Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 518–526

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Investigational study of tamoxifen phase I metabolites using chromatographic and spectroscopic analytical techniques

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ARTICLE INFO

Article history:
Received 24 September 2010
Received in revised form 3 February 2011
Accepted 10 February 2011
Available online 18 February 2011

Keywords:
Fragmentation
Identification
Liquid chromatography
Metabolites
Mass spectrometry
Tamoxifen
UV absorption

ABSTRACT

A comprehensive overview is presented of currently known phase I metabolites of tamoxifen consisting of their systematic name and molecular structure. Reference standards are utilized to elucidate the MS^n fragmentation patterns of these metabolites using a linear ion trap mass spectrometer. UV-absorption spectra are recorded and absorption maxima are defined.

Serum extracts from ten breast cancer patients receiving 40 mg tamoxifen once daily were qualitatively analyzed for tamoxifen phase I metabolites using a liquid chromatography–tandem mass spectrometry set-up. In total, 19 metabolites have been identified in these serum samples. Additionally a synthetic method for the preparation of the putative metabolite 3′,4′-dihydroxytamoxifen is described.

1. Introduction

Tamoxifen, a first generation selective estrogen receptor modulator is widely used for the treatment of early and advanced breast cancer [1,2]. Tamoxifen is a substrate for the cytochrome P450 enzyme system and flavin-containing monooxygenase (FMO). Metabolism involves hydroxylation, demethylation and N-oxidation into several metabolites that vary in toxicity and estrogenic activity [3,4]. Since most tamoxifen metabolites feature an anti-estrogenic activity towards the estrogen receptors (ER) alpha and beta higher than the parent compound, tamoxifen has been considered a prodrug.

Since 1984, 4-hydroxytamoxifen was assumed to be the active metabolite of tamoxifen [5]. The metabolite N-desmethyl-4-hydroxytamoxifen, also known as endoxifen [6] was for the first time reported in 1989 [7] and years later identified to be equally active as 4-hydroxytamoxifen though present at a much higher steady state concentration in patients [6,8,9]. There are at least 36 phase I metabolites of tamoxifen (Table 1), of which some are very similar in molecular structure to 4-hydroxytamoxifen and endoxifen. Most of these metabolites are, however, only marginally described in literature. Clinical relevance of these metabolites is unclear and additional data on estrogenic activity is lacking.

We present an extensive overview of tamoxifen phase I metabolites as known to date based on data described in literature and data as generated in this study, including mass spectrometry fragmentation patterns and UV absorption spectra. Additionally a liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) assay is presented for the analysis of the phase I metabolites in serum from breast cancer patients receiving tamoxifen once daily. Using this assay three formerly unknown metabolites are identified.

3′,4′-Dihydroxytamoxifen, a metabolite of tamoxifen which is described in the literature [10,11], but of which no reference standard is available, was synthesized. The synthetic method for the preparation of 3′,4′-dihydroxytamoxifen is described and has, to

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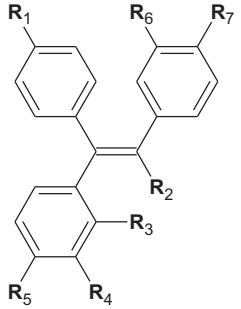
doi:10.1016/j.jpba.2011.02.009

Table 1
Trivial names, chemical structures and molecular masses of tamoxifen and its metabolites.



Trivial name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Formula	Mol. mass
Tamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	H	H	H	H	$C_{26}H_{29}NO$	371.5
Tamoxifen-$^{13}C_2$–^{15}N	O–CH_2–CH_2–^{15}N($^{13}CH_3$)$_2$	CH_2–CH_3	H	H	H	H	H	$C_{24}{}^{13}C_2H_{29}{}^{15}NO$	374.5
Tamoxifen-$^2H_5$	O–CH_2–CH_2–N(CH_3)$_2$	C^2H_2–C^2H_3	H	H	H	H	H	$C_{26}H_{24}{}^2H_5NO$	376.5
N-desmethyl-tamoxifen	O–CH_2–CH_2–NH–CH_3	CH_2–CH_3	H	H	H	H	H	$C_{25}H_{27}NO$	357.5
N,N-didesmethyl-tamoxifen	O–CH_2–CH_2–NH_2	CH_2–CH_3	H	H	H	H	H	$C_{24}H_{25}NO$	343.5
α-Hydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH(OH)–CH_3	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5
β-Hydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_2–OH	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5
2-Hydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	OH	H	H	H	H	$C_{26}H_{29}NO_2$	387.5
3-Hydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	OH	H	H	H	$C_{26}H_{29}NO_2$	387.5
4-Hydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	H	OH	H	H	$C_{26}H_{29}NO_2$	387.5
4′-Hydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	H	H	H	OH	$C_{26}H_{29}NO_2$	387.5
Tamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_3	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5
α-Hydroxytamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH(OH)–CH_3	H	H	H	H	H	$C_{26}H_{29}NO_3$	403.5
β-Hydroxytamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_2–OH	H	H	H	H	H	$C_{26}H_{29}NO_3$	403.5
3-Hydroxytamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_3	H	OH	H	H	H	$C_{26}H_{29}NO_3$	403.5
4-Hydroxytamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_3	H	H	OH	H	H	$C_{26}H_{29}NO_3$	403.5
4′-Hydroxytamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_3	H	H	H	H	OH	$C_{26}H_{29}NO_3$	403.5
2,4-Dihydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	OH	H	OH	H	H	$C_{26}H_{29}NO_3$	403.5
3,4-Dihydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	OH	OH	H	H	$C_{26}H_{29}NO_3$	403.5
3′,4′-Dihydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	H	H	OH	OH	$C_{26}H_{29}NO_3$	403.5
α,4-Dihydroxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH(OH)–CH_3	H	H	OH	H	H	$C_{26}H_{29}NO_3$	403.5
1,2-Epoxytamoxifen[a]	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	H	H	H	H	$C_{26}H_{29}NO_2$	387.5
1,2-Epoxytamoxifen-N-oxide[a]	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_3	H	H	H	H	H	$C_{26}H_{29}NO_3$	403.5
3,4-Epoxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	Epoxide	Epoxide	H	H	$C_{26}H_{27}NO_2$	385.5
3′,4′-Epoxytamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	H	H	Epoxide	Epoxide	$C_{26}H_{27}NO_2$	385.5
3,4-Epoxytamoxifen-N-oxide	O–CH_2–CH_2–NO(CH_3)$_2$	CH_2–CH_3	H	Epoxide	Epoxide	H	H	$C_{26}H_{27}NO_3$	401.5
N-desmethyl-α-hydroxytamoxifen	O–CH_2–CH_2–NH–CH_3	CH(OH)–CH_3	H	H	H	H	H	$C_{25}H_{27}NO_2$	373.5
N-desmethyl-3-hydroxytamoxifen	O–CH_2–CH_2–NH–CH_3	CH_2–CH_3	H	OH	H	H	H	$C_{25}H_{27}NO_2$	373.5
N-desmethyl-4-hydroxytamoxifen	O–CH_2–CH_2–NH–CH_3	CH_2–CH_3	H	H	OH	H	H	$C_{25}H_{27}NO_2$	373.5
N-desmethyl-4′-hydroxytamoxifen	O–CH_2–CH_2–NH–CH_3	CH_2–CH_3	H	H	H	H	OH	$C_{25}H_{27}NO_2$	373.5
N,N-didesmethyl-α-hydroxytamoxifen	O–CH_2–CH_2–NH_2	CH(OH)–CH_3	H	H	H	H	H	$C_{24}H_{25}NO_2$	359.5
N,N-didesmethyl-3-hydroxytamoxifen	O–CH_2–CH_2–NH_2	CH_2–CH_3	H	OH	H	H	H	$C_{24}H_{25}NO_2$	359.5
N,N-didesmethyl-4-hydroxytamoxifen	O–CH_2–CH_2–NH_2	CH_2–CH_3	H	H	OH	H	H	$C_{24}H_{25}NO_2$	359.5
N,N-didesmethyl-4′-hydroxytamoxifen	O–CH_2–CH_2–NH_2	CH_2–CH_3	H	H	H	H	OH	$C_{24}H_{25}NO_2$	359.5
3-Hydroxy-4-methoxy-tamoxifen	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	OH	O–CH_3	H	H	$C_{27}H_{31}NO_3$	416.5
4-Hydroxy-3-methoxy-tamoxifen (met. C)	O–CH_2–CH_2–N(CH_3)$_2$	CH_2–CH_3	H	O–CH_3	OH	H	H	$C_{27}H_{31}NO_3$	416.5
3-Hydroxy-4-methoxy-N-desmethyl-tamoxifen	O–CH_2–CH_2–NH–CH_3	CH_2–CH_3	H	OH	O–CH_3	H	H	$C_{26}H_{29}NO_3$	402.5
Metabolite E	OH	CH_2–CH_3	H	H	H	H	H	$C_{22}H_{22}O$	300.4
Metabolite Y	O–CH_2–CH_2–OH	CH_2–CH_3	H	H	H	H	H	$C_{24}H_{24}O_2$	344.4

