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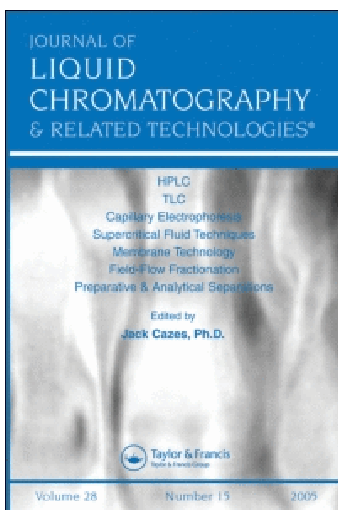

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF TOREMIFENE AND METABOLITES

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ABSTRACT

The separation of toremifene and its metabolites 4-hydroxytoremifene, N-desmethyltoremifene, N-desdimethyltoremifene and deaminohydroxytoremifene by reversed-phase high performance liquid chromatography is described. The effects of pH, buffer concentration and type and proportion of organic modifier on the retention and resolution of the compounds have been studied. This allows optimum conditions for a particular biological application to be developed by simple modification of these paramaters. For the separation of toremifene and metabolites in microsomal metabolism and in plasma, the optimum conditions were 65% (v/v) acetonitrile in 0.25M ammonium acetate-acetic acid buffer, pH 5.0-5.2.

INTRODUCTION

Toremifene (Figure 1), the chloroethyl analogue of tamoxifen, is a non-steroidal antiestrogen drug currently under clinical trial for the treatment of breast cancer (1).

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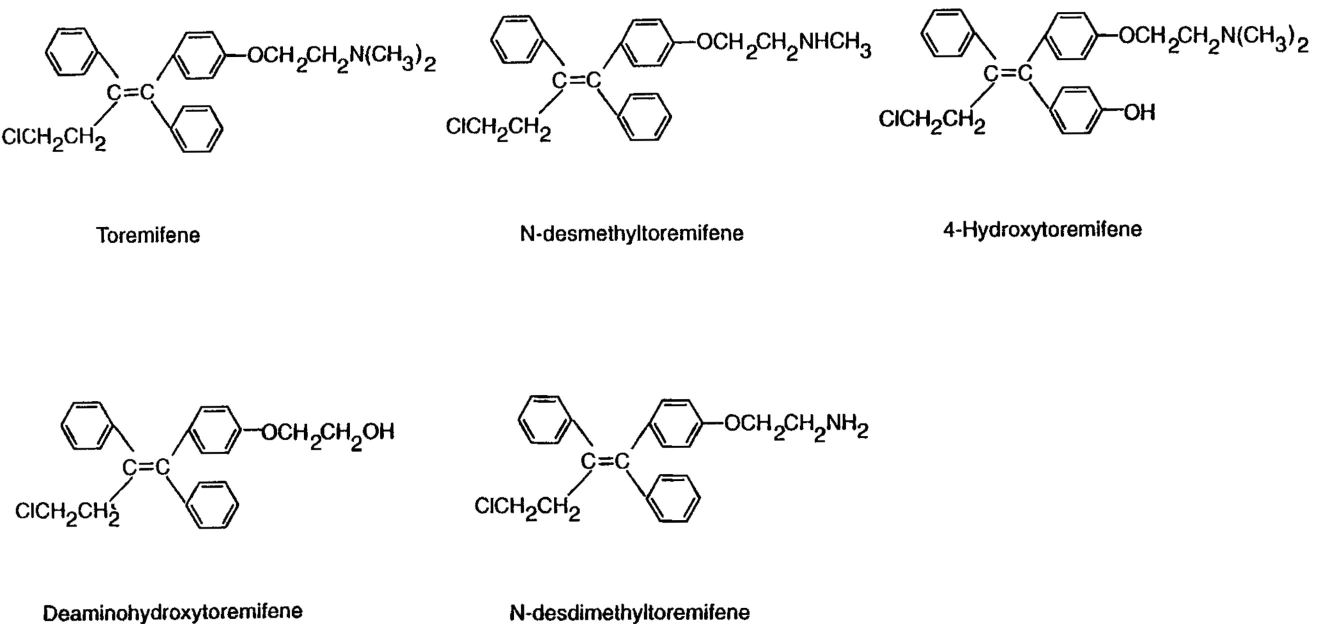

Figure 1 Structures of toremifene and its major metabolites.

