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DETERMINATION OF 2-METHYL DERIVATIVES OF TAMOXIFEN IN CELL CULTURE MEDIUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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SUMMARY

The 2-methyl derivatives of tamoxifen (2-methyltamoxifen and 2-methyl-4-hydroxytamoxifen) were extracted from a cell culture medium at pH 5 4 (Earle's Minimum Essential Medium) with an internal standard (tamoxifen) on a phenyl sorbent cartridge The compounds were then separated by high-performance liquid chromatography on a nitrile column eluted with acetonitrile–methanol–0 05 *M* sodium dihydrogenphosphate (19 4 11 6 69, v/v) containing 0 11 mmol/l disodium EDTA and determined by electrochemical detection at + 1 1 V vs Ag/AgCl/3 *M* NaCl The absolute detection limits were 50 pg for 2-methyl-4-hydroxytamoxifen and 100 pg for tamoxifen and 2-methyltamoxifen at a sensitivity of 1 nA/V

INTRODUCTION

Tamoxifen (ICI, Nalvadex, *trans*-(*Z*)-1-[4-[2-(dimethylamino)-ethoxy]-phenyl]-1,2-diphenyl-1-butene) is a triphenylethylene derivative used for the treatment of hormone-dependent breast cancer [1–4]. This non-steroidal anti-oestrogen is thought to inhibit cell proliferation not only by competing with oestradiol [5–7], but also by preventing growth factor action Both mechanisms require oestrogen receptors [8–11].

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Tamoxifen (I, Fig. 1) and up to five metabolites have been determined in female plasma [12]. Several methods described in the literature allow simultaneous assay in plasma and tissues of those molecules present in nanogram amounts. One of them, high-performance liquid chromatography (HPLC), seems to be the best method. Two detection systems have been recommended: UV detection with a detection limit of 5 ng [13] and fluorimetry with either pre-column [12,14,15] or post-column [4,16–18] photocyclization of the analytes to the corresponding phenanthrene derivatives The detection limits in fluorimetry vary between 50 and 500 pg Thin-layer chromatography with in situ conversion into a fluorescent product followed by densitometry allows detection of 950 pg [19]; gas chromatography linked to high-resolution mass spectrometry has a detection limit of 200 pg [20].

Studies have been carried out on animals, isolated organs and cell lines to elucidate the complex activity of tamoxifen and its metabolites [21]. Whereas the tamoxifen *trans*-isomer is an oestrogen antagonist, the *cis*-isomer is an agonist [22–24]. The 4-hydroxylated metabolites of *trans*-tamoxifen undergo rapid isomerization in cell cultures, which can complicate the interpretation of the results [25] The *trans* configuration is stabilized by adding a methyl moiety at position 2 (II, Fig 1) without any loss of affinity for the receptor [26]; 2-methyl-4-hydroxytamoxifen (III, Fig. 1) would appear to be an even better anti-oestrogen than 4-hydroxytamoxifen [7]. Their stability justifies the selection of such methylated compounds for the present study.

A new sensitive and selective analytical method is now proposed: the available 2-methyl derivatives [2-methyltamoxifen (II) and 2-methyl-4-hydroxytamoxifen (III)] are extracted on a phenyl bonded-phase cartridge with an

