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On-line high-performance liquid chromatographic–electrospray ionization mass spectrometric method for the study of tamoxifen metabolism

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Abstract

An on-line high-performance liquid chromatographic (HPLC)–electrospray ionization mass spectrometric (ESI-MS) method has been developed and optimized for the study of tamoxifen metabolism. Metabolism in mouse liver microsomes was chosen to demonstrate the applicability and superiority of the method, since mice metabolize tamoxifen faster and produce more metabolites than rats or humans. Mouse liver microsomal preparations were incubated with tamoxifen in the presence of NADPH and $MgCl_2$. The metabolites formed were separated and analyzed by the optimized HPLC–ESI-MS system. The separation was performed on a Res Elute-BD column (5 μm particle size, 250 × 4.6 mm I.D.) with 70% (v/v) methanol in 0.5 *M* ammonium acetate as the mobile phase. A total of eleven metabolites have been detected, some of which have not been previously reported. The metabolites identified are: tamoxifen N-oxide, N-desmethyltamoxifen, 4-hydroxytamoxifen, 4′-hydroxytamoxifen, 4-hydroxytamoxifen N-oxide, 4′-hydroxytamoxifen N-oxide, 4-hydroxy-N-desmethyltamoxifen, 4′-hydroxy-N-desmethyltamoxifen, 3,4-dihydroxytamoxifen, 3,4-epoxytamoxifen and 3,4-epoxytamoxifen N-oxide.

1. Introduction

Tamoxifen [Z-(1-(4-(2-dimethylaminoethoxy)-phenyl)-1,2-diphenyl-1-butene)] (Fig. 1) is widely used in adjuvant therapy for the treatment of breast cancer [1]. It is currently being investigated as a chemopreventative agent in healthy women who might be at greater risk of developing breast cancer, but with no evidence of organic disease [2,3]. Tamoxifen is known to induce hepatocellular tumours in rats [4–6], but not in mice [7]. It is believed that the species difference in hepatocarcinogenesis of tamoxifen may be due to differences in the rate of metabolism and different metabolites being formed by rats and mice [8]. It has been shown that metabolic activation of tamoxifen by monooxygenases led to the formation of reactive intermediates which bind covalently to rat liver microsomal proteins [9] and formed adducts with DNA [10,11]. A detailed study of tamoxifen metabolism in liver microsomes is therefore important for understanding the mechanism of heptatocarcinogenesis.

Various methods have been described for the study of tamoxifen metabolism [12–19], but many of these, particularly those using UV detection, have been shown either to have insufficient resolution, or lack the necessary sensitivity and specificity for the analysis of metabolites. The recent development of on-line high-performance liquid chromatography–electro-

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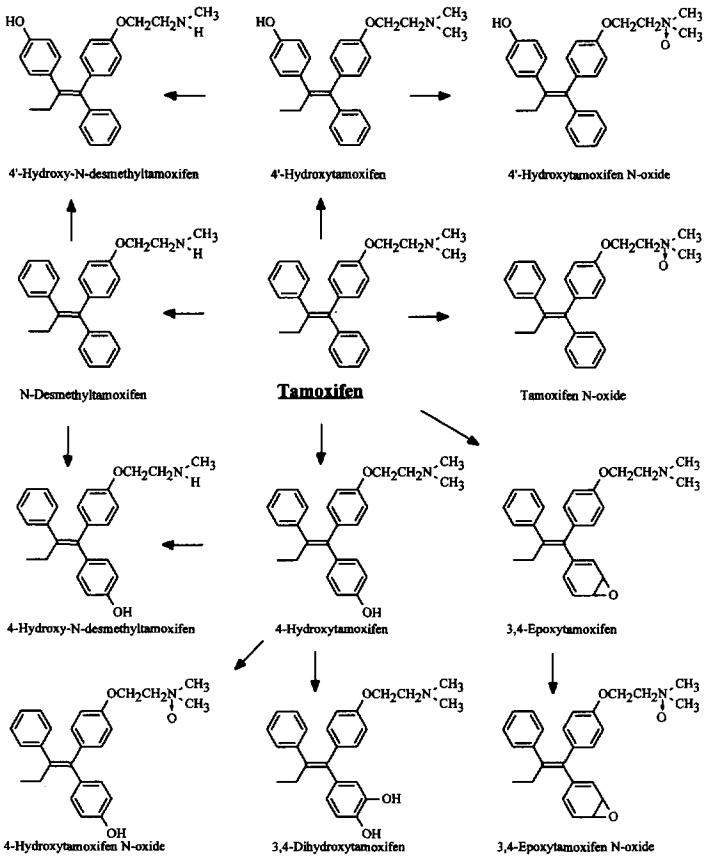

