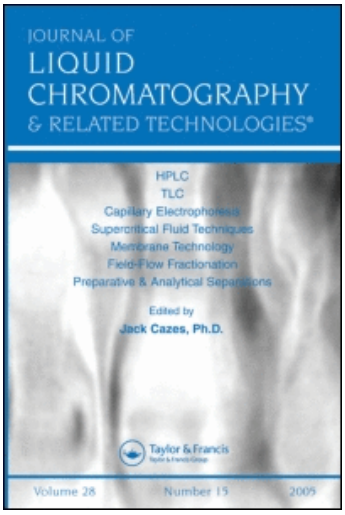

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SEPARATION OF TAMOXIFEN AND METABOLITES BY CAPILLARY ELECTROPHORESIS WITH NON-AQUEOUS BUFFER SYSTEM

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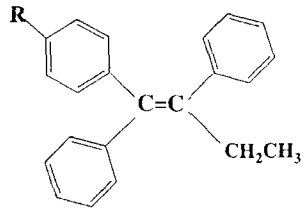
ABSTRACT

Tamoxifen and its metabolites were separated using a non-aqueous mobile phase consisting of ammonium acetate and a mixture of organic solvents, namely, methanol and acetonitrile. The effects of using other organic salts as the conducting medium and varying the composition of the mixture of acetonitrile and methanol on the separation were also investigated. Optimum separation was obtained by using 25 mM ammonium acetate in a 50:50 acetonitrile/ methanol mixture. High efficiency separations and short analysis times of less than 8 min were obtained. The reproducibility of migration time was better than 0.4 %. The detection limits of tamoxifen and its metabolites were less than 40 pg.

INTRODUCTION

Tamoxifen, a non-steroidal anti-estrogen, has been used extensively in the treatment of breast cancer in humans over a decade [1]. Tamoxifen forms several metabolites in the body, the major metabolite being N-desmethyltamoxifen. The structures of tamoxifen and its major metabolites are depicted in Fig 1.

The widespread use of tamoxifen has stimulated efforts to develop routine assays for this drug and its metabolites. Established HPLC assays involved the use of high percentage of



No.	COMPOUND	R
1	N-Desmethyltamoxifen	OCH_2CH_2NHMe
2	N-Desdimethyltamoxifen	$OCH_2CH_2NH_2$
3	Tamoxifen	$OCH_2CH_2NMe_2$
4	Metabolite Y	OCH_2CH_2OH
5	Metabolite E	OH

Fig 1 : Structures of tamoxifen and its metabolites.

organic solvents, high flow rates and long analysis times (> 50 min) owing to the hydrophobic properties of the analytes [2-6]. In some instances, ion-pairing reagents were added to improve selectivity [7]. Capillary gas chromatography with mass spectroscopic detection (GC-MS) was also used for the analysis of tamoxifens [8-9], but the major disadvantage was the need for analyte pre-derivatization before analysis.

Capillary electrophoresis (CE) has been used for pharmaceutical analysis such as the determination of components in drug formulations [10-11] and the monitoring of drugs in body fluids [12-13]. The use of CE for the analysis of tamoxifen and its metabolites has not been described previously and would pose as a challenge for the following reasons. Firstly, tamoxifen and its metabolites are very hydrophobic and hence there exists a solubility problem in aqueous buffers. Secondly, due to the similarity in structures between the tamoxifens, commonly used electrophoretic buffers would not be expected to provide adequate selectivity for their separation. Acetonitrile, methanol and ethanol have been added into the electrophoretic medium to increase the solubility of the analytes in the separation buffer as well as to increase the migration window in CE [14-15]. The disadvantage of using this type of system is the long analysis times attributable to the reduction in electroosmotic flow.

Recently, the use of a non-aqueous buffer solution for the separation of some anti-tumor agents has been reported by Naylor and co-workers [16]. In this approach, a totally organic

mobile phase such as methanol containing ammonium acetate as the electrolyte was used. An advantage of using CE with a non-aqueous medium for pharmacokinetic studies is the small sample requirement and the ability of such a system to perform sample preconcentration to increase sensitivity [16]. Furthermore, a totally organic mobile phase would allow the separation system to be conveniently coupled to a mass spectroscopic detector.