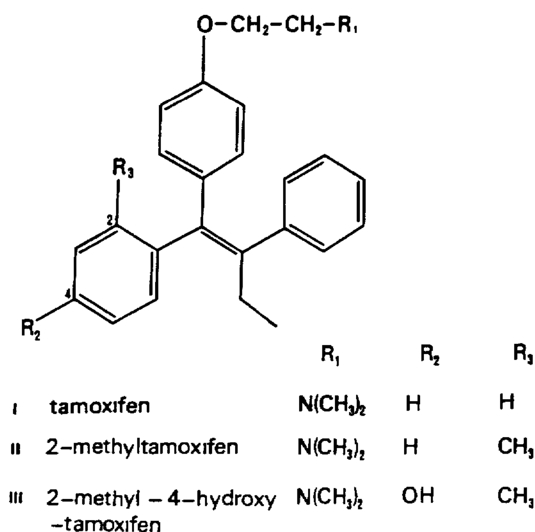

Fig 1 Structures of tamoxifen and its 2-methyl derivatives

internal standard (tamoxifen, I, which does not interfere with the determination method since the studied compounds are its methylated derivatives) and separated by HPLC on a nitrile column, which gives better resolution and efficiency than octadecyl or octyl columns. The detection method is electrochemistry, which, in contrast to fluorimetry, allows the detection of the parent molecules rather than the phenanthrene derivatives. The problems raised by pre- and post-column photocyclization [4] are therefore avoided.

This method was developed to study the uptake kinetics and metabolism of 2-methyltamoxifen and 2-methyl-4-hydroxytamoxifen in human breast cancer cell lines with (MCF7) or without (Evsa-T) oestrogen receptors grown as monolayer cultures (10% foetal calf serum with Earle's Minimum Essential Medium) This work may open new avenues in the in vitro metabolism of the methylated derivatives of tamoxifen.

EXPERIMENTAL

Tamoxifen (I), 2-methyltamoxifen (II) and 2-methyl-4-hydroxytamoxifen (III) were kindly provided by Dr. M Jarman (Drug Development Section, Institute of Cancer Research, Sutton, U.K.) Stock solutions (0 1–0 2 mg/ml, according to the derivative) were prepared in the mobile phase and kept in darkness at 4°C. Working solutions were prepared by appropriate dilutions with either mobile phase or culture medium (see below).

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 consisted of Earle's Minimum Essential Medium (Gibco, Paisley, U.K.) supplemented with L-glutamine (585 mg/l), gentamycin (50 mg/l), penicillin (100 000 U/l), streptomycin (100 mg/l) and 10% foetal calf serum (Gibco) heat-inactivated for 1 h at 56°C.

After the validation described in the next section the following conditions were adopted for the final method. The HPLC system consisted of a Perkin-Elmer Series II pump (Perkin-Elmer, Norwalk, CT, U.S.A.), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.), an Alltech Ro Sil CN column (15 cm×0.46 cm I.D.) (particle size 5 μm, pore size 8 nm) (Alltech, Deerfield, IL, U S.A.), a BAS LC-4 amperometric detector with a BAS LC-16 glassy carbon detector cell (thin-layer gasket 2 μl), an auxiliary electrode and an Ag/AgCl/3 *M* NaCl (BAS RE-1) reference electrode (E°=0.2222V at 25°C) (Bioanalytical Systems, West Lafayette, IN, U.S.A.). Data were recorded with a chart recorder (Kipp and Zonen, Delft, The Netherlands).The HPLC system was eluted isocratically with acetonitrile–methanol–0.05 *M* sodium dihydrogenphosphate (19.4.11.6.69, v/v) containing 0.11 mmol/l disodium EDTA at a flow-rate of 1 ml/min, at room temperature (18–22°C).

Extraction was effected in either culture medium or mobile phase solutions

spiked with III (100 ng/ml), II and I (200 ng/ml). A 0.5-ml volume of solution was filtered through a 50-mg solid-phase phenyl sorbent cartridge (Bond Elut) (Analytichem International, Harbor City, CA, U.S.A.) preconditioned with 1 ml of methanol and 1 ml of water. Washed with 1 ml of water, 1 ml of acetonitrile–water (1 1, v/v) and 0.2 ml of acetonitrile, the cartridge was then eluted with 5 ml of 6 *M* acetic acid in acetonitrile. The eluate was immediately evaporated under reduced pressure. The dry residue was reconstituted in 1 ml of mobile phase; 20 μl were injected into the chromatographic system.

RESULTS AND DISCUSSION

The development involved two aspects: the chromatographic conditions, which allowed a one-step separation of the analytes, and the successful extraction of the analytes from the culture medium.

