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Mass spectrometric characterization of urinary toremifene metabolites for doping control analyses

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ABSTRACT

Toremifene is a selective estrogen receptor modulator included in the list of prohibited substances in sport by the World Anti-doping Agency. The aim of the present study was to investigate toremifene metabolism in humans in order to elucidate the structures of the most abundant urinary metabolites and to define the best marker to detect toremifene administration through the analysis of urine samples. Toremifene (Fareston®) was administered to healthy volunteers and the urine samples were subjected to different preparation methods to detect free metabolites as well as metabolites conjugated with glucuronic acid or sulphate. Urinary extracts were analyzed by LC–MS/MS with triple quadrupole analyzer using selected reaction monitoring mode. Transitions for potential metabolites were selected by using the theoretical $[M+H]^+$ as precursor ion and m/z 72 or m/z 58 as product ions for N,N-dimethyl and N-desmethyl metabolites, respectively. Toremifene and 20 metabolites were detected in excretion study samples, excreted free or conjugated with glucuronic acid or sulphate. Structures for most abundant phase I metabolites were proposed using accurate mass measurements performed by QTOF MS, based on fragmentation pattern observed for those metabolites available as reference standards. Several metabolic pathways including mono- and di-hydroxylation, N-desmethylation, hydroxymethylation, oxidation, dehalogenation and combinations were proposed. All metabolites were detected up to one month after toremifene administration; the most abundant metabolites were detected in the free fraction and they were metabolites resulting from dehalogenation. Several of the metabolites elucidated in this work have not been reported until now in the scientific literature.

1. Introduction

Toremifene (2-[4-[(Z)-4-chloro-1,2-diphenylbut-1-enyl] phenoxy]-N,N-dimethylethanamine) is a selective estrogen receptor modulator (SERM) that is structurally similar to tamoxifen. The only difference between toremifene and tamoxifen structures is the chlorine atom in C4 which is present in toremifene (Fig. 1) and absent in tamoxifen.

Anti-estrogen drugs are effective for prevention and as adjuvant treatment of estrogen-dependent breast cancer, for the treatment and prevention of post-menopausal osteoporosis and cardiovascular disease. Anti-estrogenic substances may cause an increase of the endogenous production of androgens and increase endogenous testosterone by stimulating the release of gonadotrophines. There are no well-established clinical indications for anti-estrogens in men [1,2]. In athletes the use of antiestrogenic compounds may compensate an extensive abuse of anabolic androgenic steroids [3,4]. For these reasons, the use of agents with anti-estrogenic activity has been banned in sports by the World Anti-doping Agency (WADA) [5], and anti-doping control laboratories have to be able to detect the administration of the drug.

In doping control, identification of an exogenous compound is typically sufficient to declare a sample as adverse analytical finding for most of doping agents. Therefore, metabolic studies are compulsory in order to find the metabolites that are detectable for longer period of time after administration, which are the most adequate markers for the detection of the drug misuse.

Usefulness of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) in doping control analyses have been demonstrated in several studies [6–8] with enhanced separation of