[a] Epoxide is formed from water added to the ethylenic double bond.

the best of our knowledge, not been reported in the literature hitherto.

The presented data may be useful to further elucidate the clinical pharmacologic role of metabolism in tamoxifen-treated breast cancer patients.

2. Materials and methods

2.1. Chemicals and reagents

Tamoxifen, tamoxifen-$^2H_5$, 3-hydroxytamoxifen, 4-hydroxytamoxifen, 4′-hydroxytamoxifen, α-hydroxytamoxifen, β-hydroxytamoxifen, N-desmethyl-tamoxifen, N-desmethyl-4-hydroxytamoxifen, N-desmethyl-4′-hydroxytamoxifen and tamoxifen-N-oxide were purchased from Toronto Research Chemicals (North York, Ontario, Canada). 3,4-Dihydroxytamoxifen was synthesized following the procedure described in literature [12]. 3′,4′-Dihydroxytamoxifen was synthesized according to the procedure as described below. Acetonitrile was obtained from Biosolve (Valkenswaard, The Netherlands). LiChrosolv water for chromatography was purchased from Merck (Darmstadt, Germany). Ammonium formate was from Acros Organics (Geel, Belgium). Tamoxifen-$^{13}C_2$–^{15}N and dry tetrahydrofuran (THF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were commercial compounds of the highest purity available.

2.2. Treatment of patients

Included patients used 40 mg of tamoxifen daily, for treatment of metastatic breast cancer. Patients had to use tamoxifen for at least 3 weeks to ensure steady state concentrations. Other inclusion

criteria were age over 18 years, good WHO performance score (zero or one), female gender, and adequate haematological, renal and hepatic functions. All patients provided written informed consent prior to study entrance. The study protocol was approved by the institutional review board and registered by the Dutch trial registry (http://www.trialregister.nl/, number NTR 1751).

2.3. General procedures for the synthesis and structural confirmation of 3′,4′-dihydroxytamoxifen

Analytical thin layer chromatography (TLC) was performed on aluminium plates with Merck Kieselgel 60F254 and visualised by UV irradiation (254 nm) or by staining with solution of phosphomolibdic acid. Flash column chromatography was carried out using Merck Kieselgel 60 (230–400 mesh) under pressure. Liquid chromatography was performed using a Shimadzu instrument using a dual wavelength detector (254 and 300 nm) with a NUCLEODUR® C4 gravity column (150 mm × 21 mm I.D., 5 μm; Macherey-Nagel) using a flow rate of 25 mL min^{-1} and a mixture of water with 0.1% trifluoroacetic acid (TFA, eluent A) and acetonitrile with 0.1% TFA (eluent B) as the mobile phase. To elute the compounds from the column, gradient elution was used. At time zero a ratio of A–B (10:90, v/v) was pumped through the column. Linear gradients were applied from time zero to 5 min to a A–B composition of 30:70 (v/v) and from 5 to 40 min to 80:20 (v/v).

All reactions were carried out under an argon atmosphere, and those not involving aqueous reagents were carried out in oven-dried glassware. Fourier transform infrared spectra were obtained on a Bruker Tensor 27FT equipped with an ATR. ^{1}H NMR spectra were recorded in $CDCl_3$ and CD_3OD at ambient temperature on a Bruker DRX-400 spectrometer at 400 MHz with residual protic solvent as the internal reference ($CDCl_3$, δ_H = 7.26 ppm; CD_3OD, δ_H = 3.31 ppm); chemical shifts (δ) are given in parts per million (ppm), and coupling constants (J) are given in Hertz (Hz). The proton spectra are reported as follows: δ (multiplicity, coupling constant J, number of protons). ^{13}C NMR spectra were recorded in $CDCl_3$ and CD_3OD at ambient temperature on the same spectrometer at 100 MHz, with the central peak of $CDCl_3$ (δ_C = 77.0 ppm) and CD_3OD (δ_C = 49.15 ppm) as the internal reference. DEPT135 experiments are used to aid in the assignment of signals in the ^{13}C NMR spectra. The naming s, d, t, q behind the ^{13}C signal assigns the hydrogen multiplicity of the respective carbon (no H = s; 1H = d; 2H = t; 3H = q).

Synthesis products were identified by high resolution mass spectra taken on a Thermo Electron LTQ Orbitrap equipped with an electrospray ionization source operating in the positive ion mode. All data were recorded using LCquan™ software (Thermo Fisher Scientific).