For the chromatographic development, UV detection was used because the time required to equilibrate the detector cell after changing the mobile phase was much shorter (20 min) than with the electrochemical detector (3 h) For the extraction development, electrochemical detection (ED) was used because it was much more sensitive (50–100 pg for the detection limit at a 1 nA/V sensitivity) than UV detection (2–4 ng at a 0.02 A.U./V sensitivity).

Chromatographic analysis

At first, pure standard solutions of methylated derivatives (II,III) were kept without light protection. Light is responsible for the conversion of those photosensitive standards into several unidentified analytes. These analytes were supposed to be phenanthrenes produced by photocyclization [4,14,15,17–19]. They enabled us to test the efficiency and resolution of the three optimized systems (Table I). They corresponded to the unassigned peaks in Fig. 2

For that development, a UV detector (Gilson, Middleton, WI, U.S.A.) set at 254 nm was used. The three evaluated columns, octadecyl [12,15,17,27], octyl [13] and nitrile [14,16,18], were characterized by very different parameters (Table II). The lipophilic ODS column was the least adequate, with a low theoretical plate number and poor resolution. The octyl and nitrile columns were found to have the same theoretical plate numbers but, in spite of the excellent resolution of system 2, the nitrile column and system 3 were chosen for their shorter analysis time, 20 min instead of 55 min. In each system, a better resolution between the standards and their conversion products was achieved with a mixture of methanol and acetonitrile in the mobile phase rather than either methanol or acetonitrile alone.

In order to evaluate the influence of pH and ionic strength on system 3, the mobile phase was phosphate buffer–methanol (1 · 1, v/v). The buffer pH was adjusted with 0.015 *M* H_3PO_4, NaH_2PO_4 and Na_2HPO_4 solutions. The ionic

TABLE I

THE THREE HPLC SYSTEMS

	System 1	System 2	System 3
Column	Spherisorb ODS (10 cm×0 3 cm I D) Chrompack International (Middelburg, The Netherlands)	Zorbax C_8 (15 cm×0 46 cm I D) Dupont (Wilmington, DE, U S A)	Alltech Ro Sil CN (15 cm×0 46 cm I D) Alltech Assoc (Deerfield, IL, U S A)
Mobile phase (v/v)	Acetonitrile 30, methanol 30, water 40, diethylamine 0 5, H_3PO_4 to adjust pH to 4 0	Acetonitrile 30, methanol 17 5, 0 02 *M* NaH_2PO_4 52, diethylamine 0 5, acetic acid 6	Acetonitrile 25, methanol 15, 0 03 *M* NaH_2PO_4 30, 0 053 m*M* disodium EDTA 30
pH of the mobile phase	4 0	4 1	5 4
Flow-rate	0 7 ml/min	1 5 ml/min	1 0 ml/min
Chromatogram	Fig 2a	Fig 2b	Fig 2c