It has similar estrogenic / antiestrogenic properties to tamoxifen but, in contrast to tamoxifen , has been shown not to cause hepatocellular carcinoma in rats in long term high dose feeding experiments (1, 2). We are interested in studying the metabolism and pharmacokinetics of toremifene in order to find out whether the difference in carcinogenesis between the two compounds can be explained in terms of metabolism and pharmacokinetics. This requires a simple and efficient analytical method. There are only two high performance liquid chromatographic (HPLC) methods reported for the separation of toremifene and metabolites (3, 4). We describe here a novel reversed-phase system for the separation of toremifene and metabolites on a Hypersil-ODS column. The retention behaviours of these compounds in the acetonitrile-ammonium acetate buffer system have been studied in detail to allow simple optimization of the system for a particular biological application. The applications of the method were demonstrated by the separation of toremifene and metabolites in rat liver microsomes following incubation in the presence of NADPH and in spiked human plasma.

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EXPERIMENTAL

Materials and Reagents

Toremifene and metabolites were gifts from Farmos Gronp Ltd. (Oulu, Finland). Ammonium acetate, glaical acetic acid, $MgCl_2$, NaOH, Hepes and dimethyl sulphoxide (DMSO) were AnalaR grade from BDH (Poole, Dorset, U.K.). NADPH was from Sigma Chem. Co. (poole, Dorset, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Toremifene Metabolism in Rat Liver Microsomes

Rat liver microsomes (1mg protein) were incubated with toremifene (50μM) in 0.05M Hepes-NaOH buffer (pH 7.4) in the presence of NADPH (0.5mM) and

$MgCl_2$ (5mM) at 37° for 15min. The reaction was stopped by vortex-mixed with 2 volumes of methanol / DMSO (4:1 v/v). The supernatant after centrifugation was analysed by HPLC.

Extraction of Toremifene and Metabolites in Plasma

Plasma (200μl) was vortex-mixed with 400μl of methanol / DMSO (4:1 v/v) for 30 sec. The mixture was centrifuged at 5000×g for 10min and the supernatant was analysed by HPLC.

High Performance Liquid Chromatography

A Varian Ltd. (Walton-on-Thames, Surrey, U.K.) model 9010 liquid chromatograph was used with a Varian 9050 UV-Vis detector set at 280nm. A Rheodyne 7125 injector (Cotati, CA, U.S.A.) fitted with a 200μl loop was used for sample injection. The column was Hypersil-ODS (5μM particle size, 250×4.6mm I.D.) and the mobile phase was acetonitrile in ammonium acetate buffer at various pH and molarity. The flow-rate was 1ml / min.

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RESULTS AND DISCUSSION

The Effect of Buffer Concentration on the Retention and Resolution of Toremifene and Metabolites

The retention and resolution of toremifene and its metabolites are significantly affected by the molar concentration of ammonium acetate buffer used in the mobile phase. The variation of the capacity factor (k') with the buffer concentration is shown in Figure 2. The optimum buffer concentration for the rapid and complete separation of toremifene, 4-hydroxytoremifene, N-desmethyltoremifene and deaminohydroxytoremifene was between 0.25 and 0.38M. At concentrations below