2.4. Characterization of tamoxifen and phase I metabolites

2.4.1. MS^n fragmentation

From the available reference standards fragmentation (MS^n) spectra were acquired. Standard solutions in methanol: 3.5 mM ammonium formate buffer pH 3.5 (1:1, v/v) were continuously infused into an LTQ XL Linear Ion Trap mass spectrometer equipped with an electrospray ionization source operating in the positive ion mode. Multiple reaction monitoring (MRM) spectra were acquired with LCquan™ software (Thermo Fisher Scientific). Positive ions were created at atmospheric pressure using an ion spray voltage of 4000 V and sheath, auxiliary and ion sweep gas at 45, 25 and 2 arbitrary units, respectively. The capillary temperature was set at 375 °C.

2.4.2. Liquid chromatography–ultraviolet detection

HPLC–UV experiments were performed using the same chromatographic parameters as described under Section 2.4 but using an Accela chromatography system (Thermo Fisher Scientific) instead, consisting of a quaternary solvent delivery system, online degasser, autosampler, column oven and a photodiode array detector.

2.5. Analysis of tamoxifen and its phase I metabolites in patient serum samples

Serum samples (50 μL) were prepared by the addition of a volume of 150 μL acetonitrile. The mixture was vortex mixed for 10 s followed by centrifugation for 10 min at 11,300 × g. The clear supernatant was diluted 1:1 (v/v) with 3.5 mM ammonium formate buffer pH 3.5. The sample was vortex mixed for 10 s and subsequently transferred to an amber colored autosampler vial. The final extracts were stored at 2–8 °C until analysis. To correct for signal fluctuation as a result of sample preparation of fluctuation in the mass spectrometry response, a stable isotope labeled internal standard might be added to the serum extract. Deuterated tamoxifen and deuterated tamoxifen metabolites are often used for this purpose [8].

HPLC experiments were performed on a LC-20AD Prominence binary solvent delivery system with a column oven, DGU-20A3 online degasser and a SIL-HTc controller (Shimadzu, Kyoto, Japan).

Mobile phase A was prepared by adjusting a 3.5 mM ammonium formate solution to pH 3.5 with a 98% formic acid solution. Mobile phase B consisted of acetonitrile. Mobile phases A and B were pumped through a Synergi Hydro 110 Å column (150 mm × 2.0 mm I.D., 4 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.4 mL min^{-1}. To elute the compounds from the column, gradient elution was used. At time zero a ratio of A–B (70:30, v/v) was pumped through the column. Linear gradients were applied from time zero to 12 min to a A–B composition of 30:70 (v/v) and from 12.1 to 16 min to 20:80 (v/v). The column was reconditioned from 16.1 to 19.0 min with the original composition (70:30, v/v). The separation was performed at 40 °C. Volumes of 15 μL were injected using an autosampler thermostatted at 7 °C. The total run time was 19 min. The autosampler needle was rinsed with methanol before and after each injection.

Multiple reaction monitoring (MRM) chromatograms were acquired on a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization source operating in the positive ion mode at atmospheric pressure using an ion spray voltage of 4000 V and sheath, auxilliary and ion sweep gas at 45, 25 and 2 arbitrary units, respectively. The capillary temperature was set at 375 °C. The quadrupoles were operating at unit resolution (0.7 Da).

3. Results and discussion

3.1. Synthesis and structural confirmation of 3′,4′-dihydroxytamoxifen

The synthesis of 3′,4′-dihydroxytamoxifen is based on a McMurry reaction between the alkylated 4-hydroxybenzophenone and 3,4-methylenedioxopropiophenone, followed by selective removal of the methylenedioxy ring [13] (Fig. 1). In the first step, 4-hydroxybenzophenone was alkylated using 1,2-dibromoethane and NaH as base. The McMurry coupling of the alkylated ketone and 3,4-methylenedioxopropiophenone in the presence of $TiCl_4$/Zn gave 1-[4-(2-bromoethoxy)phenyl]-1-phenyl-2-(3,4-methylendioxyphenyl)-1-butene as a mixture of E and Z isomers in good yield (76%) which were converted into the amines in a moderated yield (49%) by refluxing with dimethylamine in methanol. By selective removal of the methylenedioxy ring using BCl_3 in CH_2Cl_2 at room temperature [14], 3′,4′-dihydroxytamoxifen was obtained as a mixture of E and Z isomers.

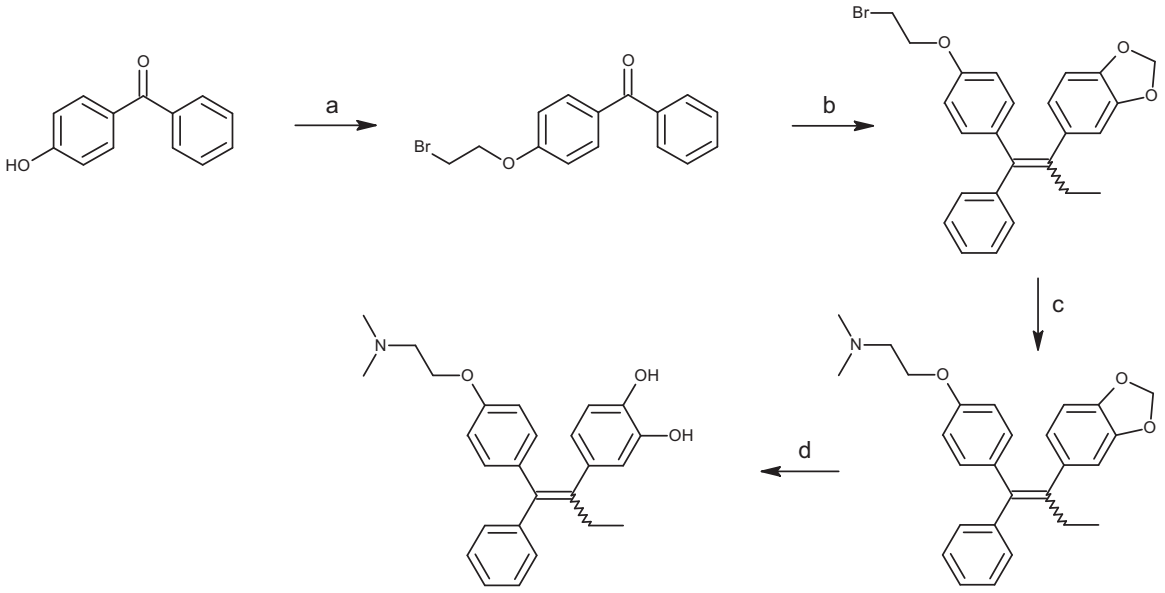


Fig. 1. Synthesis of 3′,4′-dihydroxytamoxifen. Reagents and conditions: (a) 1,2-dibromoethane, NaH, THF, reflux, on, 15%; (b) $TiCl_4$, Zn, 3,4-methylenedioxopropio phenone, THF, reflux, 2.5 h, 76%; (c) NMe_2 in MeOH, 60 °C, 96 h, 49%; (d) BCl_3 in CH_2Cl_2, 1,2-dichloroethane, 48 h, 20%.