In this work, optimum conditions based on the use of a totally organic separation medium was determined for the separation of tamoxifen and some of its major metabolites. In addition, the effects of various organic salts and different compositions of organic mixtures used in the electrophoretic medium on the separation were investigated.

EXPERIMENTAL

A home-built CE system was used to conduct the experiments. An Isco Model CV^4 UV detector (Lincoln, NE, USA) with wavelength set at 254 nm was employed for the detection of peaks. Chromatographic data were collected with a Hewlett Packard Model 3394A integrator (Avondale, Palo Alto, USA) or a Perkin Elmer Model R100 chart recorder (Illinois, USA). A Spellman Model CZE1000R220 high power voltage supply (Plainview, NY, USA) capable of delivering up to 30 kV (with reversible polarity) and fused silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA) of dimension 50 µm i.d., 44 cm effective length and 55 cm overall length were used in the experiments.

Tamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) and its metabolites N-desdimethyltamoxifen, N-desmethyltamoxifen, metabolite E and metabolite Y were synthesized by colleagues from the same department. Standards were used without further purification. Sample solutions were prepared in the concentration range of 150-200 µg/mL. The structures of tamoxifen and the metabolites are shown in Fig 1.

Ammonium acetate, tetrabutylammonium bromide, tetrabutylammonium hydrogen sulphate and tetrapentylammonium bromide were obtained from Sigma Chemical Comapny (St Louis, MO, USA). The ammonium salts were first dissolved in methanol and appropriate volumes of acetonitrile were subsequently added to the required percentage (v/v). The two buffer reservoirs were capped to prevent evaporation. Methanol and acetonitrile of HPLC grade were purchased from BDH (Poole, England). Sample was injected at a height of 5 cm for 10 sec. The volume of injection was calculated to be 4 nL by using the equation [17] :

$$q = (\rho g \Pi r^4 \Delta h t_i) / (8 \eta L)$$

where q is the amount injected, ρ is the density of the sample solution, g is the acceleration due to gravity, r is the radius of the capillary column, Δh is the injection height (5 cm) raised and t_i is the time of injection, η is the viscosity of the sample solution and L is the length of the column.

RESULTS AND DISCUSSION

Since the tamoxifens are hydrophobic in nature and neutral at all pH conditions, they would not be expected to be separated by varying the buffer pH alone. In fact, even with the addition of SDS (MEKC) and γ-cyclodextrin to the micellar solution (γ-cyclodextrin modified MEKC), only a single peak was observed, as shown in Fig 2(a) and (b). This could be due to the similarity of the structures of tamoxifen and its metabolites. The addition of organic modifiers into the electrophoretic buffer has been shown to decrease electroosmotic flow and increase solubility of sample components in the electrophoretic buffer [14-15]. The effect of adding 10 % (v/v) of acetonitrile into a electrophoretic buffer consisting of SDS and γ-cyclodextrin is shown in Fig 2(c). Although there is an improvement in resolution, the peaks observed were broad and the total analysis time was increased to more than 20 mins.

Since tamoxifen and its metabolites are more readily soluble in organic solvents, the use of a non-aqueous separation buffer was considered. When 25 mM ammonium acetate was added to 100 % methanol to form the electrophoretic medium, the peaks were better resolved but analysis times were long (ca 30 min) and the peaks were broad. Based on the consideration that acetonitrile is a better solvent for the tamoxifens and its viscosity is less than methanol, the addition of acetonitrile was expected to shorten the migration times. The effect of varying the percentage of acetonitrile in methanol on the migration times of the tamoxifens is illustrated in Fig 3. From the plot, it can be observed that a marked decrease in migration times of the tamoxifens was observed after the addition of 5 % acetonitrile. The sharp decrease in migration time could be partly due to a drop in viscosity of the electrophoretic medium. Another reason could be that the solutes are more soluble in acetonitrile than in methanol, thus the addition of acetonitrile increased solubility of the analytes in the separation medium. Increase in acetonitrile concentration above 50 % resulted in a more gradual decrease in migration time. At 50 % acetonitrile, satisfactory separation was achieved. The use of 100 % acetonitrile as the separating medium was attempted without success since the solubility of ammonium acetate in acetonitrile was too low.