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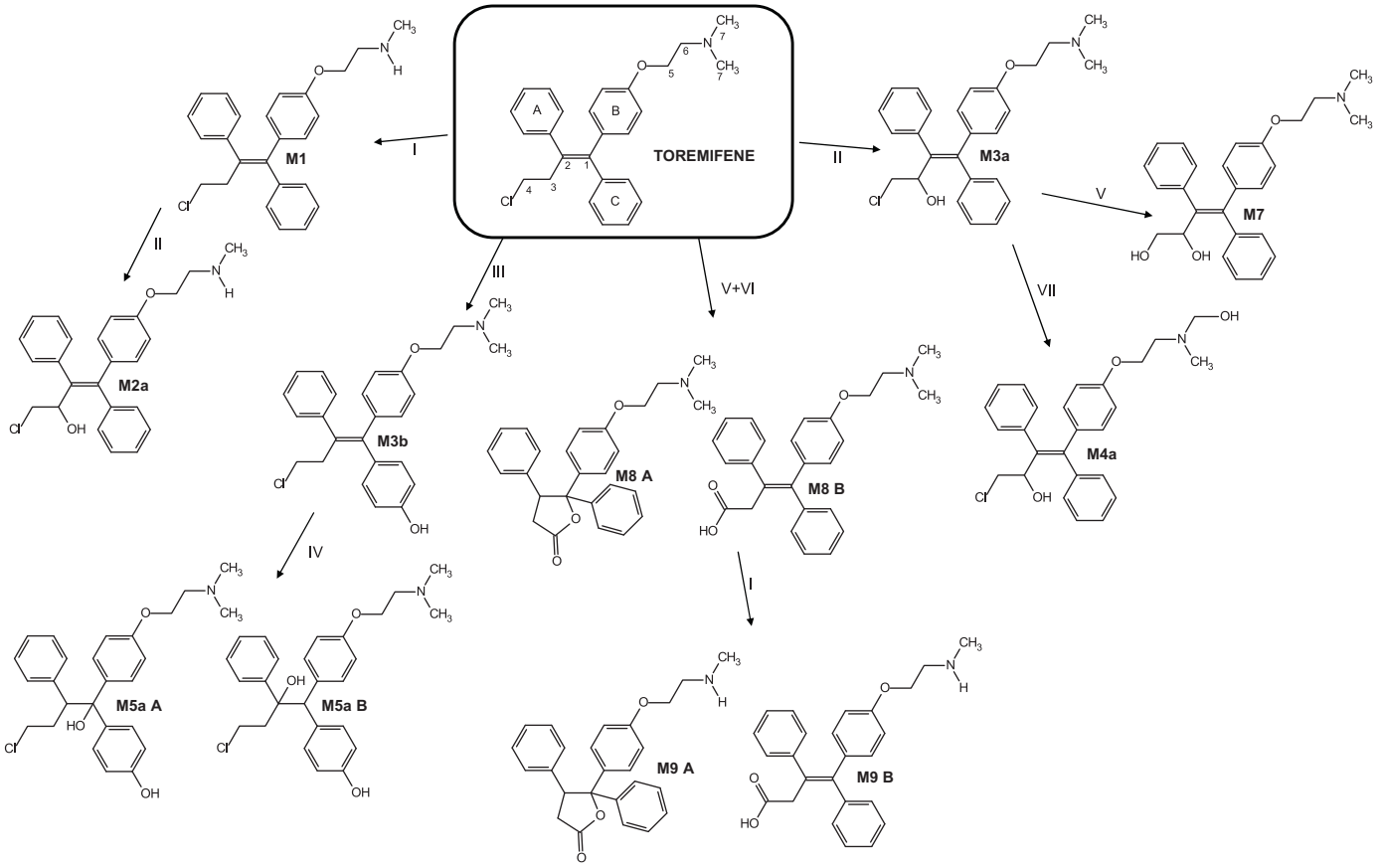


Fig. 1. Proposed metabolic pathways: N-demethylation (I); 3-hydroxylation (II); C ring para-hydroxylation (III); 1-hydroxylation (A) or 2-hydroxylation (B) (IV); dehalogenation (V); oxidation + cyclization (A) or oxidation + dehydratation (B) (VI) and 7-hydroxylation (VII); and proposal structures of most abundant toremifene metabolites. The alkylic chains of toremifene have been numbered in order to facilitate the description of structure elucidation; the numeration does not follow IUPAC rules.

analytes, shorter sample pretreatment, and detection of substances that are not identified by gas chromatography–mass spectrometry (GC–MS). Additionally, the use of accurate mass measurements has been reported to be useful in several areas of the anti-doping control field like the qualitative detection of doping agents, preventive doping analysis or metabolic studies [9,10]. The combination of LC–MS/MS and accurate mass measurements is a powerful tool for metabolic studies of those compounds which the proton affinity is high enough for electrospray ionization (ESI). The use of this approach allowed for the detection and elucidation of several previously unreported metabolites for some doping agents [11,12].