A detailed description of the synthesis, including quality control and structural identification of intermediate products is described in Electronic supplementary/addendum part of the manuscript.

3.2. Characterization of tamoxifen and phase I metabolites

3.2.1. Phase I metabolites of tamoxifen

Systematic names, chemical structures and molecular masses of tamoxifen phase I metabolites are presented in Table 1 and partly in [15]. Phase I metabolism of tamoxifen is extensive but comprises predominantly demethylation and oxidation by CYP enzymes or oxidation by FMO. A combination of N-oxidation, (di)hydroxylation and (di)demethylation leads to a large set of potential metabolites. Epoxides are formed from dihydroxylated tamoxifen or from the addition of water on the ethylenic double bond. Two metabolites are known to have a trivial name which are: endoxifen [6] and droloxifene [16] for N-desmethyl-4-hydroxytamoxifen and 3-hydroxytamoxifen, respectively. Additionally, metabolites C, E and Y (Tables 1 and 2) are described in various publications [3,17–19]. These do not have a trivial name, but metabolites C and Y are also known under their systematic names: 4-hydroxy-3-methoxytamoxifen [19] and deaminohydroxytamoxifen [18], respectively. Their chemical structures are included in Table 1. Additional metabolites might be expected when demethylation, hydroxylation or N-oxide formations take place. However, Table 1 presents the selected metabolites of tamoxifen that have been described in the literature or have been recovered from serum of breast cancer patients receiving tamoxifen (Section 3.3).

3.2.2. MS^n fragmentation

Standard solutions of reference compounds were continuously infused into a linear ion trap mass spectrometer. Subsequently, MS, MS^2 and MS^3 spectra were recorded. Reference compounds (Table 2) include tamoxifen phase I metabolites and two stable-isotope-labeled tamoxifen analogues. The fragmentation patterns of tamoxifen are exemplified by the structure of tamoxifen and its metabolite 4-hydroxytamoxifen.

Two stable-isotope-labeled tamoxifen analogues were used to identify fragments from MS^n analyses by comparing the fragmentation spectra of tamoxifen with the spectra from the isotope-labeled analogues. Fragments from $^2H_5$-tamoxifen (Table 1) with a mass increase of 5 contain the terminal ethyl group as the deuterium atoms are positioned in this group. Likewise, fragments from $^{13}C_2$–^{15}N-tamoxifen (Table 1) with a mass increase of 3 contain the ether-linked N,N-dimethylethanamine group as this group contains both the carbon and nitrogen isotopes.

The remaining fragments that could not be identified by tamoxifen or its isotope-labeled analogues, were identified by comparing the MS^n fragmentation spectra of tamoxifen with the spectra from hydroxylated tamoxifen metabolites (e.g. 4-hydroxytamoxifen and 4′-hydroxytamoxifen). Fragments from hydroxylated tamoxifen with a mass increase of 16 contain the hydroxyl group. The exact position of the hydroxyl group (i.e. the 3- or 4-position) could not be deduced from the spectra as this requires fragmentation of the phenyl group while the hydroxyl group remains attached. This is an unlikely fragmentation and was not observed in the MS^n fragmentation analyses.

Figs. 2 and 3 present the fragmentation pathways of tamoxifen and 4-hydroxytamoxifen in which the capital characters at each structure correlate to the same characters on the associated fragmentation spectra (Figs. 4 and 5). Fig. 4A–D represents fragmentation spectra of precursor ions $[M+H]^+$ with m/z 372, 327, 249 and 207, respectively. Fig. 5E, F, C and G represents fragmentation spectra of precursor ions $[M+H]^+$ with m/z 388, 343, 249 and 223, respectively. Fragment C is a fragment that can originate from both tamoxifen and 4-hydroxytamoxifen. Note that the theoretically deduced fragmentation masses, in some cases deviate from the mass (m/z) in the fragmentation spectra by one or two units. This is hypothesized to be due to the addition or loss of protons during fragmentation and is depicted in the figures. Additionally in fragments B–D and F–G double bonds are postulated based on the molecular masses and their fragmentation patterns. The mass range in the fragmentation spectra (Figs. 4 and 5) is dependent on the mass of the precursor ion. This is due to a limitation of the linear ion trap, commonly known as the “one-third rule”. This rule arises from the decreased stability of fragment ions with m/z values less than 30% of the m/z of the precursor ion selected for fragmentation by collision-induced dissociation [20]. In a triple quadrupole mass spectrometer as used for the analysis of serum samples (Section 3.3), the analyzed mass range is independent of the precursor ion's m/z value. The dominant product ion of tamoxifen when fragmented in a triple quadrupole mass spectrometer is the ether-

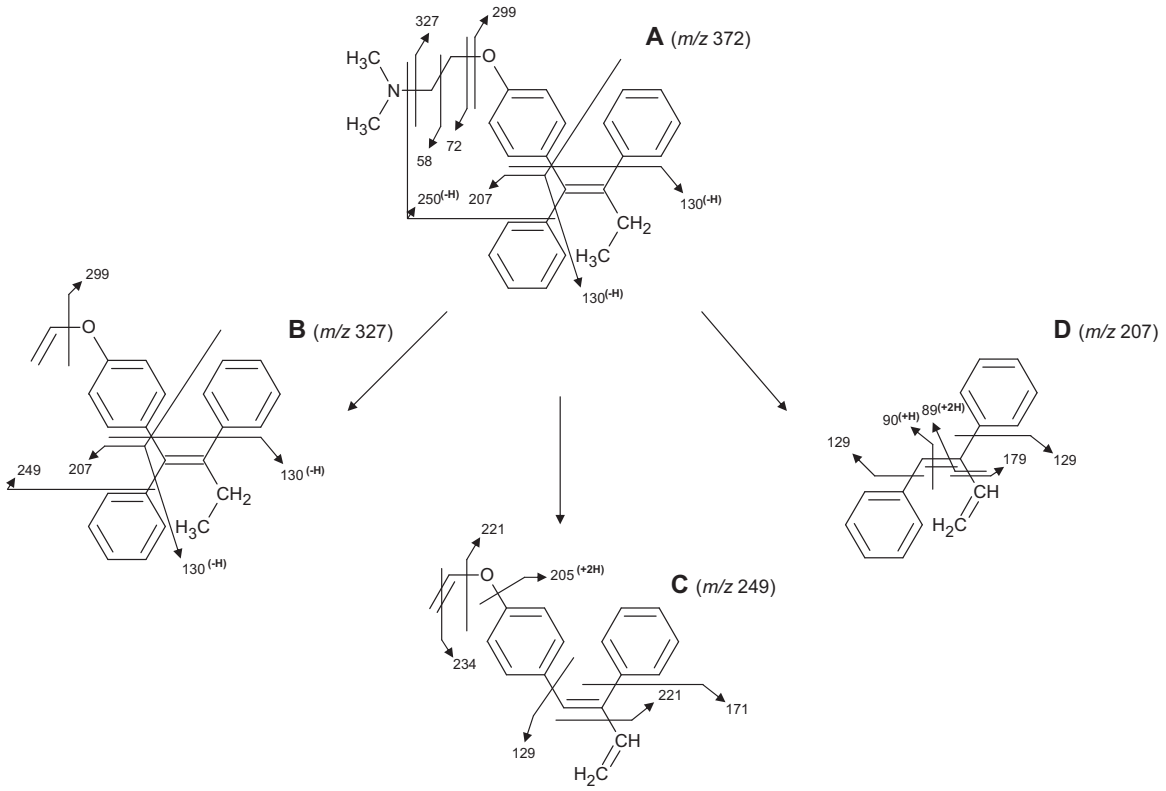


Fig. 2. Proposed mass spectrometry fragmentation pathways of tamoxifen (A; *m*/*z* 372).