Fig. 1. Possible metabolic pathways of tamoxifen in mouse liver microsomes.

spray ionization mass spectrometry (HPLC–ESI-MS), with vastly improved sensitivity and specificity, provided an opportunity for the detailed study of tamoxifen metabolism. We have previously reported some preliminary results on the use of on-line HPLC–ESI-MS for the identification of tamoxifen metabolites [20]. We describe here further development and optimization of the HPLC–ESI-MS system, particularly on ESI-MS, for the separation and detection of tamoxifen metabolites (Fig. 1) and report the identification of several new metabolites in mouse liver microsomal incubates.

2. Experimental

2.1. Materials and reagents

Tamoxifen, 4-hydroxytamoxifen and N-desmethyltamoxifen were gifts from Dr. J. Topham (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) Tamoxifen N-oxide was prepared according to a published procedure [21]. NADPH was from Sigma (Poole, Dorset, UK). Ammonium acetate, HEPES, $MgCl_2$, NaOH and dimethyl sulphoxide (DMSO) were AnalaR grade from Merck (Poole, Dorset, UK). Methanol was HPLC grade from Fisons Scientific Equipment (Loughborough, Leicestershire, UK).

2.2. Tamoxifen metabolism in mouse liver microsomal preparation

Mouse liver microsomes were prepared as described previously [20]. The microsomes (0.6 mg protein) were preincubated at 37°C for 5 min in an incubation mixture of 2 ml containing 0.05 *M* HEPES–NaOH buffer, pH 7.4, NADPH (0.5 m*M*) and $MgCl_2$ (5 m*M*). The reaction was

started by the addition of tamoxifen (50 μM) and then continued for 30 min. The reaction was terminated by vortex-mixing with 4 ml of methanol–DMSO (4:1, v/v). The supernatant, after centrifugation at 2000 g for 20 min, was analyzed by HPLC and on-line HPLC–ESI-MS.

2.3. High-performance liquid chromatography

A Varian 9012 Solvent delivery system (Varian, Walton-on-Thames, Surrey, UK) was used with a Varian 9065 Polychrom diode-array detector set at 280 nm. Sample injections (100 μl) were via a Varian 9100 autosampler. Tamoxifen and its metabolites were separated isocratically on a Varian Res Elute-BD column (5 μm particle size, 250 × 4.6 mm I.D.), using a mobile phase of 70% methanol in 0.5 M ammonium acetate, at a flow-rate of 1 ml/min.

2.4. On-line HPLC–ESI-MS

A VG Quattro BQ tandem quadrupole mass spectrometer (Fisons Instruments, Manchester, UK) fitted with API electrospray source was used. Capillary and high-voltage electrode potentials were 0.38 and 3.78 kV, respectively. The flow leaving the HPLC column was split in the ratio of 1:6. The remainder was passed into a Varian 9050 variable-wavelength UV–Vis detector set at a wavelength of 280 nm. Sample injections (100 μl) were via a Rheodyne 7125 injector (Cotati, CA, USA) fitted with a 200-μl loop. Optimum source temperature was determined to be 150°C. Lens 1 and Lens 2 were both set at 80–85 V. Ion spectra were acquired in the positive ion mode, over the mass range of 300 to 500 atomic mass unit (amu), at a rate of 1 scan every 2 s. All spectra were acquired as continuum data.