In the case of toremifene, pharmacokinetic and pharmacodynamic studies with detection of toremifene and its metabolites in plasma and faeces, have been reported [13–25]. Few analytical methods for the urinary detection of toremifene administration have been developed [3,14,26–29]. Different metabolic pathways have been described as the most characteristic for toremifene and related compounds such as tamoxifen and clomifene including, hydroxylation, N-desmethylation, N,N-didesmethylation, deaminohydroxylation, deaminocarboxylation, N-oxide formation, among others [3,4,17,30–42]. However, no systematic study on toremifene metabolites appearing in urine after drug administration has been performed.

The aim of the present work was to study the human urinary metabolic profile of toremifene, including free metabolites as well as metabolites conjugated with glucuronic acid and sulphate; to optimize an analytical assay based on reference material; to study real samples to screen for the presence of all potential metabolites and to elucidate the structure of the most abundant metabolites. The final goal was to suggest the best markers to detect the misuse of toremifene in doping control analyses.

2. Experimental

2.1. Chemicals and reagents

Reference standard of toremifene (Fig. 1) was supplied by Sigma–Aldrich (St. Louis, MO, USA). N-desmethyltoremifene (**M1** in Fig. 1) was purchased by Synfine Research Inc. (Ontario, Canada) p-hydroxytoremifene (**M3b** in Fig. 1) was synthesized by AMRg-CSIC (Barcelona, Spain) based on the synthesis of p-hydroxytamoxifen described by Gauthier et al. [43] and Yu et al. [44] with slightly modifications. Methyltestosterone, used as internal standard, was supplied by Sigma (St. Louis, MO, USA).

Tert-butyl methyl ether (TBME, HPLC grade), ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade), potassium carbonate, sodium hydroxide, di-sodium hydrogen phosphate, sodium hydrogen phosphate, sulphuric acid, sodium chloride, 25% ammonia, ammonium chloride (all analytical grade) were purchased from Merck (Darmstadt, Germany). β-Glucuronidase from *Escherichia coli* was obtained from Roche Diagnostics (Mannheim, Germany). Detectabuse™ extraction columns were purchased from Biochemical Diagnostics, Inc. (Edgewood, NY, USA). Milli Q water was obtained

by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. Sample preparation procedures

Free fraction of metabolites. The extraction of free urinary toremifene metabolites was based on a previously described procedure [45,46]. Briefly, 100 ng mL^{-1} of methyltestostenone was added to 5 mL aliquots of urine samples and the pH was made alkaline with 100 μL of 5.3 M ammonium chloride solution (adjusted to pH 9.5 with ammonia). Then, sodium chloride (1 g) was added to promote salting-out effect and the samples were extracted with 8 mL of ethyl acetate by shaking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layers were evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 100 μL of a mixture of deionised water:acetonitrile (50:50, v|v).

Glucuronide fraction of metabolites. Samples (5 mL) were adjusted to pH 7 with 1.5 mL of sodium phosphate buffer (0.2 M, pH 7) and were extracted with 5 mL of TBME by shaking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layer containing free metabolites was discarded. The small volume of organic solvent still present on top of the aqueous phase was evaporated under stream of nitrogen. After addition of ISTD solution, the aqueous phase was passed through a Detectabuse™ column previously conditioned with 2 mL methanol and 2 mL water. The column was washed with 2 mL water and the analytes were eluted with 2 mL methanol. The methanolic extract was evaporated to dryness under a stream of nitrogen in a water bath at 50 °C and reconstituted with 1 mL of sodium phosphate buffer (0.2 M, pH 7). Enzymatic hydrolysis was performed by adding 30 μL of β-glucuronidase from *E. coli* and incubating the mixture at 55 °C for 1 h. After the sample reached the ambient temperature 250 μL of 5% K_2CO_3 solution was added and the mixture was extracted with 5 mL of TBME by shaking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The dry extracts were reconstituted with 100 μL of a mixture of deionised water:acetonitrile (50:50, v|v).