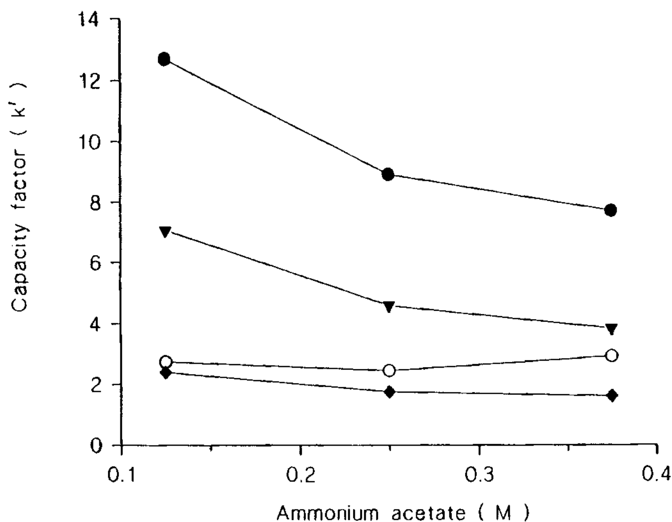

Figure 2 The effect of ammonium acetate buffer molar concentration on the capacity factors (k') of toremifene and metabolites. The mobile phasd was maintained at pH 5.16 and contained 65% acetonitrile. ● = Toremifene, ▲ = N-desmethyltoremifene, O = deaminohydroxytoremifene and ◆ = 4-hydroxytoremifene.

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0.2M excessive retention of N-desmethyltoremifene and particularly of toremifene was observed with the consequence of peak broadening. Ammonium acetate is an excellent general purpose mobile phase additive for improving the efficiency of reversed-phase columns (5). The observed trend of decreasing k' values with the increased in ammonium acetate concentrations is in common with other compounds studied (5-8).

The Effect of pH on the Retention and Resolution of Toremifene and Metabolites

The pH of the mobile phase can greatly influence the retention of compounds, especially those which tend to ionize in solution, in reversed-phase HPLC. The k'

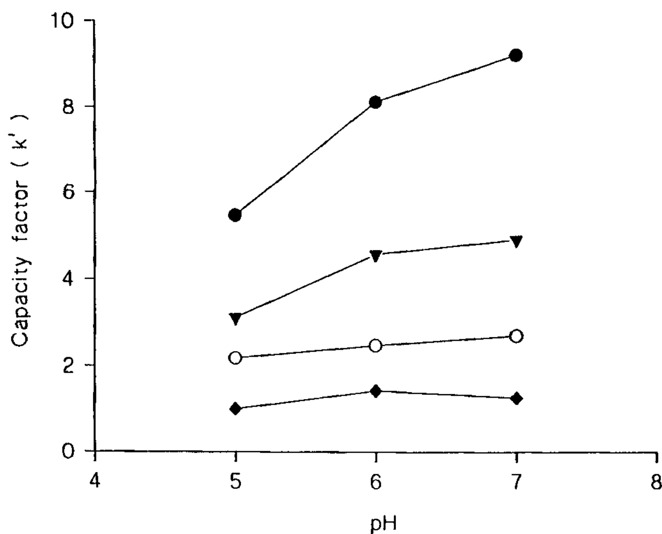

Figure 3 The effect of mobile phase pH on the capacity factors of toremifene and metabolites. The eluent was 65% acetonitrile in 0.25M ammonium acetate buffer at the various pH studied. ● = Toremifene, ▲ = N-desmethyltoremifene, ○ = deaminohydroxytoremifene and ◆ = 4-hydroxytoremifene.

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values of toremifene and N-desmethyltoremifene increased significantly with increasing mobile phase pH (Figure 3). This is because increasing the pH suppressed the protonation of the amino nitrogen of these compounds. This increased their hydrophobicity and therefore their retention. The effect of pH on the retention of deaminohydroxytoremifene was negligible. This is to be expected since with the lose of the amino group this compound is essentially neutral. For 4-hydroxytoremifene, there was an initial increased of retention with increasing pH up to a value of about 6. This was followed by a decreased in k' as the pH increased. These results are consistent with the fact that 4-hydroxytoremifene has two ionizable groups, *i.e.* dimethylamino and phenol groups. The former ionizes at low while the latter at high