3. Results and discussion

The aim of the present study is to develop a highly efficient, sensitive and specific on-line HPLC–ESI-MS method applicable to the investigation of tamoxifen metabolism in liver microsomes, especially for the detection and identification of new metabolites. This requires optimization of the HPLC separation as well as the ESI-MS detection. Our previous study showed that tamoxifen and its metabolites chromatographed most effectively on a base-deactivated ODS column. We have compared several columns of this type and have chosen the Res Elute-BD column for the present study, because it gave excellent resolution, particularly of the more polar metabolites. High resolution is important, as many of the metabolites are present in isomeric forms. High resolution is also necessary to compensate for a slight loss in resolution, caused by splitting the HPLC effluent before it enters the ESI-MS.

Two important variables need to be optimized in order to achieve the highest possible ESI-MS sensitivity of detection. These are the flow-rate of the HPLC effluent entering the ion source, and the source temperature. Optimizations of the flow-rate of the mobile phase entering the ion source and the source temperature for maximum signal transmission for tamoxifen and its derivatives were therefore investigated. Figs. 2a and 2b show the relationships between signal intensity and temperature over a range (100–200 μl/min) of flow-rates for 4-hydroxytamoxifen and N-desmethyltamoxifen, respectively. Flow-rate and source temperature were clearly linked to give maximal signal intensity; higher temperatures were required for high flow-rates. Optimum temperatures for 100, 150 and 200 μl/min were 90–110, 130–150 and 160–170°C, respectively, depending on the compound studied. Source temperature of 160°C and above, needed for a flow-rate of 200 μl/min, was difficult to maintain because it required the source heater to be set as high as 180°C to overcome cooling. At low flow-rate (100 μl/min), the signal intensities of two of the metabolites (4-hydroxytamoxifen and N-desmethyltamoxifen) were reduced compared to those with flow-rates of 150 μl/min and 200 μl/min (Figs. 2a and 2b). Flow-rates under 100 μl/min and above 200 μl/min were studied under similar conditions, and showed a marked reduction in signal intensity. From these results, it was concluded that maximum peak intensity for tamoxifen and its derivatives was obtained

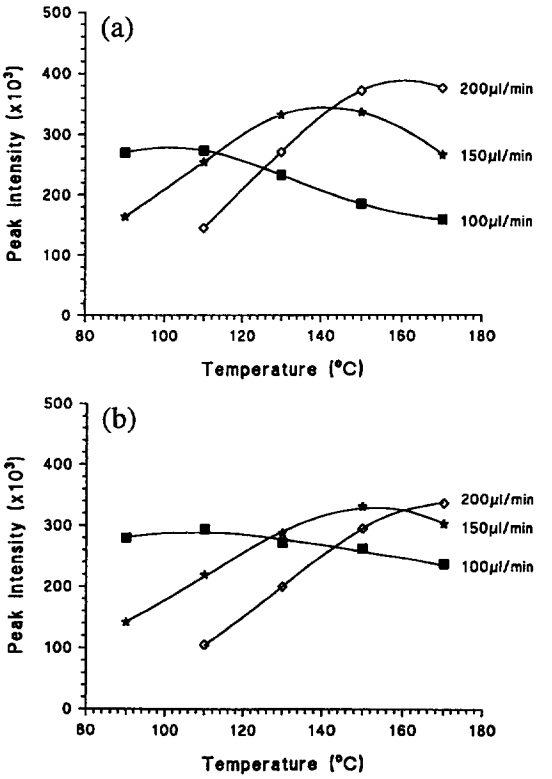

Fig. 2. Relationships between pseudomolecular ion signal intensity and temperature over a range of flow-rates for: (a) 4-hydroxytamoxifen (m/z 388), (b) N-desmethyltamoxifen (m/z 358). The mobile phase was 70% (v/v) methanol in 0.5 M ammonium acetate at flow-rates of 100, 150 and 200 μl/min.

when the flow-rate was between 150–200 μl/min with the source temperature between 140–160°C.