Sulphated fraction of metabolites. The recovery of sulphated metabolites was accomplished using a procedure previously described [47]. The extracts obtained after Detectabuse™ extraction, using the procedure described in the previous paragraph, were reconstituted with 4 mL of ethyl acetate/methanol/sulphuric acid (80:20:0.06, v|v|v) and incubated at 55 °C for 2 h. The samples were neutralized with 60 μL of 1 M NaOH and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and 250 μL of 5% K_2CO_3 solution were added. The extraction was performed with 5 mL of TBME by shaking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The dry extracts were reconstituted with 100 μL of a mixture of deionised water:acetonitrile (90:10, v|v).

2.3. LC–MS/MS study of urinary metabolic profile of toremifene

Chromatographic separation was carried out on a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA) using an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 45 °C. The mobile phase consisted of deionized water with 0.01% formic acid (solvent A) and acetonitrile with 0.01% formic acid (solvent B). Separation was performed at a flow rate of 0.4 mL min^{-1} and using a gradient pattern: from 0 to 1 min, 5% B; from 1 to 16 min, to 90% B; during 1.6 min, 90% B; from 17.6 to 17.8 min, to 5% B; from 17.8 to 23 min, 5% B. The mobile phases were filtered daily using filters of 0.22 μm. The sample volume injected was 5 μL.

The liquid chromatograph was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation, Milford, MA.) with an electrospray (Z-spray) ionization source with positive ion mode ESI. Source conditions were fixed as follows: capillary voltage, 3 kV; lens voltage, 0.2 V; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1200 L/h. Negative ionization mode was tested, using the same conditions, except the capillary voltage was set at 2.5 kV. High-purity nitrogen was used as desolvation gas and argon was used as collision gas.

For detection of toremifene metabolites, different fractions (free, glucuronide- or sulpho-conjugated metabolites) of urine samples from excretion studies were analyses by LC–MS/MS in selected ion reaction monitoring (SRM) mode. Transitions were selected using the protonated molecular ion $[M+H]^+$ of the potential metabolite as the precursor ion, which, depending on the specific structure, yield product ions at *m/z* 72, 58, or 44. The targeted metabolic pathways are described in Table 1, and included mono- and di-hydroxylation, hydroxy-methoxylation, N-desmethyl and N,N-didemesmethyl, among others. ESI-parameters (ionization mode, cone voltage and collision energy) and precursor ion-product ion pairs were optimized with available reference material (toremifene, M1and M3b) using direct infusion of individual standard solutions of the compounds (10 μg mL^{-1}) at 10 μL min^{-1} with mobile phase (50:50, A:B) at 200 μL min^{-1}. Cone voltage was set at 35 V and collision energy used was 30 eV.

2.4. Accurate mass measurement and characterization of toremifene metabolites

Accurate mass experiments were carried out using a hybrid quadrupole time-of-flight (QTOF Premier, Waters) mass spectrometer, and UPLC (Acquity, Waters) was interfaced with the system via positive ion mode ESI. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 600 L/h and the cone gas flow to 50 L/h. A cone voltage of 40 V and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set at 350 °C and the source temperature at 120 °C. TOF MS resolution was approximately 10,000 (FWHM) at *m/z* 556. MS and MS/MS spectra were acquired over a *m/z* range of 50–1000. For both MS and MS/MS, argon (99.995%) was used as collision gas with a pressure of approximately 8×10^{-3} mbar in the collision cell. The MCP detector potential was set to 1700 V. Scan times of 0.2 s/spectrum were chosen.