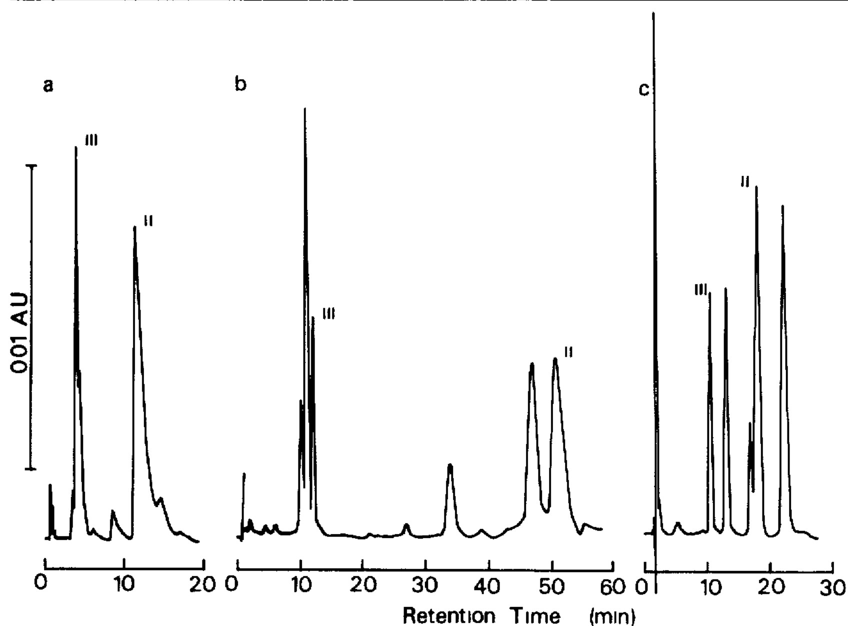

Fig 2 Chromatograms of a degraded solution of II and III on an octadecyl column (a), an octyl column (b) and a nitrile column (c) with UV detection (254 nm) The unassigned peaks are the conversion products of the standards under light effect

TABLE II

PARAMETERS OF THE THREE HPLC SYSTEMS

HPLC system	Column	Number of theoretical plates		Resolution	Capacity factor		t_0 (s)
		III	II		III	II	
1	Octadecyl	1742	481	6 1	7 33	23 17	29
2	Octadecyl	2401	3397	17 0	8 80	39 80	60
3	Nitrile	3136	3318	7 5	6 00	11 00	90

strength was established with 0.005–0.2 M NaH_2PO_4 solutions; accurate values were then calculated by means of a previously developed algorithm [28]. The pH was set at 5.4, as a compromise between speed of analysis (20 min), retention time for derivative III allowing separation from more polar compounds co-extracted from biological media and easier oxidation at less acidic pH (Fig. 3a). The higher the ionic strength, the shorter the retention times, mainly between $\mu=0$ 05 and $\mu=0.005$ a slight change in ionic strength had a considerable influence on retention times, which varied from 10 to 30 min (Fig 3b)

The mobile phase used to check the linearity of system 3 had to be modified because of the introduction of I as internal standard for extraction. This new mobile phase had a higher ionic strength, which was counterbalanced by a greater proportion of aqueous phase to keep the same retention times. This modification led to better electrochemical stability.

Voltamperometric analysis

ED parameters of derivatives II and III were determined out of chromatographic line from static voltammograms taken in 10^{-4} M solutions of mobile phase of system 3 (pH 5.4), at 23°C, with a glassy carbon electrode (Metrohm, Herisau, Switzerland) referenced to a saturated calomel electrode ($E°=0$ 2676 V at 25°C, KCl). The $E_{1/2}$ values were +0.74 V for II and +1.07 V for III Therefore, the working electrode polarization potential was set at +1.10 V vs. Ag/AgCl/3 M NaCl ($E°=0$ 2222V at 25°C).

HPLC (CN column) electrochemical system linearity

The standard calibration curves of derivatives III and II were linear over the concentration ranges 5–80 ng/ml (i.e. 5, 20, 40, 60 and 80 ng/ml) ($y=4.7x-0$ 6, $r=1.0$) and 30–240 ng/ml (i e. 30, 60, 120, 180 and 240 ng/ml) ($y=15.8x-0.2$; $r=1.0$), respectively. The intra-assay coefficients of variation were, at the lower limit, 5% and, at the upper limit, 1% for both derivatives ($n=3$ injections for each concentration). The respective absolute detection limits were 50 and 300 pg or 0.12 and 0 78 pmol (injected amount giving a signal-to-noise ratio of 3) at a 2 nA/V sensitivity; 50 μl were injected