The voltage-induced ionization of the pseudomolecular ions within the electrospray source also requires optimization for maximum sensitivity of detection. The factors requiring optimization are the voltages of the sampling cone (lens 1) and the skimmer (lens 2). Preliminary results indicated that maximum peak signal transmission was seen when both lens 1 and lens 2 were set at 70–90 V. Further investigations of these values by fixing each lens, in turn, at 80 V and varying the value of the other lens between 20 and 140 V were carried out. Variation of one lens relative to the other fixed at 80 V resulted in bell-shaped curves, with the optimum at 80–90 V. A significant difference could be seen between the effect

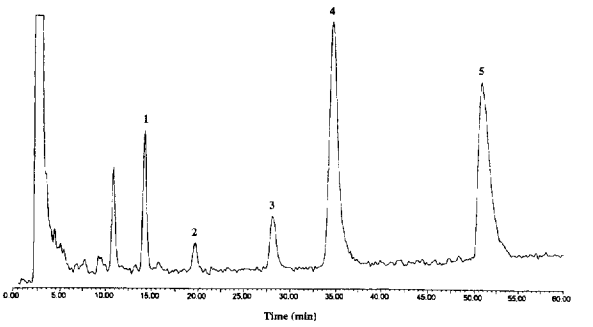

Fig. 3. HPLC separation of tamoxifen and its metabolites. Tamoxifen was incubated with a mouse liver microsome preparation for 30 min, and the resulting metabolites were separated isocratically by HPLC on a Varian Res Elute-BD column (5 μm particle size, 250 × 4.6 mm I.D.), using a mobile phase of 70% methanol in 0.5 M ammonium acetate at a flow-rate of 1 ml/min. Peaks were detected using a wavelength of 280 nm. Peaks: 1 = 4-hydroxytamoxifen, 2 = 4′-hydroxytamoxifen, 3 = N-desmethyltamoxifen, 4 = tamoxifen N-oxide, 5 = tamoxifen.

of the two lenses. Altering lens 1 led to a graph with a very clear optimum value, between 80 and 90 V, suggesting that the value of this lens was

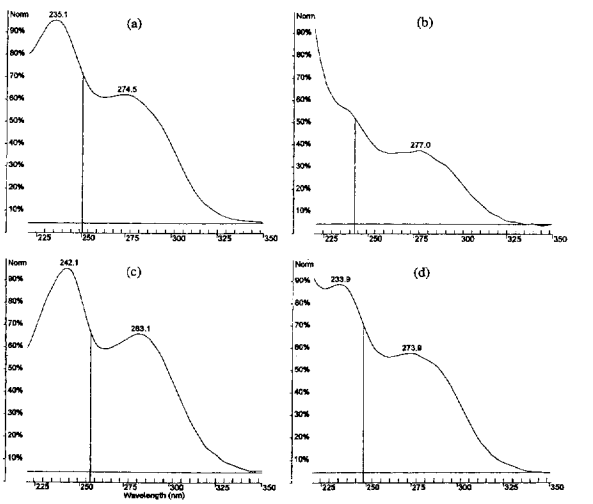

Fig. 4. UV absorption spectra of tamoxifen and its metabolites. Tamoxifen was incubated with a mouse liver microsome preparation for 30 min and the resulting metabolites separated by HPLC, as described in the section Methods. UV absorption spectra were acquired between 200 and 350 nm using a diode-array detector. The spectra are as follows: (a) = tamoxifen, (b) = N-desmethyltamoxifen, (c) = 4-hydroxytamoxifen, (d) = tamoxifen N-oxide.