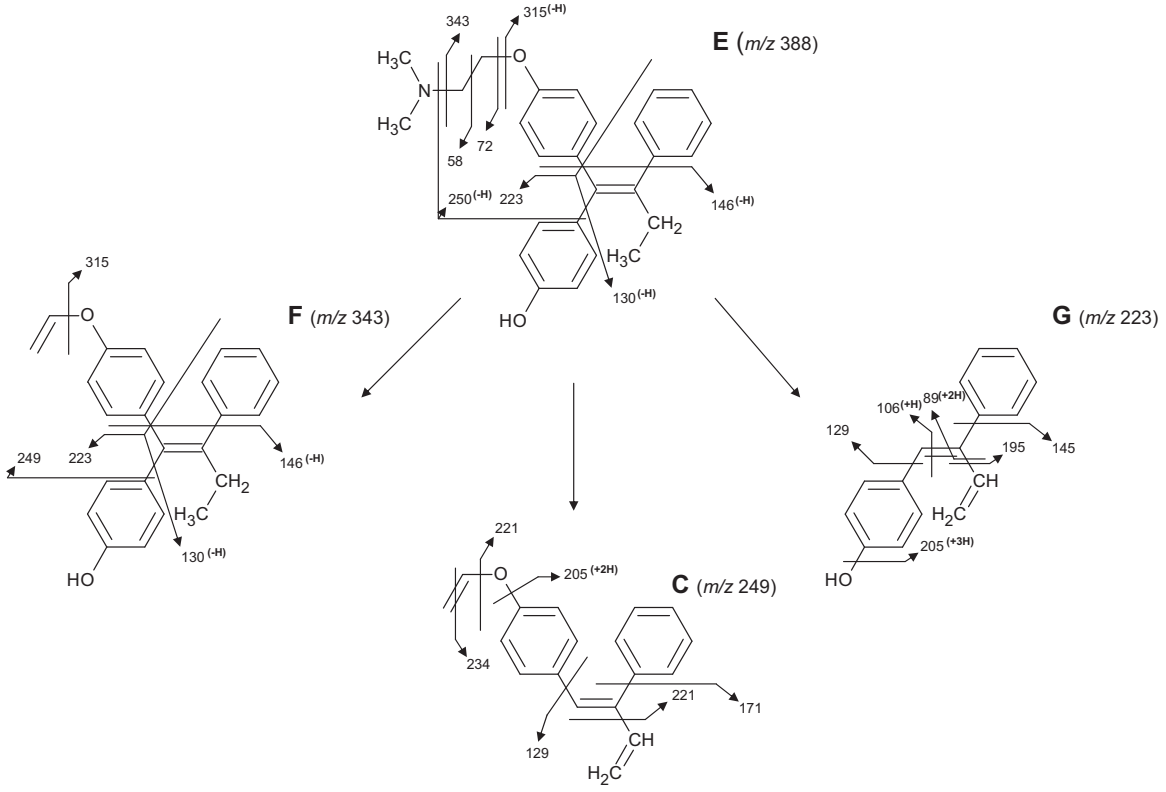


Fig. 3. Proposed mass spectrometry fragmentation pathways of 4-hydroxytamoxifen (E; *m*/*z* 388).