Calibration of the *m/z* axis was performed using the built-in single syringe pump, directly connected to the interface. Calibration was conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.1 M sodium hydroxide: 10% formic acid diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 10 μL min^{-1}. For automated accurate mass measurement, the lockspray probe was used, using as lockmass a solution of leucine enkephaline 2 μg mL^{-1} in acetonitrile/water (50:50) pumped at 30 μL min^{-1} through the lockspray needle using a Reagent Delivery Module from Waters. The protonated molecule of leucine enkephaline at *m/z* 556.2771 was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time.

Chromatographic separation was carried out on the same conditions (column and mobile phases) as described above using a different gradient pattern: from 0 to 1 min, 20% B; from 1 to 2 min, to 25% B; from 2 to 3 min, to 30% B; from 3 to 5 min, to 40% B; from 5 to 17.6 min, to 90% B; during 0.4 min, 90% B; from 18 to 18.4 min,

Table 1
Selected transitions of proposed metabolic pathways. Code and retention time (RT) of metabolites detected (free and/or conjugated with glucuronid acid or sulphate).

Metabolic pathways	Transition	Code	RT (mim)	Free	Glucuronides	Sulphates
Toremifene	406 > 72	Toremifene	9,86	+		
N-desmethylation	392 > 58	M1	9,72	+		
N-desmethylation + hydroxylation	408 > 58	M2a	7,65	+		
		M2b	8,31		+	+
Hydroxylation	422 > 72	M3a	7,75	+		
		M3b	8,43		+	+
		M3c	8,85		+	
Dihydroxylation	438 > 72	M4a	7,98	+		
		M4b	6,99	+		
		M4c	6,68	+	+	
		M4d	6,80		+	
		M4e	7,82		+	
		M4f	8,25		+	
Dihydroxylation + reduction	440 > 72	M5a	6,88	+	+	
		M5b	7,07	+	+	
Hydroxylation + hydroxymethylation	452 > 72	M6a	8,52		+	
		M6b	8,95		+	
		M6c	9,74			+
Dehalogenation	404 > 72	M7	5,11	+		
	402 > 72	M8	6,84	+		
	388 > 58	M9	6,72	+		
N,N-didesmethylation	378 > 44		n.d.			
Desmethylation + hydroxylation + hydroxymethylation	438 > 58		n.d.			
Hydroxy quinine	386 > 72		n.d.			
Hydroxylation + oxidation	420 > 72		n.d.			

to 20% B; from 18.4 to 23 min, 20% B. Cone voltage was adjusted at 35 V. Collision energies of 15 and 45 eV were used.

2.5. Excretion study samples

Urine samples obtained in excretion studies involving the administration of toremifene to healthy volunteers were obtained. The clinical protocol was approved by the Local Ethical committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). A single dose of 60 mg of toremifene (Fareston®) was administered to two healthy volunteers by oral route. In the first study, the urine samples were collected before administration and up to 82 h after administration. In the second study, urine samples were collected before administration and up to 10 days after administration and a spot sample was collected one month after toremifene administration. In order to compare toremifene metabolites with those of tamoxifen, a urine was collected from 0 to 24 h after oral administration of tamoxifen (20 mg Tamoxifeno Ratiopharm EGF). Urine samples were stored at −20 °C until analysis and they were analyzed for all the metabolites.

3. Results and discussion

3.1. CID for toremifene metabolites available as reference standards

Electrospray ionization working parameters were optimized for toremifene and metabolites available as reference material, i.e., p-hydroxytoremifene (**M3b**) and N-desmethyltoremifene (**M1**). Positive and negative ion modes were tested. Abundant signal was obtained in positive ionization mode; no signal was obtained in negative mode. $[M+H]^+$ were obtained for all compounds, adduct ions like $[M+Na]^+$ were not observed. The cone voltage was optimized to maximize the intensity of the $[M+H]^+$ and it was set to 35 V.

