by overnight incubation at 4°C with a suspension of dextran (0.005%) and charcoal (0.5%) in MEM (DCC method) [FCS-MEM + DCC (1 : 1.5, v/v)].

Plastic flasks (50 ml, 25 cm^2) and dishes (diameter: 3.5 cm) were from different origins: Falcon (Becton Dickinson, Plymouth, U.K.), Nunclon (Gibco) and Sterilin (Teddington, U.K.). Glass material was borosilicate grade.

Determination of adsorption with radiolabeled tamoxifen and 4-hydroxytamoxifen

Working solutions of each tritiated compound were prepared at room temperature (22°C) by diluting the stock solutions with the culture medium (supplemented with 0, 1 or 10% of DCC treated FCS) to a concentration of 10^{-8} M (0.1% final in organic solvent) in glass containers; aliquots of 2.5 ml were immediately transferred to plastic Petri dishes ($n = 6$) (time 0) and incubated at 37°C. Samples of 100 μl were taken at times $t = 0$, 0.5, 1, 3, 7 and 24 h. Radioactivity of stock and working solutions was determined with a Beckman LS 2800 (U.S.A.).

Determination of adsorption of tamoxifen and 2-methyl derivatives

The HPLC-ED equipment and the extraction method were described by Chamart et al. (1989).

Stock solutions (0.05–0.1 mg/ml according to the derivative) were prepared in glass flasks with the mobile phase used for the HPLC separation [acetonitrile-methanol-0.05 M sodium dihydrogen phosphate (19.4 : 11.6 : 69, v/v) containing 0.11 mmol/l disodium EDTA] and kept in darkness at 4°C. Working solutions ($2.4–4.8 \times 10^{-7}$M according to the derivative) (0.03% final of organic solvent) of each compound were obtained by diluting the stock solutions with water or culture medium (supplemented with 0 or 4% of DCC

treated FCS) directly in the plastic (Falcon) or glass flask to be studied (time 0). They were then incubated at 37 or 22°C for 0.25, 1 or 3 h. To avoid the extraction stage preliminary to the HPLC analysis, the following procedure was applied: in a glass flask, a solution with the same composition as the mobile phase was reconstituted from the incubated medium used as water to which the other components of the mobile phase were added in appropriate amounts; 20 μl were injected into the chromatographic system.

Desorption of adsorbed tamoxifen

Immediately after determination of the adsorption, the medium was removed from the flask and replaced by the HPLC mobile phase. After a