The effect of varying concentrations of ammonium acetate on the migration time and separation is shown in Fig 4. Increasing the concentration of ammonium acetate increased

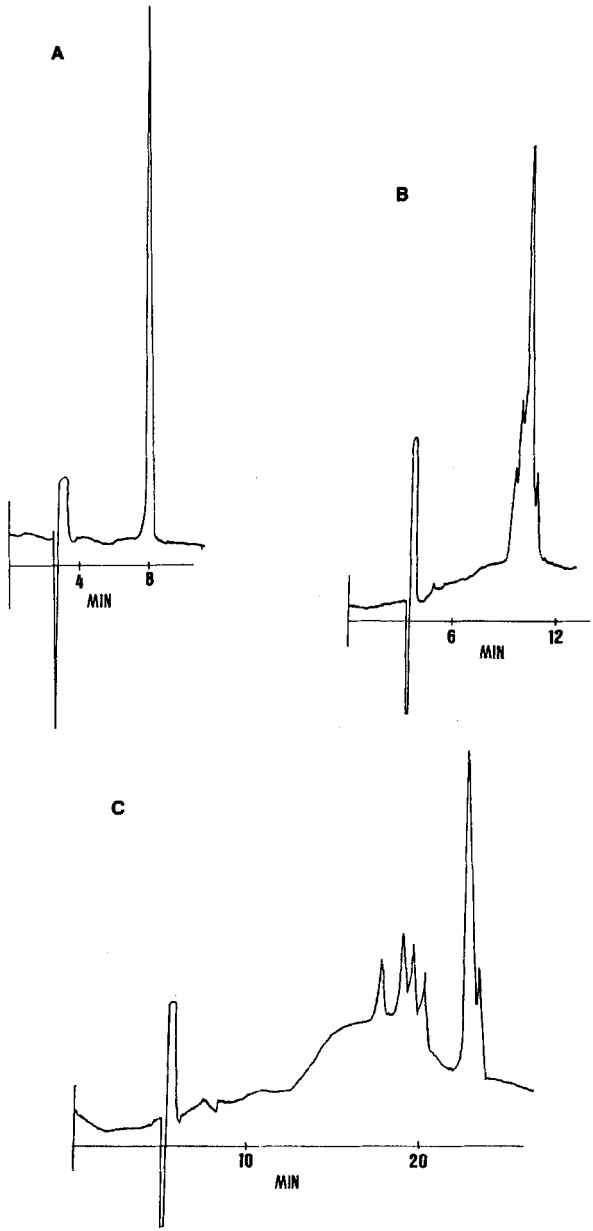


Fig 2 : Typical electropherogram of the separation of tamoxifen and its metabolites.
Electrophoretic conditions :
(a) 25 mM phosphate / borate at pH 8.0 and 50 mM SDS; voltage : 18 kV; detection wavelength : 254 nm; column : 50 µm i.d. fused silica, 44 cm effective length and 55 cm overall length.
(b) 25 mM phosphate / borate at pH 8.0, 60 mM SDS and 20 mM γ-cyclodextrin. Other conditions as in (a).
(c) 25 mM phosphate/ borate at pH 8.0, 60 mM SDS, 20 mM γ-cyclodextrin and 10 % acetonitrile; voltage : 14 kV. Other conditions as in (a).