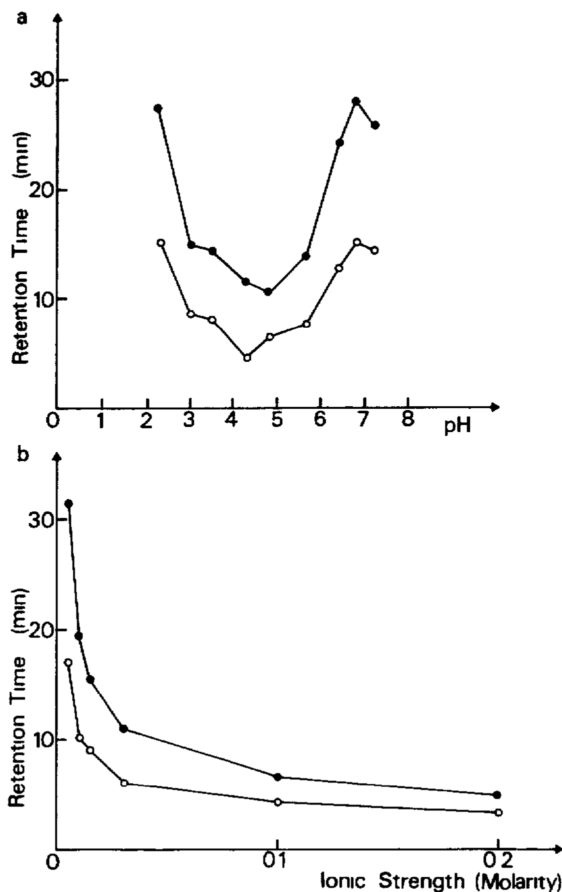

Fig 3 Effect of pH (a) and ionic strength (b) on the retention times on a nitrile column (●) II, (○) III

Extraction

Two extractive techniques were compared: liquid–liquid and liquid–solid extractions Several organic solvents were tried: diethyl ether, hexane, toluene and cyclohexane. A 1-ml volume of culture medium containing 100 ng/ml III and 200 ng/ml II was agitated for 5 min with 5 ml or 4×5 ml of organic solvent. After centrifugation (2000 *g*, 20°C, 5 min) and evaporation of the organic layer under reduced pressure, the residue was reconstituted in 1 ml of mobile phase, and 20 μl were injected The efficiencies of organic solvent extractions are presented in Table III. Cyclohexane gave a low and non-reproducible recovery Though the molecules were not ionized at pH 10.0 the results were not improved with culture medium alkalinized with ammonium hydroxide. Diethyl ether [12,13,15,16,27] and hexane [4,17,19] were rejected because of non-volatile impurities detected at the working potential, which interfered with III.

TABLE III

EFFICIENCIES OF LIQUID-LIQUID AND LIQUID-SOLID EXTRACTION

Extraction from cell culture medium (supplemented Minimum Essential Medium) spiked with 100 ng/ml III and 200 ng/ml II The mean recoveries were the results of two assayed samples injected twice (the toluene extract was sampled only once)

Culture medium pH	Extraction process	Extraction efficiency (mean ± S D) (%)	
		III	II
7 4	Cyclohexane (5 ml)	62 ± 2	45 ± 5
7 4	Cyclohexane (4×5 ml)	67 ± 14	72 ± 4
10 0	Cyclohexane (5 ml)	66 ± 4	61 ± 5
7 4	Toluene (5 ml)	83	66
7 4	Octadecyl sorbent (500 mg)	Non-eluted	Non-eluted
7 4	Octyl sorbent (100 mg)	48 ± 22	33 ± 17
7 4	Ethyl sorbent (500 mg)	13 ± 5	14 ± 9
7 4	Phenyl sorbent (500 mg)	84 ± 7	73 ± 10

Toluene was not selective enough, as the extract was found to contain impurities poorly separated from III.