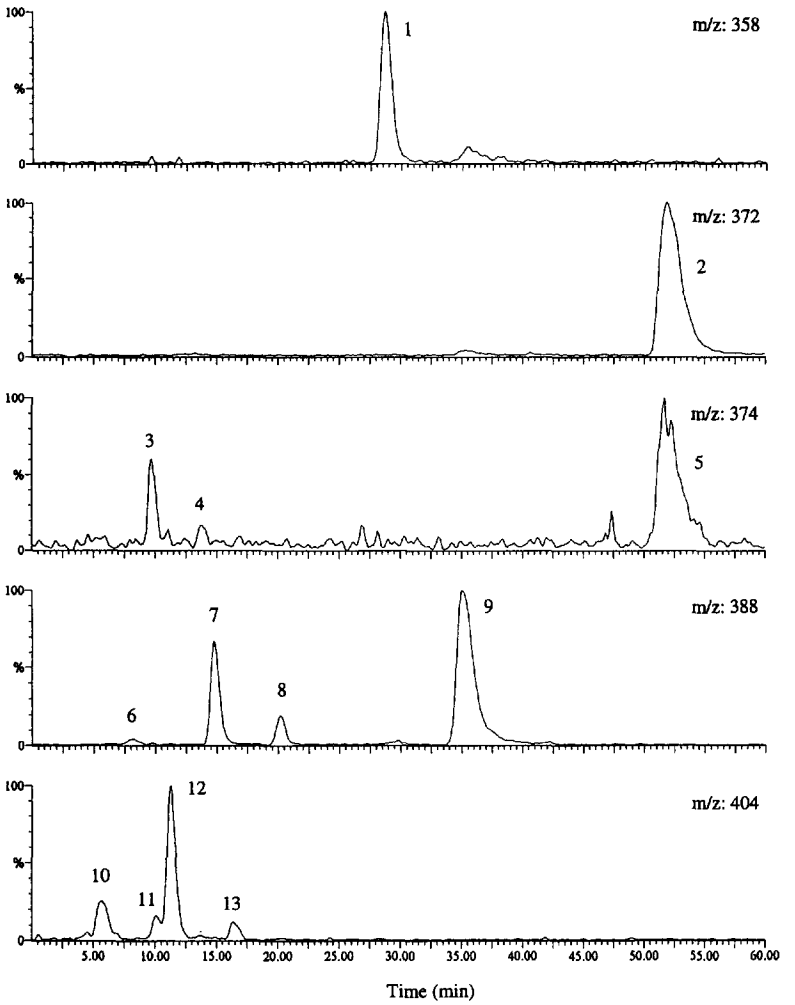

Fig. 5. On-line HPLC–ESI-MS separation of tamoxifen and its metabolites. Tamoxifen was incubated with a mouse liver microsome preparation for 30 min, and the resulting metabolites separated by isocratically HPLC. A Varian Res Elute-BD column (5 μm particle size, 250 × 4.6 mm I.D.) was used, with a mobile phase of 70% methanol in 0.5 *M* ammonium acetate at a flow-rate of 1 ml/min. Detection was on-line, using a VG Quattro BQ electrospray MS. Mass chromatograms were acquired over the mass range 300–500 amu. Peaks: 1 = N-desmethyltamoxifen, 2 = tamoxifen, 3 = 4-hydroxy-N-desmethyltamoxifen, 4 = 4′-hydroxy-N-desmethyltamoxifen, 5 = isotope signal of *m/z* 372, 6 = 3,4-epoxytamoxifen, 7 = 4-hydroxytamoxifen, 8 = 4′-hydroxytamoxifen, 9 = tamoxifen N-oxide, 10 = 3,4-epoxytamoxifen N-oxide, 11 = 3,4-dihydroxytamoxifen, 12 = 4-hydroxytamoxifen N-oxide, 13 = 4′-hydroxytamoxifen N-oxide.

more critical for signal transmission. Altering lens 2 resulted in a much broader peak shape, suggesting that the optimum for this lens was over a greater range and hence has much less effect on signal transmission. It was therefore concluded that the optimum values for lens 1 and lens 2 are between 80–85 V. Using these optimized voltages, together with the optimized flow-rate and source temperature, the detection limit for tamoxifen was 0.2 ng/ml at a signal-to-noise ratio of 5, which is at least 10 times more sensitive than the fluorescence detection methods.