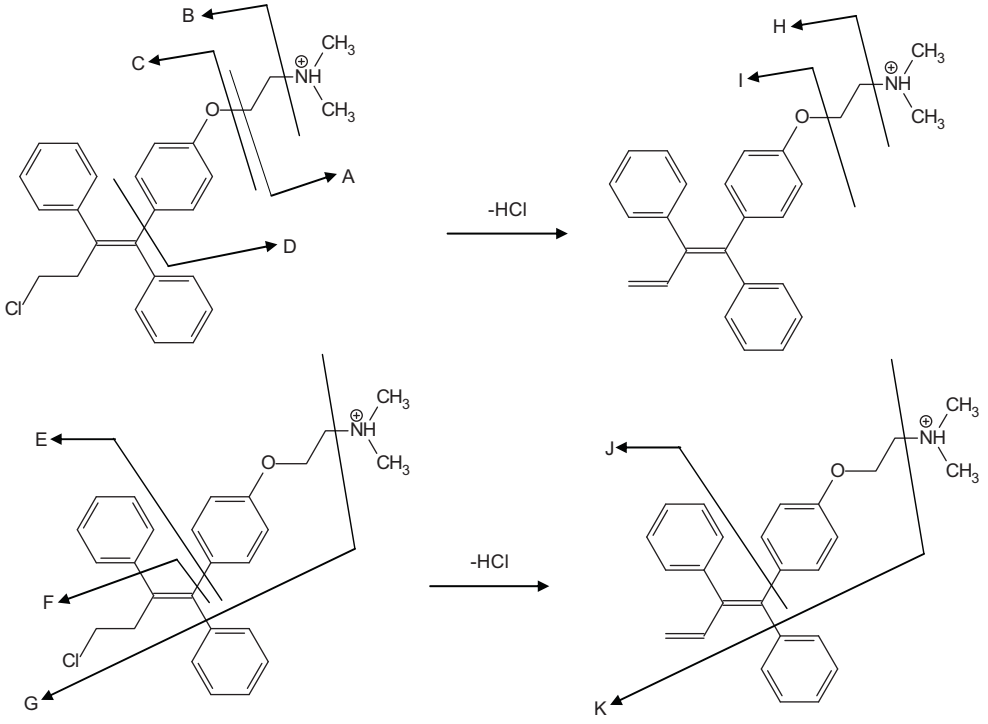
Collision Induced Dissociation (CID) of model compounds was studied at two collision energies (15 and 45 eV). At 15 eV, poor fragmentation was observed being the most abundant ion $[M+H]^+$ (see supplementary information). Due to toremifene structure, the CID at low collision energy mainly involved the alkylic chains. Only one product ion showed a relative abundance higher than 5%. For N,N-dimethylated compounds this product ion was obtained at m/z 72 and can be explained as the result of the cleavage of the bond of O-C5 (Fig. 2). In an analogous way, product ion at m/z 58 is characteristic of N-desmethylated metabolites. The remaining structure hardly undergoes fragmentation. Additional product ions showed relative abundances lower than 5%. Several neutral losses and product ions were common to all model compounds and therefore they can be useful for establishing relationships between product ion spectra and structure. Neutral loss of HCl $[M+H-35.9703\ Da]^+$ and N-chain ($[M+H-45.0578\ Da]^+$ in N,N-dimethylated metabolites or $[M+H-31.0422\ Da]^+$ in N-desmethylated) were observed. No losses of water were observed in **M3b** spectrum, probably due to the stability of the hydroxyl group in the phenolic moiety.

Other common product ions for the three model compounds were also observed. Some of these ions showed a mass difference of 16 Da (15.9949 Da) when studying **M3b** or 14 Da (14.0157 Da) with **M1** (see Fig. 2). As it is shown in Fig. 2, some ions contained the 3 aromatic rings (m/z 298.1319 and 334.1115), several ions contained two of rings (m/z 205.1011, 241.0757, 247.1082 and 283.0921) and one ion contained one of the rings (m/z 163.0299). The presence of C ring in these ions could be established due to the mass increase when studying **M3b**. Ion at m/z 166.1240 was observed only in **M3b** product ion spectrum. The hydroxyl group present in *para*-position in C ring could influence in the formation of this product ion.