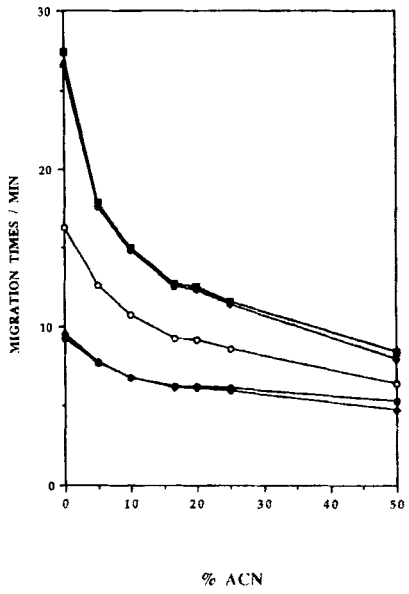


Fig 3 : Variation of migration time with percentage of acetonitrile in methanol
Legend : ♦ : N-desmethyltamoxifen; ● : N-desdimethyltamoxifen; o : tamoxifen; ◇ : metabolite Y; ■ : metabolite E.
Electrophoretic conditions : 25 mM ammonium acetate in organic buffer of varying acetonitrile in methanol. Other conditions as in Fig 2(a).

the ionic strength of the separation medium. It has been shown that electroosmotic flow decreased with increase in ionic strength [15], which subsequently led to longer migration times. No further improvement in resolution was obtained with the addition of more than 25 mM of ammonium acetate. Hence, the optimum separation condition of 25 mM ammonium acetate in 50:50 % acetonitrile / methanol was chosen which offered the best separation and shortest analysis time. A typical electropherogram obtained in such a condition is shown in Fig 5. Analytes which contain hydroxyl groups, such as metabolites E and Y, exhibited stronger interactions with methanol, and thus migrated out later. Efficiencies for this system ranged from 140 000 to 240 000 plates per meter whereas in typical HPLC analysis, the number of theoretical plates would be less than 4000. Another advantage of using the non-aqueous medium is the low current observed which resulted in reduced Joule heating, and consequently, less peak broadening. Reproducibilities in migration times was better than 0.4 % over 5 consecutive runs. The effect of various organic salts on the selectivity was also

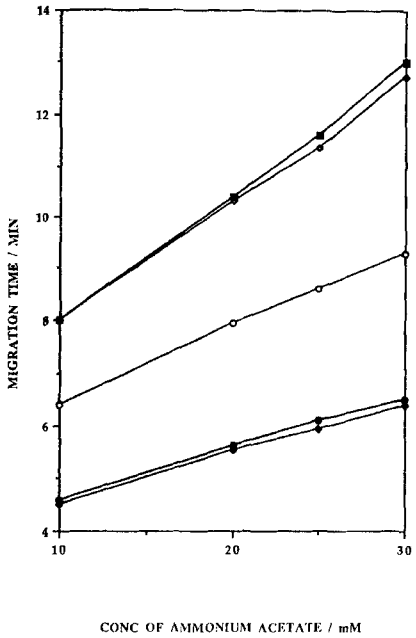


Fig 4 : Variation of migration time with concentration of ammonium acetate.
Legend : ♦ : N-desmethyltamoxifen; ● : N-desdimethyltamoxifen; o : tamoxifen; ◇ : metabolite Y; ■ : metabolite E.
Electrophoretic conditions : varying concentration of ammonium acetate in 25:75 (v/v) % acetonitrile:methanol mixture. Other conditions as in Fig 2(a).

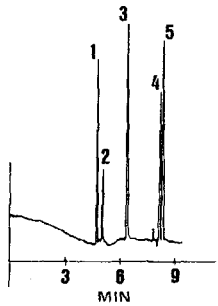


Fig 5 : A typical electropherogram of tamoxifen and its metabolites.
Electrophoretic conditions : 25 mM ammonium acetate in 50:50 acetonitrile:methanol. Other conditions as in Fig 2(a).
Peak identification : 1 : N-desmethyltamoxifen; 2 : N-desdimethyltamoxifen; 3 : tamoxifen; 4 : metabolite Y; 5 : metabolite E.