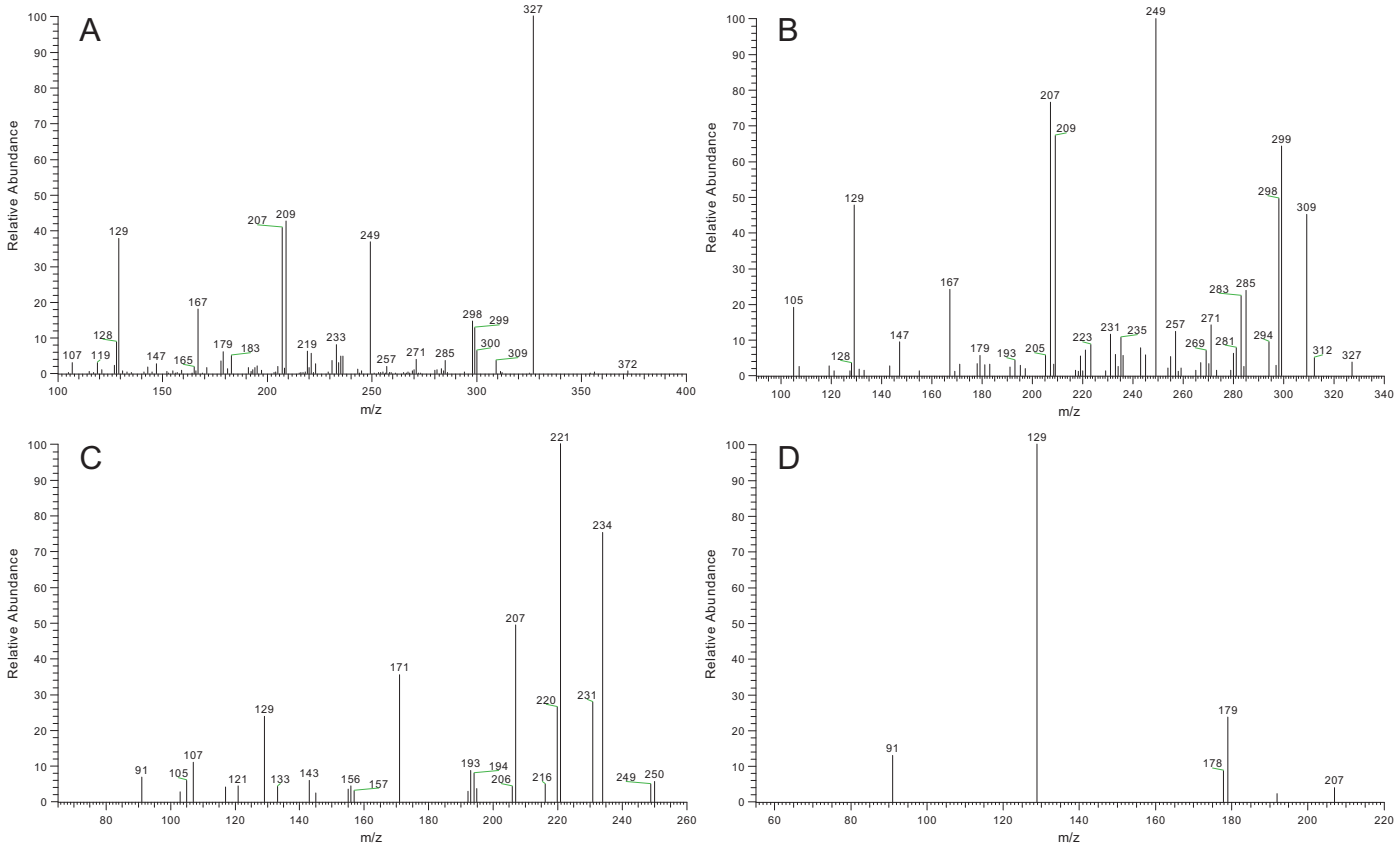


Fig. 4. Mass spectrometry fragmentation spectra of tamoxifen. Precursor ions: (A) *m*/*z* 372, (B) *m*/*z* 327, (C) *m*/*z* 249, and (D) *m*/*z* 207.

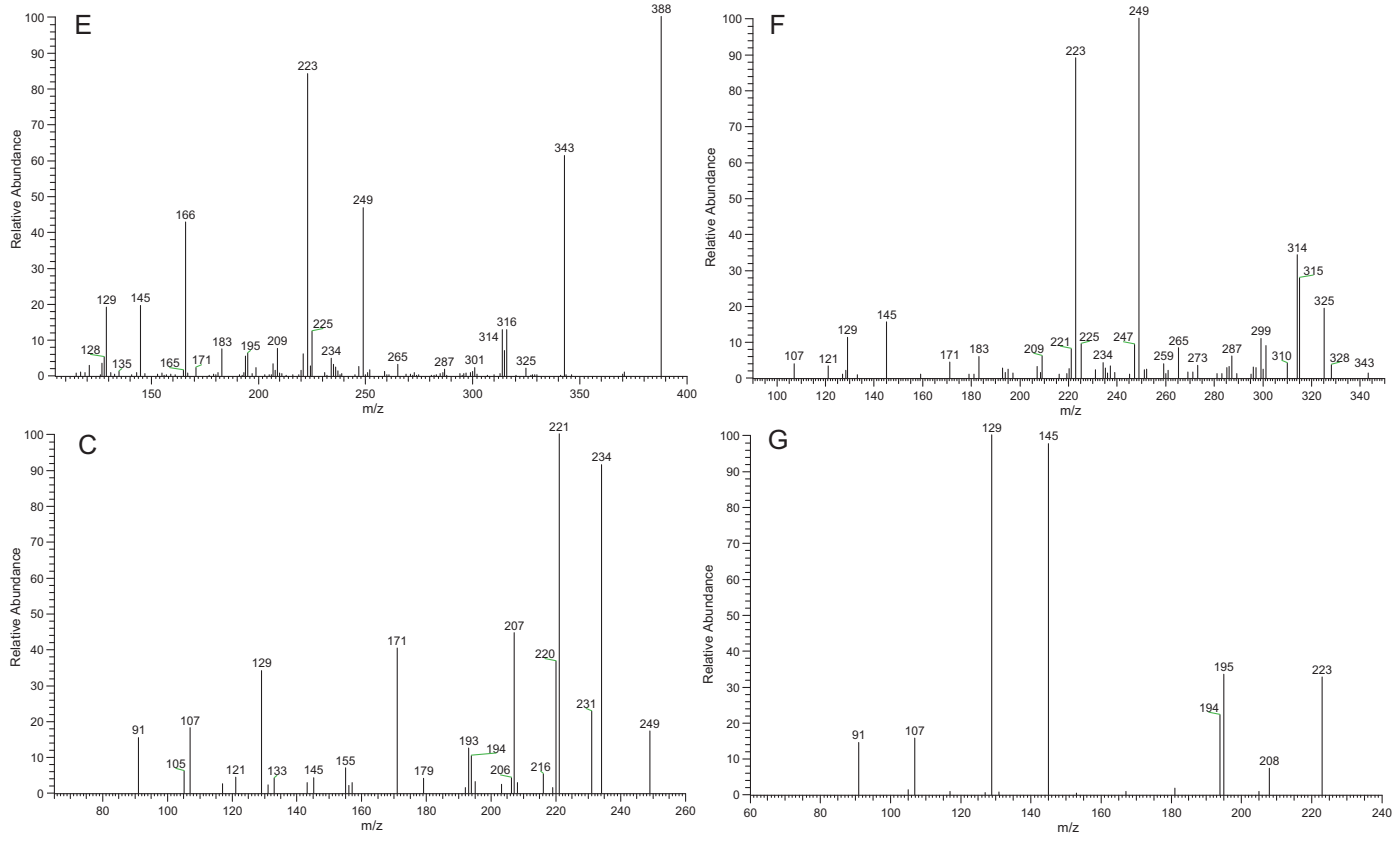


Fig. 5. Mass spectrometry fragmentation spectra of 4-hydroxytamoxifen. Precursor ions: (E) *m*/*z* 388, (F) *m*/*z* 343, (C) *m*/*z* 249, and (G) *m*/*z* 223.

Table 2
Retention times, absorption maxima, steady-state serum concentrations and most abundant MS^2 fragments of tamoxifen and its metabolites.