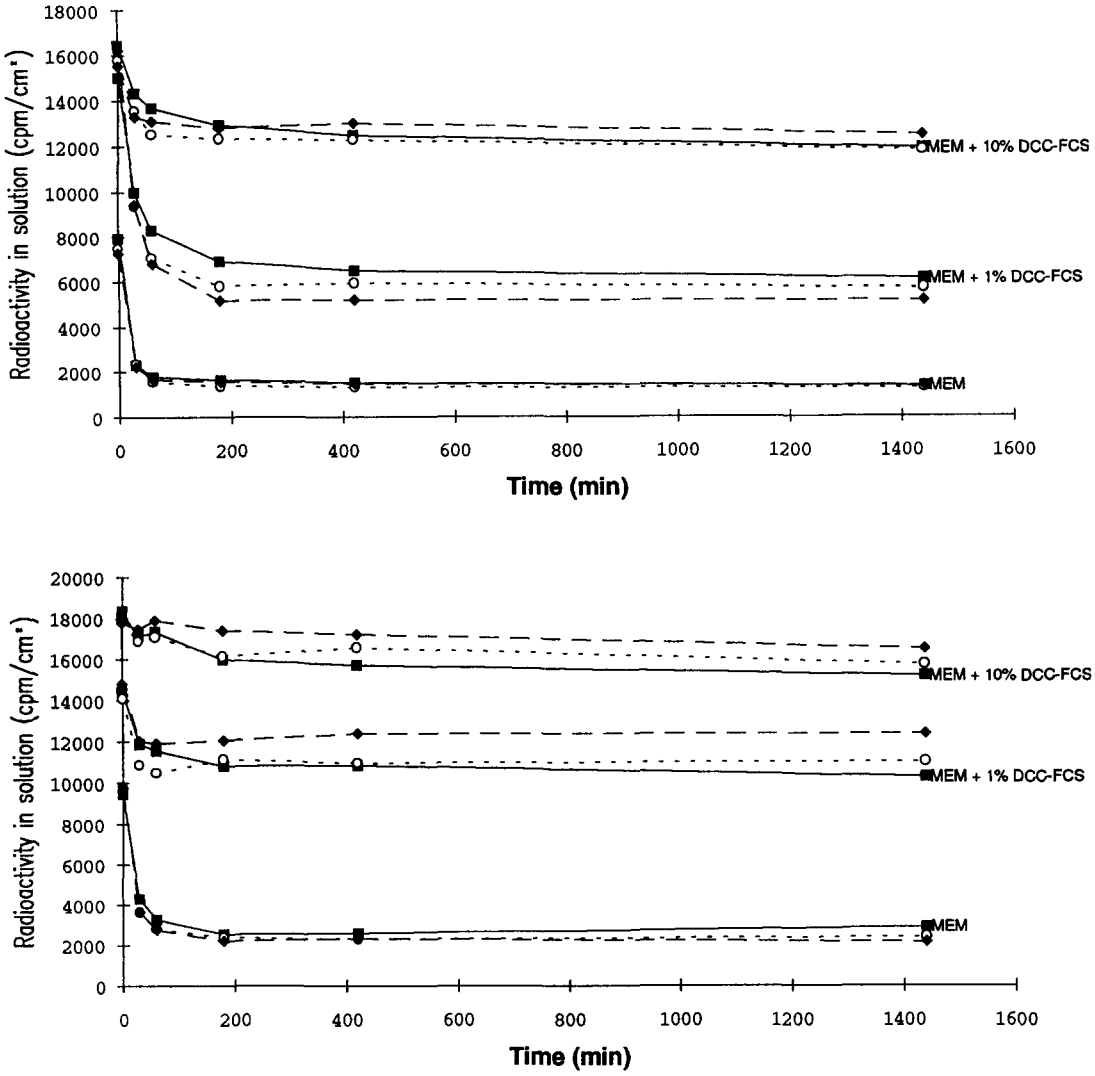

Fig. 1. Evolution of the radioactive signal in solution corrected to account for the decrease of contact surface between the dish and the solution due to successive samplings of 100 μl at times t. [*N-methyl*-^{3}H]Tamoxifen (upper panel) and 4-[^{3}H]hydroxytamoxifen (lower panel) in solution (theoretically 10^{-8} M) in the culture medium supplemented with 0, 1 or 10% of DCC treated FCS were incubated at 37°C in plastic dishes [Falcon (■), Nunclon (○) or Sterilin (◆); diameter: 3.5 cm]. Each data point represents the mean of six determinations.

contact period of 20 min (22°C), 20 μl were injected into the chromatographic system.

Results and Discussion

Adsorption of [N-methyl-^{3}H]tamoxifen and 4-[^{3}H]hydroxytamoxifen (10^{-8} M)

Glass surfaces, 22°C The radioactivity of the working solution in culture medium supplemented with 0, 1 or 10% of DCC treated FCS was compared to the activity of the radiolabeled drug in ethanolic solution in which no adsorption can occur. When there was no serum in the medium, [*N-methyl*-^{3}H]tamoxifen was strongly adsorbed on the glass container ($59 \pm 1\%$ in solution; $t = 24.358$, $df = 10$, $p < 0.001$); 4-[^{3}H]hydroxytamoxifen was adsorbed to a lesser extent ($84 \pm 7\%$ in solution; $t = 5.146$, $df = 10$, $p < 0.001$) (Student's t-test). With 1 and 10% of steroid-depleted serum in the medium no adsorption on glass was recorded (101 ± 2 and $97 \pm 2\%$ in solution, respectively, for [*N-methyl*-^{3}H]tamoxifen; 102 ± 3 and $100 \pm 3\%$ in solution, respectively, for 4-[^{3}H]hydroxytamoxifen).