Fig. 3 shows the HPLC separation of tamoxifen metabolites formed following incubation of tamoxifen with mouse liver microsomes. The

peaks were detected with a photodiode-array detector, and the characteristic UV spectra of tamoxifen and its major metabolites, 4-hydroxytamoxifen, N-desmethyltamoxifen and tamoxifen N-oxide, are shown in Fig. 4. Although a photodiode-array detector could be used for the identification of major metabolites based on their spectral characteristics, it was insufficiently sensitive for the detection and identification of minor metabolites, which may be more important biologically. This could be achieved with the optimized HPLC–ESI-MS system.

Fig. 5 shows the mass chromatograms of the metabolites detected in the mouse liver microsomal incubate. N-Desmethyltamoxifen (peak 1, m/z 358) and tamoxifen (peak 2, m/z 372) were identified by their retention times in comparison to authentic standards.

Peaks 3 and 4 (m/z 374) were identified as 4-hydroxy-N-desmethyltamoxifen and 4′-hydroxy-N-desmethyltamoxifen, respectively. They were derived from 4-hydroxytamoxifen and 4′-hydroxytamoxifen by N-demethylation. 4-Hydroxy-N-desmethyltamoxifen has been identified previously [16] as a metabolite, while detection of 4′-hydroxy-N-desmethyltamoxifen has hitherto not been reported.

Peak 5 (m/z 374) is not a true HPLC peak. It is the isotopic ion of the m/z 372 signal.

There were four peaks with the $[M + H]^+$ ion at m/z 388. These peaks were identical to, and consistent with, our previous findings: they were 3,4-epoxytamoxifen (peak 6), 4-hydroxytamoxifen (peak 7), 4′-hydroxytamoxifen (peak 8) and tamoxifen N-oxide (peak 9). 3,4-Epoxytamoxifen can be prepared by incubating tamoxifen with the horseradish peroxidase–H_2O_2 system [22]. It had an identical retention time to peak 6.

The tamoxifen N-oxide peak was the most intense, because N-oxidation is known to be catalyzed by the flavin-containing monooxygenase [23], and this enzyme is particularly active in mouse liver microsomes [20].

The high activity of mouse microsomal flavin-containing monooxygenase also led to the N-oxidation of 4-hydroxytamoxifen and 4′-hydroxytamoxifen with the formation of 4-hydroxytamoxifen N-oxide (peak 12, m/z 404) and 4′-hydroxytamoxifen N-oxide (peak 13, m/z 404), respectively. Confirmation of structures was obtained by treating 4- and 4′-hydroxytamoxifen with H_2O_2, when the same N-oxides were formed. Peak 11 (m/z 404) was characterized as 3,4-dihydroxytamoxifen. An identical compound could be generated by incubation of 3-hydroxytamoxifen with mouse liver microsomes.

Peak 10 (m/z 404) was identified as 3,4-epoxytamoxifen N-oxide. This is based on the observation that the N-oxidation reaction was highly active in mouse liver microsomes, and metabolites with a dimethylamino group tend to form N-oxide derivatives. An identical compound was detected when tamoxifen was metabolized by the horseradish peroxidase–H_2O_2 system [22].

The compounds N-desdimethyltamoxifen and diaminohydroxytamoxifen, which have been detected in human plasma [19], were not metabolites in mouse liver microsomal metabolism.

The present study clearly demonstrated the power of the on-line HPLC–ESI-MS system for the study of drug metabolism, especially in the identification of new metabolites. A total of 11 metabolites have been detected. The new metabolites of tamoxifen characterized in this study are 4′-hydroxytamoxifen N-oxide, 4′-hydroxy-N-desmethyltamoxifen and, possibly, 3,4-epoxytamoxifen N-oxide (Fig. 1).

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