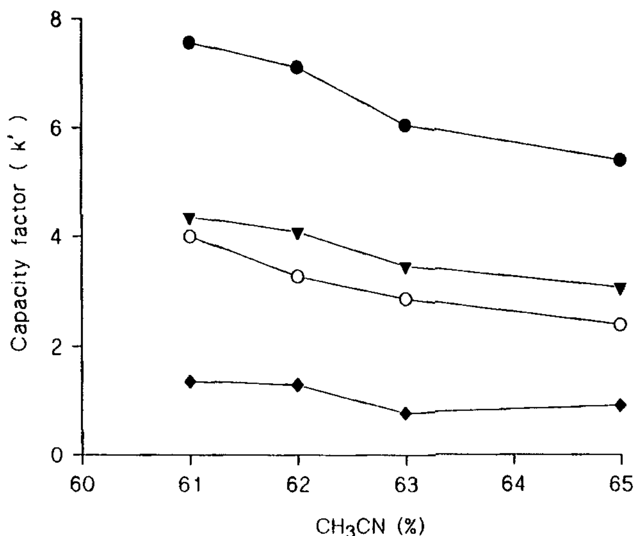

Figure 4 The effect of mobile phase acetonitrile content on the capacity factors (k') of toremifene and metabolites. The eluent was 0.25M ammonium acetate buffer, pH 5.16 containing the various concentration of acetonitrile.
● = Toremifene, ▲ = N-desmethyltoremifene,
○ = deaminohydroxytoremifene and ◆ = 4-hydroxytoremifene.

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pH. The compound is therefore less hydropholic at either low or high pH and is most hydropholic at pH around 6 when it was retained the longest.

Figure 3 clearly shows that the optimum pH for the rapid separation of toremifene and its major metabolites is between 5.0 and 5.2.

The Effect of organic Modifier Concentration on the Retention and Resolution of Toremifene and Metabolites

Two organic modifiers, acetonitrile and methanol, were investigated for the separation of toremifene and metabolites. Acetonitrile was found to be better than methanol in terms of speed of separation and column efficiency. The effect of

acetonitrile concentration on the k' values of toremifene and metabolites (Figure 4) is that expected for reversed-phase chromatography. The k' values decreased with increasing acetonitrile content in the mobile phase. The optimum concentration of acetonitrile was between 64-65%.

The Optimum Solvent System for the Separation of Toremifene and Metabolites

From the results obtained above it becomes obvious that for the fast and efficient separation of toremifene and its major metabolites on the Hypersil-ODS column a mobile phase of 65% (v/v) in 0.25M ammonium a cetate, pH 5.1-5.2 is required. The separation of a standard mixture consisted of 4-hydroxytoremifene, deaminohydroxytoremifene, N-desdimethyltoremifene, N-desmethyltoremifene and toremifene is shown in Figure 5, with the elution order as in the order of the compounds listed. The elution order is that expected for reversed-phase HPLC.

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Applications of the Separation

Two examples of application of the method are given here. The separation of toremifene and metabolites in rat liver microsomes following incubation at 37°C in the presence of NADPH is shown in Figure 6. The metabolites detected were 4-hydroxytoremifene, deaminohydroxytroemifene, N-desdimethyltoremifene and N-desmethyltoremifene, cllearly indicated that the major pathway of toremifene metabolism in this species is the demethylation reactions.

Figure 7 shows the separation of deaminohydroxytoremifene (0.25μg /mL), N-desdimethyltoremifene (0.5 μg / mL), N-desmethyltoremifene (1 μg / mL) and toremifene (2.5 μg / mL) in a human plasma sample spiked with these compounds. No interfering peaks were detected when control plasma samples were analysed.