Trivial name	t_r (min)	λ_{max} (nm)	Reference standard	Conc. (ng mL^{-1}) in serum[b]	Most abundant MS^2 fragments	Literature reference
Tamoxifen	12.40	243	Y	200	327, 249, 207, 129	[6]
N-desmethyl-tamoxifen	11.76	243	Y	261	327, 249, 207, 129	[6]
N,N-didesmethyl-tamoxifen	11.09	–	N	20.6	–	[3,6]
α-Hydroxytamoxifen	5.59	235	Y	0.327	325, 247, 205, 145	[21,22]
β-Hydroxytamoxifen	5.28	235	Y	–	325, 247, 205, 145	[21,22]
2-Hydroxytamoxifen	–	–	N	–	–	[21]
3-Hydroxytamoxifen	8.47	235	Y	0.482	343, 249, 223, 145, 129	[3]
4-Hydroxytamoxifen	8.23	243	Y	3.85	343, 249, 223, 145, 129	[6]
4′-Hydroxytamoxifen	9.13	241	Y	6.95	343, 249, 223, 145, 129	[23]
Tamoxifen-N-oxide	14.42	243	Y	26.4	370, 327, 225, 129	[24]
α-Hydroxytamoxifen-N-oxide	–	–	N	–	–	[22]
β-Hydroxytamoxifen-N-oxide	–	–	N	–	–	[22]
3-Hydroxytamoxifen-N-oxide	–	–	N	–	–	[25]
4-Hydroxytamoxifen-N-oxide	–	–	N	–	–	[22]
4′-Hydroxytamoxifen-N-oxide	–	–	N	–	–	[26]
2,4-Dihydroxytamoxifen	–	–	N	–	–	[21]
3,4-Dihydroxytamoxifen	(*Z*): 9.69	249	Y	–	359, 332, 239, 161, 129	[3,10]
3′,4′-Dihydroxytamoxifen	(*Z*): 10.7	243	Y	–	359, 332, 239, 161, 129	[10,11]
α,4-Dihydroxytamoxifen	–	–	N	–	–	[27]
1,2-Epoxytamoxifen[a]	–	–	N	–	–	[22]
1,2-Epoxytamoxifen-N-oxide[a]	–	–	N	–	–	[22]
3,4-Epoxytamoxifen	–	–	N	–	–	[3]
3′,4′-Epoxytamoxifen	–	–	N	–	–	[28]
3,4-Epoxytamoxifen-N-oxide	–	–	N	–	–	[26]
N-desmethyl-α-hydroxytamoxifen	5.21	–	N	1.24	–	[10]
N-desmethyl-3-hydroxytamoxifen	8.05	–	N	3.09	–	[29]
N-desmethyl-4-hydroxytamoxifen	7.83	243	Y	22.9	343, 316, 223, 145, 129	[6,9]
N-desmethyl-4′-hydroxytamoxifen	8.69	240	Y	12.1	343, 316, 223, 145, 129	[26]
N,N-didesmethyl-α-hydroxytamoxifen	4.78	–	N	0.388	–	[3]
N,N-didesmethyl-3-hydroxytamoxifen	7.55	–	N	0.0527	–	This study
N,N-didesmethyl-4-hydroxytamoxifen	7.38	–	N	0.471	–	This study
N,N-didesmethyl-4′-hydroxytamoxifen	8.24	–	N	0.708	–	This study
3-Hydroxy-4-methoxy-tamoxifen	–	–	N	–	–	[25]
4-Hydroxy-3-methoxy-tamoxifen (met. C)	–	–	N	–	–	[19]
3-Hydroxy-4-methoxy-N-desmethyl-tamoxifen	–	–	N	–	–	[30]
Metabolite E	–	–	N	–	–	[3,17]
Metabolite Y	–	–	N	–	–	[17,18]

(*Z*), Zusammen isomer; Y, yes; N, no.

[a] Epoxide is formed from water added to the ethylenic double bond.

[b] Steady-state serum concentrations are given of tamoxifen and metabolites from patients receiving 40 mg tamoxifen once daily. '–' indicates that metabolites were not quantified (S/N < 10). Concentrations are calculated using a calibration curve of the reference standard or, if no reference standard was available, based on a calibration curve of a reference standard detected at the same mass transition. HPLC conditions are described in the text.

linked side chain (*m*/*z* 72 for tamoxifen) which is therefore selected for LC–MS/MS analyses.

3.2.3. UV absorption

On-line LC–UV was performed on all reference compounds and UV absorption spectra were recorded on-line using a photodiode array detector. For metabolites of which no reference standard was available, a processed serum sample from patients with steady-state serum concentrations receiving 40 mg tamoxifen once daily was injected onto the LC–UV system. Concentrations of these metabolites, however, appeared to be too low to record absorption spectra. Absorption maxima, presented in Table 2, are very similar for the various metabolites. Demethylation and oxidation on the aliphatic amine appear to have little influence on the absorption maxima. Hydroxylation is neither expected to significantly influence the conjugated system of the three phenyl groups oriented