Plastic Petri dishes, 37°C Measurements of the radioactivity in solution were corrected to account for the decrease in contact surface between the dish and the solution due to successive samplings of 100 μl at times t. Fig. 1 shows the evolution of the radioactive signal left in solution as a function of time, culture medium composition and brand of plastic. Values at time 0 were lower in the absence of serum because adsorption had already occurred in the glass container (cf. preceding section). At a given time, in a given medium, each molecule was adsorbed to the same extent on the three plastics tested (Falcon, Nunclon, Sterilin). Under all conditions, the steady state was reached after 7 h (steady state was defined as no statistically significant difference for values between successive times). The adsorption phenomenon was inversely correlated to the percentage of steroid-depleted serum in the culture medium ([*N-methyl*-^{3}H]tamoxifen: $r = -0.97 \pm 0.03$; 4-[^{3}H]hydroxytamoxifen: $r = -0.71 \pm 0.02$; mean $\pm$ SD ($n = 3$) of r determined at 7 h for each plastic). [*N-methyl*-^{3}H]Tamoxifen, less polar than its metabolite 4-[^{3}H]hydroxytamoxifen, was adsorbed in a higher proportion ($df = 10$, $p < 0.001$) (Student's t-test).

The real concentration in solution in the Petri dish resulted from combined adsorptions on the glass flask and on both the plastic pipet and dish. The determined concentration was systematically biased by the last sampling made with a plastic tip. Table 1 gives the percentage (mean $\pm$ SD, $n = 6$) of radioactive drug actually in solution in the plastic dish (Falcon) relative to the initial concentration put in the glass flask. The initial adsorption rates were 100, 91 and 33 pg/min for [*N-methyl*-^{3}H]tamoxifen and 104, 50 and 22 pg/min for 4-[^{3}H]hydroxytamoxifen in solution in the culture medium supplemented, respectively, with 0, 1 and 10% of steroid-depleted serum.

Adsorption of tamoxifen and 2-methyl derivatives ($2.4–4.8 \times 10^{-7}$ M)

A solution of tamoxifen (3.4×10^{-7} M) in water was not adsorbed on a glass flask after 15 minutes of contact, at either 22 or 37°C (101 ± 2 and $100 \pm 4\%$ ($n = 4$) in solution, respectively); the use of siliconed glassware significantly impaired the concentration (22°C: $92 \pm 2\%$ in solu-

TABLE 1

Percentage (mean $\pm$ SD of six determinations) of [N-methyl-^{3}H]tamoxifen and 4-[^{3}H]hydroxytamoxifen actually in solution in the plastic dish (Falcon) relative to the initial concentration (10^{-8} M) put in the glass flask

Culture medium	[*N-methyl*-^{3}H]Tamoxifen				4-[^{3}H]Hydroxytamoxifen			
	0 h	0.5 h	1 h	3 h	0 h	0.5 h	1 h	3 h
MEM	47 ± 2	14 ± 1	10 ± 1	10 ± 1	61 ± 4	28 ± 1	21 ± 1	16 ± 1
MEM + 1% stripped FCS	89 ± 3	59 ± 2	49 ± 2	41 ± 2	93 ± 4	77 ± 2	74 ± 2	70 ± 4
MEM + 10% stripped FCS	96 ± 2	85 ± 3	81 ± 2	76 ± 3	98 ± 2	91 ± 3	92 ± 3	85 ± 4

tion, 37°C: 92 ± 4% in solution) (df = 6, $p < 0.05$) (Student's t-test) and was thus avoided.

In the absence of serum, an unidentified component of the culture medium favoured the adsorption of tamoxifen on glass (78 ± 8% in solution, $n = 4$) ($t = 5.578$, df = 6, $p < 0.001$) (Student's t-test); a test without Hepes, one of the most concentrated components of the medium (5960 mg/l), did not change the result (76 ± 5% in solution, $n = 4$). However, the presence of steroid-depleted serum (4%) restored the initial concentration (102 ± 2% ($n = 4$)).

Table 2 gives the percentage (mean ± SD, $n = 4$) of tamoxifen or 2-methyl derivatives left in solution after contact with glass at 22°C or with plastic at 37°C. The adsorption depended on the material (glass or plastic) (df = 6, $p < 0.001$) and the proportion of steroid-depleted serum in the culture medium (0 or 4%) (df = 6, $p < 0.001$); under the same conditions (time, medium and recipient), the adsorption of tamoxifen and 2-methyltamoxifen was similar. The addition of a methyl moiety to tamoxifen did not modify the extent of adsorption (Table 2).