Several solid-phase extraction cartridges (Bond Elut) were then investigated: octadecyl (500 mg; 6 0 ml), octyl (100 mg; 1.0 ml), ethyl (500 mg, 2.8 ml) and phenyl (500 mg; 2.8 ml) non-polar sorbents. The cartridge was first conditioned with 5 ml of methanol and 5 ml of water. A 1-ml volume of either culture medium or mobile phase spiked with standards (100 ng/ml III and 200 ng/ml II) was filtered through the cartridge (0.5 ml through the octyl), which was then washed with 2 ml of water, 1 ml of acetonitrile–water (1 · 1, v/v) and 0.5 ml of acetonitrile, and finally eluted with 4 ml of 2 *M* acetic acid in acetonitrile. The eluate was immediately evaporated under reduced pressure. The dry residue was reconstituted in 1 ml of mobile phase, and 20 μl were injected The post-adsorption or wash solutions did not contain the analytes and were discarded. The efficiencies of cartridge extractions from culture medium are given in Table III. Recoveries were low with the octyl and ethyl sorbents and equal to zero with the octadecyl phase [18], even with 1 *M* perchloric acid as a stronger eluent The phenyl cartridge yielded a good recovery and produced an electrochemically clean extract with a very small amount of co-extracted contaminants Though the standards in the mobile phase were adsorbed onto the four tested sorbents, they could not be eluted under conditions suitable for the culture medium.

Phenyl cartridge extraction

Fig. 4 shows the effect of sorbent mass on the extraction efficiency of the mobile phase (pH 5.4) spiked with standards. When the sorbent had been

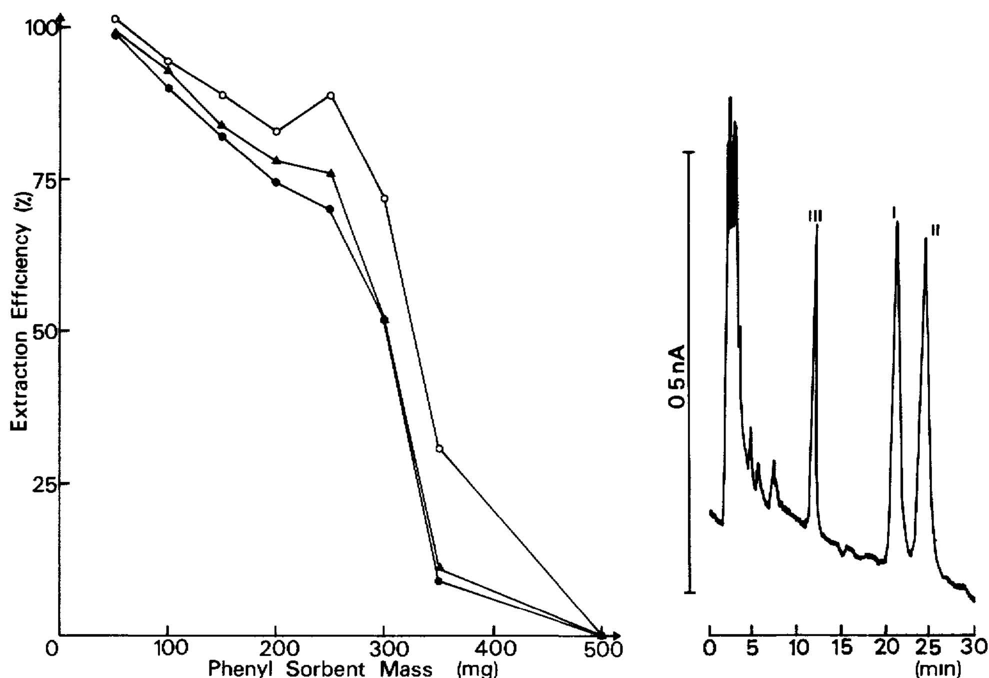

Fig 4 Influence of the phenyl sorbent mass on the extraction efficiency from the mobile phase spiked with standards (▲) I, (●) II, (○) III

Fig 5 Chromatogram of an extract of 0 5 ml of culture medium (supplemented Minimum Essential Medium) at pH 5 4 spiked with 100 ng/ml III and 200 ng/ml I and II, effected on a 50-mg phenyl sorbent cartridge, reconstituted in 1 ml of acetonitrile-methanol-0 05 *M* sodium dihydrogenphosphate (19 4 11 6 69, v/v) mobile phase containing 0 11 mmol/l disodium EDTA, 20 μl were injected into a nitrile column

reduced from 500 to 50 mg, the recovery reached 100 ± 3% for analyte III, 97 ± 4% for I and 96 ± 6% for II (mean ± S.D. of six determinations).