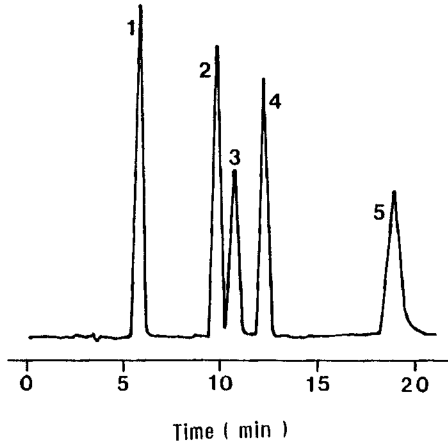

Figure 5 Separation of a standard mixture of toremifene and metabolites. Column, Hypersil-ODS; mobile phase, 65% (v/v) acetonitrile, pH 5.16; flow-rate, 1mL / min; detector, UV 280nm. Peaks: 1 = 4-hydroxytoremifene, 2 = deaminohydroxytoremifene, 3 = N-desdimethyltoremifene, 4 = N-desmethyltoremifene, and 5 = toremifene.

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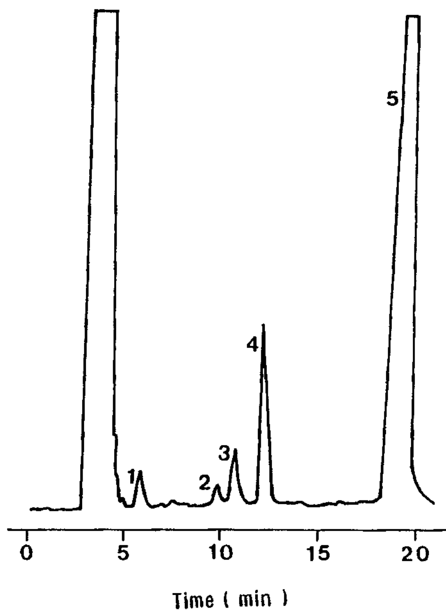

Figure 6 Separation of toremifene and metabolites in rat liver microsome metabolism. HPLC conditions and peak identification as in Figure 5.

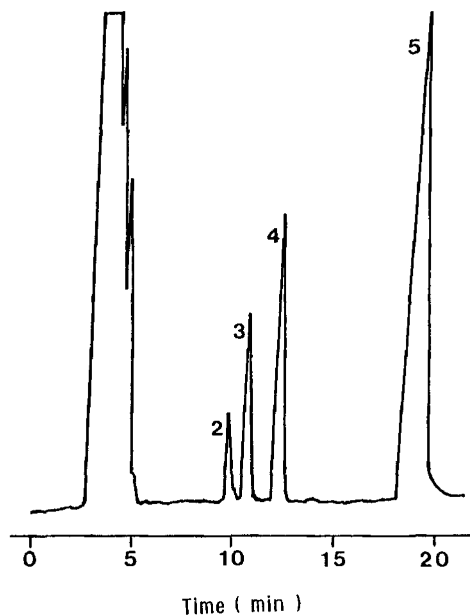

Figure 7 Separation of toremifene and metabolites in plasma spiked with deaminohydroxytoremifene (0.25μg / mL), N-desdimethyltoremifene (0.5 μg / mL), N-desmethyltoremifene (1 μg / mL)and toremifene (2.5 μg / mL . HPLC and peak identification as in Figure 5.

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Since toremifene is given to patients at relatively high dose (200mg / m^2 daily) the present method is sensitive enough for the detection of toremifene and metabolites in plasma.

We expect the flexibility of the method will allow other toremifene metabolites in tissues, urine or faecal samples to be analysed by simple modification of the mobile phase system, *e.g.* by adjusting the buffer concentration, pH and / or organic modifier content in the eluent.

The method is currently being used in our laboratory for the study of toremifene metabolism and pharmacokinetics in rat liver. The results will than be compared to

those obtained with tamoxifen. It is hoped that such a study will lead to a better understanding of the mechanism of carcinogenesis caused by tamoxifen and the lack of it by toremifene.

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