On plastic, the adsorption was stabilised after 3 h. Comparison of Tables 1 and 2 at time $t = 3$ h shows a stronger adsorption of tamoxifen at lower concentration ($t = 8.332$, df = 8, $p < 0.001$) (Student's t-test).

Desorption of adsorbed tamoxifen, 22°C

Desorption of the adsorbed tamoxifen with the HPLC mobile phase yielded 102 ± 12% ($n = 2$) from glass and 86 ± 2% ($n = 2$) from plastic (Falcon).

The decrease of concentration of these basic triphenylethylene derivatives is due to their rapid adsorption from the solution onto glass or plastic surfaces. Any decomposition phenomenon (different from the radioactive decomposition negligible in this experiment) would not impair the radioactive signal and no decomposition product is detected by HPLC.

The lack of effect of temperature in water (22 or 37°C) and the incomplete desorption from plastic lead to the hypothesis of a covalent link (chemisorption) between these basic drugs and the positive charge of the plastic surface, in addition to physisorption which is due to electrostatic interactions (Gerasimov et al., 1974; Atkins, 1982).

The adsorption phenomenon affected the concentration of solutions of tamoxifen, 4-hydroxytamoxifen or 2-methyl derivatives of tamoxifen more or less dramatically depending on the composition of the solvent, the nature of the container, the derivative and its concentration. The worst conditions resulted from incubating the less polar molecules at low concentration in a culture medium not supplemented with serum in a plastic recipient. Serum exerted a stabilizing effect probably through its protein content because the triphenylethylene compounds are highly (> 98%) (Lien et al., 1989; Iino et al., 1991) or irreversibly (Mani and Kupfer, 1991) bound to proteins. The presence of a cell monolayer should therefore be expected to decrease the adsorption and to increase the availability of the drug.

In experiments studying these triphenylethylene derivatives, plastic ware should be replaced by glassware for the preparation of solutions

TABLE 2

Percentage (mean ± SD of 4 determinations) of tamoxifen (3.4×10^{-7} M), 2-methyltamoxifen (4.8×10^{-7} M) and 2-methyl-4-hydroxytamoxifen (2.4×10^{-7} M) left in solution after contact with glass at 22°C or plastic at 37°C

Derivatives	Culture medium	Glass 22°C 0.25 h	Plastic 37°C		
			0.25 h	1 h	3 h
Tamoxifen	MEM	78 ± 8	22 ± 2	21 ± 3	19 ± 2
	MEM + 4% stripped FCS	102 ± 2	83 ± 5	83 ± 5	79 ± 4
2-Methyltamoxifen	MEM	73 ± 6	24 ± 4	25 ± 3	16 ± 2
	MEM + 4% stripped FCS	101 ± 1	86 ± 3	77 ± 2	78 ± 3
2-Methyl-4-hydroxytamoxifen	MEM	83 ± 6	28 ± 3	27 ± 3	22 ± 2
	MEM + 4% stripped FCS	98 ± 5	80 ± 5	75 ± 2	80 ± 4

(pipets, flasks, etc.). Replacing the plastic dish by a glass dish for cell culture would be an undesirable technical regression in terms of the user's convenience (plastic is sterile and disposable) and of cellular growth (adhesion).

The role played by the proteins of the serum is of utmost importance for the 'stability' of the drug concentration in solution. The total suppression of the serum from the culture medium generates a very poor recovery (10–20%) of these triphenylethylenic antioestrogens after only 1 h of contact between solution and plastic dish. To make up for such a lack, other proteins can be added to the medium when the serum is suppressed (Issandou et al., 1990; Katzenellenbogen and Norman, 1990) on the condition that they do not irreversibly bind these non-steroidal drugs (Mani and Kupfer, 1991).

For more precision on the actual available dose, the active principle should be determined at least at the beginning (0.5–1 h) of the incubation in the culture medium and possibly after incubation in the culture medium, in the cell or on the surface of the dish.

Therefore, in cell culture experiments, it is essential to evaluate the actual concentration of drug in solution to avoid misleading conclusions based on overestimated values.

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