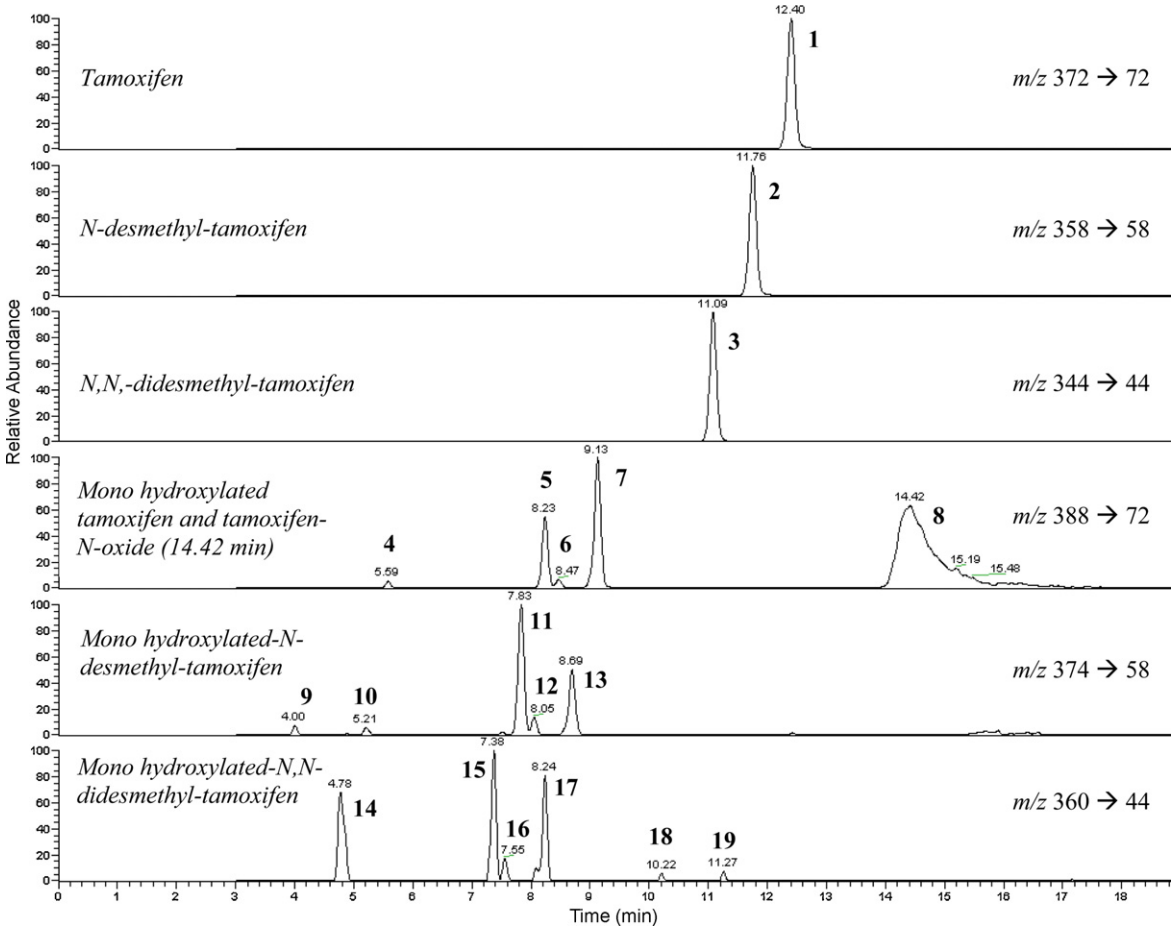


Fig. 6. LC–MS/MS chromatogram of tamoxifen phase I metabolites recovered from serum of a patient with steady state serum concentrations receiving 40 mg tamoxifen once daily. (1) tamoxifen (t_r = 12.40 min); (2) N-desmethyl-tamoxifen (t_r = 11.76 min); (3) N,N-didesmethyl-tamoxifen (t_r = 11.09 min); (4) α-hydroxytamoxifen (t_r = 5.59 min); (5) 4-hydroxytamoxifen (t_r = 8.23 min); (6) 3-hydroxytamoxifen (t_r = 8.47 min); (7) 4′-hydroxytamoxifen (t_r = 9.13 min); (8) tamoxifen-N-oxide (t_r = 14.42 min); (9) unidentified metabolite (t_r = 4.00 min); (10) N-desmethyl-α-hydroxytamoxifen (t_r = 5.21 min); (11) N-desmethyl-4-hydroxytamoxifen (t_r = 7.83 min); (12) N-desmethyl-3-hydroxytamoxifen (t_r = 8.05 min); (13) N-desmethyl-4′-hydroxytamoxifen (t_r = 8.69 min); (14) N,N-didesmethyl-α-hydroxytamoxifen (t_r = 4.78 min); (15) N,N-didesmethyl-4-hydroxytamoxifen (t_r = 7.38 min); (16) N,N-didesmethyl-3-hydroxytamoxifen (t_r = 7.55 min); (17) N,N-didesmethyl-4′-hydroxytamoxifen (t_r = 8.24 min); (18) unidentified metabolite (t_r = 10.22 min); (19) unidentified metabolite (t_r = 11.27 min).

around the central ethylene group and accordingly had negligible influence on the absorption maximum.

3.3. Analysis of tamoxifen and its phase I metabolites in patient serum samples

Tamoxifen phase I metabolites cover a wide range in polarity. As many tamoxifen metabolites have identical precursor and product ion masses, baseline separation of these metabolites is required for identification purposes. A C18 stationary phase end-capped with polar groups was the column of choice to ensure maximum selectivity. Based on expected transitions and literature data, mass transitions were chosen corresponding to known and expected phase I metabolites. Reference standards (Table 2) and serum extracts from ten patients with steady-state serum concentrations receiving 40 mg tamoxifen once daily were analyzed for metabolite identification using a chromatographic system adjusted from [8]. Results are shown in Fig. 6 and Table 2. Metabolites in serum were identified by comparison with reference standards or based on their molecular mass, product ion masses and relative retention times (i.e. N,N-didesmethyl-tamoxifen, N-desmethyl-α-hydroxytamoxifen, N-desmethyl-3-hydroxytamoxifen and four hydroxylated didemethylated metabolites). The influence of polarity of metabolites on the relative retention time is clearly demonstrated in Fig. 6. When comparing the retention times of tamoxifen, N-desmethyl-tamoxifen and N,N-didesmethyl-tamoxifen, a decrease in retention time of 5% is seen for the loss of each methyl group. The first four eluting peaks (Fig. 6, peaks 4–7) in the 388/72 *m/z* window, namely the mono-hydroxylated metabolites are identified by use of reference compounds (Fig. 6). The pattern in which these four hydroxylated metabolites elute is also seen in the 374/58 *m/z* window (Fig. 6, peaks 10–13) with a decrease in retention time of 5% and in the 360/44 *m/z* window (Fig. 6, peaks 14–17) with again a decrease in retention time of 5%. Based on the pattern in which these metabolites (Fig. 6, peaks 10–17) elute and their mass transition these metabolites are identified as mono-hydroxylated-demethylated (374/58 *m/z*) and mono-hydroxylated-didemethylated (360/44 *m/z*) tamoxifen metabolites (Fig. 6 and Tables 1 and 2).

Many of the presented metabolites in Tables 1 and 2 were at concentrations below the LLOD (±0.05 ng mL^{-1}). Mass transitions for these metabolites were chosen based on expected transitions and literature data. These metabolites are the 2-hydroxytamoxifen, hydroxylated N-oxides, dihydroxylated metabolites, epoxides, metabolites C, E and metabolite Y.

Furthermore, as can be seen in Fig. 6, an unidentified metabolite (peak 9, t_r: 4.00 min) is present in the 374/58 *m/z* window, typical for a tamoxifen molecule which is both demethylated and either monohydroxylated or N-oxidated. Two unidentified metabolites (peak 18, t_r: 10.22 and peak 19, t_r: 11.27 min) are present in the

360/44 m/z window, typical for tamoxifen molecules which are both didemethylated and either monohydroxylated or N-oxidated. The concentrations of these metabolites were too low to scan MS^n spectra and thus the exact position of the hydroxyl group could not be assigned. All the chromatographic peaks in Fig. 6 were not seen in blanks and were present in all patient serum samples though the relative intensities varied.

4. Conclusion

A comprehensive overview is presented of currently known phase I metabolites of tamoxifen consisting of their systematic name and molecular structure. Reference standards were used to elucidate the MS^n fragmentation patterns of tamoxifen and metabolites using a linear ion trap mass spectrometer. UV-absorption spectra are recorded and absorption maxima are defined.

Serum extracts from ten breast cancer patients with steady-state serum concentrations receiving 40 mg tamoxifen once daily were analyzed for metabolite identification using a liquid chromatography–tandem mass spectrometry set-up. Ten metabolites were identified in the serum samples by comparison with reference standards (liquid chromatography–mass spectrometry), and 9 metabolites were identified based on literature data and their MS fragmentation patterns. Of the 19 identified metabolites, three have, to the best of our knowledge, never been described in the literature before. These are: N,N-didesmethyl-3-hydroxytamoxifen, N,N-didesmethyl-4-hydroxytamoxifen and N,N-didesmethyl-4′-hydroxytamoxifen. Various metabolites were described in the literature but were not recovered from serum. One of these is 3′,4′-dihydroxytamoxifen [10,11]. For identification purposes, this compound was synthesized, and the synthetic method described herein. Despite careful screening, 3′,4′-dihydroxytamoxifen was not detected in serum from breast cancer patients receiving tamoxifen. The presented data may be useful to further elucidate the role of metabolism in tamoxifen-treated breast cancer patients. Little is known about the estrogenic activity of tamoxifen metabolites. A detailed investigation should provide profound insight in both the estrogenic activity of tamoxifen metabolites, as well as their concentration variations within and between patients. Of the 36 known phase I metabolites only 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen are very commonly studied. Other hydroxylated and/or demethylated tamoxifen metabolites might, however, very well have strong affinities towards the estrogen receptor. The hereby presented study provides a strong bases for further investigations regarding the structural elucidation of new tamoxifen metabolites, their estrogenic activity and inter as well as intra patient steady-state serum concentration fluctuations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.02.009.

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