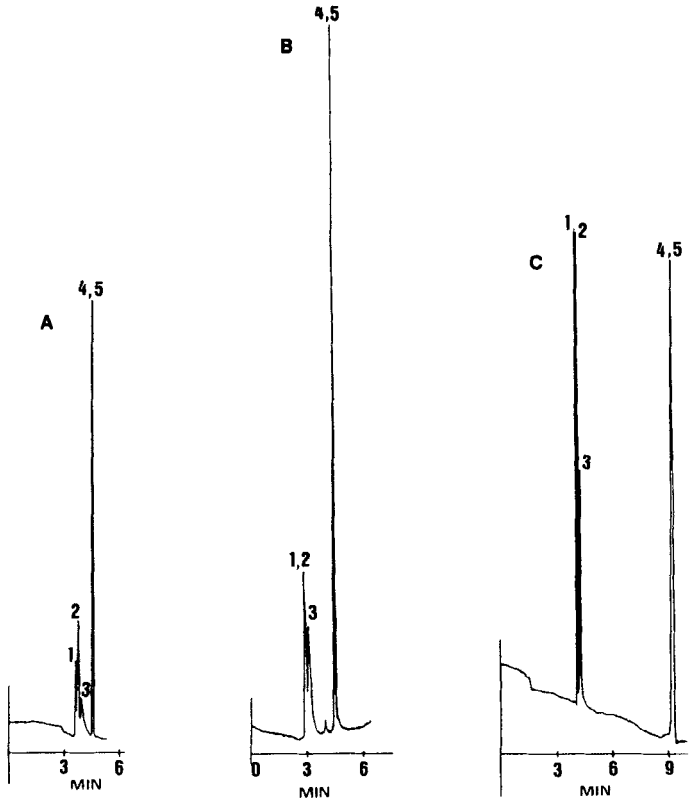


Fig 6 : Comparison of different organic salts on the selectivity and efficiency.
(a) tetrabutylammonium bromide
(b) tetrapentylammonium bromide
(c) tetrabutylammonium hydrogen sulphate
Electrophoretic conditions : 20 mM of the organic salts in 33:67 acetonitrile:methanol. Other conditions as in Fig 2(a). Peak identification as in Fig 5.

investigated. Instead of ammonium acetate, three other salts ie, tetrabutylammonium bromide, tetrabutylammonium hydrogen sulphate and tetrapentylammonium bromide were used. Typical electropherograms obtained using different salts are shown in Fig 6. Fig 6(a) and (b) show the electropherograms obtained using tetrabutylammonium bromide and tetrapentylammonium bromide. It was observed that although short analysis times were obtained, slight peak tailing and broadening were evident. In Fig 6(a) and (c), where the only difference is the presence of different anions in the buffer, tetrabutylammonium hydrogen

TABLE I

Detection Limits of Tamoxifen and Its Metabolites.

No.	Compound	Detection Limit*	
		pg	ppm
1.	N-desmethyltamoxifen	4	1
2.	N-desdimethyltamoxifen	20	5
3.	Tamoxifen	8	2
4.	Metabolite Y	40	10
5.	Metabolite E	8	2

* based on S/N = 2

sulphate seemed to offer slightly higher efficiency separations than tetrabutylammonium bromide. However, none of the tetra-alkylammonium salts provided selectivity as high as that obtained by ammonium acetate.

The detection limits for tamoxifen and it metabolites were determined and the results are tabulated in Table I. Although the concentration detection limit were only in the 1-10 ppm range, the mass sensitivity was higher than those obtained by HPLC with post column derivatization and fluorescence detection (50-500 pg) [2,3], UV detection (5 ng) [18], GC-MS (200 pg) [19] or recently, electrochemical detection (50-100 pg) [6].

CONCLUSION

A non-aqueous electrophoretic separation medium was employed for the separation of tamoxifen and its metabolites. Different organic solvents or a mixture of them and various organic salts could be used for the separation. High efficiencies, short analysis times and relatively low detection limits were obtained. The sensitivity and specificity of the system, the use of low volume of sample and the possiblity of coupling this technique to mass spectroscopic detection makes CE potentially a very powerful technique for the analysis of pharmaceuticals and their metabolites.

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