At high collision energy (45 eV), in N,N-dimethylated metabolites (toremifene and p-hydroxytoremifene, **M3b**), the most abundant product ion was also observed at m/z 72. The other product ions obtained were less abundant than 30% (see supplementary information). For N-desmethyltoremifene, **M1**, most of the ions obtained exhibited relative abundances higher than 30%. Additionally, several common ions were also observed at 45 eV depending on the presence of an aromatic hydroxyl group (Table 2). Some of these ions showed a mass difference of 15.9949 Da and therefore can be considered as characteristics of *para* hydroxylation in C ring. The presence or absence of these ions (m/z) can be used as a marker of aromatic hydroxylation in structure elucidation of toremifene metabolites.

Table 2
Ions observed at 45 eV for toremifene, N-desmethyltoremifene and p-hydroxytoremifene.

Toremifene and N-desmethyltoremifene			p-hydroxytoremifene		
Ion (*m*/*z*)	Error (mDa)	Molecular formula	Ion (*m*/*z*)	Error (mDa)	Molecular formula
281.0974	+0.8	$C_{21}H_{13}O$	297.0913	−0.3	$C_{21}H_{13}O_2$
265.0993	−2.4	$C_{21}H_{13}$	281.0978	+1.2	$C_{21}H_{13}O$
253.1004	−1.3	$C_{20}H_{13}$	269.0960	−0.6	$C_{20}H_{13}O$
252.0934	−0.5	$C_{20}H_{12}$	252.0937	−0.2	$C_{20}H_{12}$
241.1014	−0.3	$C_{19}H_{13}$	257.0946	−2.0	$C_{19}H_{13}O$
228.0914	−2.5	$C_{18}H_{12}$	244.0909	+2.1	$C_{18}H_{12}O$
215.0844	−1.7	$C_{17}H_{11}$	231.0793	−1.7	$C_{17}H_{11}O$
207.0813	+0.3	$C_{15}H_{11}O$	223.0721	−3.8	$C_{15}H_{11}O_2$
203.0852	−0.9	$C_{16}H_{11}$	203.0852	−0.9	$C_{16}H_{11}$
202.0774	−0.9	$C_{16}H_{10}$	202.0774	−0.9	$C_{16}H_{10}$
191.0867	+0.6	$C_{15}H_{11}$	191.0870	+0.9	$C_{15}H_{11}$
178.0766	−1.7	$C_{14}H_{10}$	178.0766	−1.7	$C_{14}H_{10}$
165.0689	−1.5	$C_{13}H_9$	165.0692	−1.2	$C_{13}H_9$



	Toremifene	N-desmethyltoremifene (**M1**)	p-hydroxytoremifene (**M3b**)
$[M+H]^+$	406.1880	392.1784	422.1868
A	72.0783	58.0629	72.0787
B	361.1383	361.1370	377.1264
C	334.1115	334.1141	350.1139
D	n.d.	n.d.	166.1240
E	241.0757	241.0763	257.0722
F	163.0299	163.0303	179.0246
G	283.0921	283.0910	283.0804
-HCl	370.2177	361.1270	386.2101
H	325.1579	325.1392	341.1505
I	298.1319	298.1392	314.1274
J	205.1011	205.1008	221.0946
K	247.1082	247.1131	247.1057

Fig. 2. Proposed fragmentation pattern of toremifene and metabolites available as reference standards (p-hydroxytoremifene and N-desmethyltoremifene) at 15 eV.