The pH of the adsorbed solution influenced the recovery· by decreasing the culture medium pH from 7.4 to 5.4, the efficiency was increased by 10% for derivatives I ($t=4.325$, $df=8$, $p<0.01$) and II ($t=5.835$, $df=8$, $p<0\ 001$) (Student's *t*-test). (At pH 7.4: III = 89 ± 5%; I = 77 ± 2%; II = 78 ± 2%. At pH 5.4: III = 90 ± 4%; I = 85 ± 3%; II = 87 ± 3%; mean ± S.D. of five determinations.) Lowering the pH to 3.0 did not improve the efficiency further

Variations in the eluent volume (2–10 ml of 6 *M* acetic acid in acetonitrile) did not give significantly different results; 5 ml were sufficient to elute completely the largest tested amount (5 μg of III and 10 μg of I and II).

A culture medium blank was extracted according to the described process and no peaks were detected at the retention times of the analytes. Fig. 5 is a typical chromatogram obtained under the above conditions.

TABLE IV

CALIBRATION OF THE EXTRACTION METHOD FROM CELL CULTURE MEDIUM (SUPPLEMENTED MINIMUM ESSENTIAL MEDIUM) AT pH 5 4

The mean recoveries are the results of six assayed samples

III			I (internal standard)		II		
Amount added (ng)	Absolute recovery (%)	Relative (to I) recovery (%)	Amount added (ng)	Absolute recovery (%)	Amount added (ng)	Absolute recovery (%)	Relative (to I) recovery (%)
1 00	93±4	98±7	1 5	95±6	2 1	96±7	101±10
5 0	94±5	102±6	7 5	94±7	10 6	91±6	98± 6
50	94±3	103±5	75	92±5	106	91±5	97± 4
250	92±7	100±4	376	93±4	530	92±6	99± 2
500	94±2	101±4	752	93±3	1061	91±2	98± 2
2500	98±4	103±4	3760	95±4	5304	94±4	99± 2
5000	97±2	101±2	7520	97±3	10608	94±3	97± 1
$y=0\ 97x-3\ 70$			$y=0\ 97x-14\ 48$		$y=0\ 94x-8\ 64$		
$r=1\ 00$			$r=1\ 00$		$r=1\ 00$		

The extraction method gave a linear recovery (Table IV) over the concentration range from 2 ng/ml to 10 μg/ml for derivative III and from 4 ng/ml to 20 μg/ml for I and II. With the usual working solution, containing 100 ng/ml III and 200 ng/ml I and II, the within-day (six samples in one day) and the between-day (four days) coefficients of variation (C V.) were, respectively, 3 and 5% for III, 5 and 5% for I and 5 and 4% for II. The results, expressed as a ratio to the internal standard, had within-day and between-day C.V. of 3 and 4% for III and 2 and 2% for II.

At the upper limit of quantification, the within-day variation was 2% for III, 3% for I and 3% for II. At the lower limit it was 4% for III, 6% for I and 7% for II (Table IV).

The absolute detection limit was 50 pg for derivative III and 100 pg for I and II at a 1 nA/V sensitivity. For tamoxifen (I) this ED method was as sensitive (100 pg) as the most sensitive methods in fluorimetry (50–500 pg) found in the literature [4,14,16–18] Similar analytical data do not exist for the 2-methyl derivatives of tamoxifen.

The next stage of this work will be an assessment of 2-methyl-tamoxifen and its metabolites at the intra-cellular level to learn more about the uptake kinetics in human breast cancer cell cultures Extension to clinical practice for the measurement of tamoxifen (with another internal standard), derivatives and metabolites is another possibility.

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