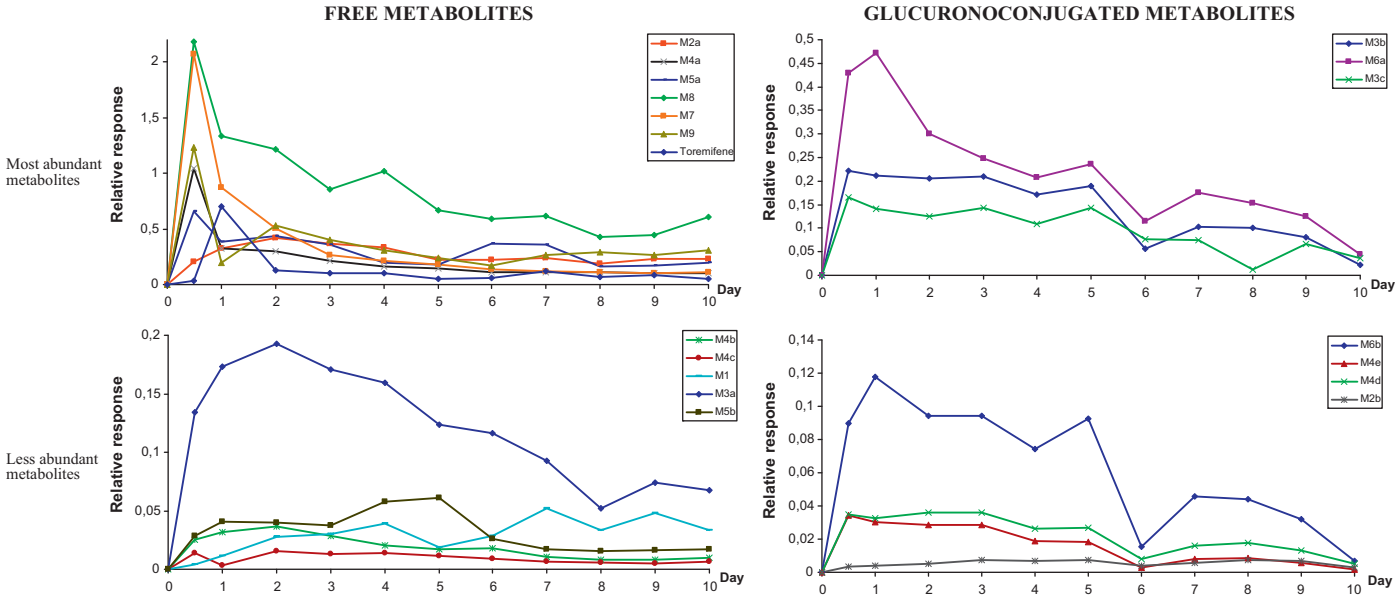


Fig. 3. Excretion ratios of toremifene metabolites. Relative responses for most abundant free and conjugated metabolites (top) and less abundant metabolites (bottom). Relative responses were obtained as comparison between the areas of analytes (using transitions described in Table 1) and the area of ISTD (303 > 97).

3.2. Detection of metabolites in urines from excretion studies

For the detection of toremifene metabolites, extracts of urines samples from excretion studies were analyzed by LC–MS/MS in SRM mode by monitoring the transitions of potential metabolites excreted in free form. The characteristic fragmentation pattern shown by toremifene and its metabolites available as standards, with main fragment ions resulting from the alkylic chains, allow the use of SRM analysis for the detection of new metabolites. As indicated in experimental section, transitions were selected by calculating the protonated molecular ion $[M+H]^+$ of the potential metabolite as precursor ion to the product ions at *m/z* 72, for N,N-dimethylated metabolites, at *m/z* 58 for N-desmethylated metabolites, and at *m/z* 44 for N-didesmethylated metabolites.

Metabolic pathways studied were based on those metabolites reported in previous studies for toremifene [3,13,14,20,21,24,26,28–30] and other related compounds, such as tamoxifen and clomifene [3,4,17,31–41]. All previously reported metabolic pathways and their combination were included in SRM method, with the main exception of those proposed after metabolic removal of the amine function because of no ionization in positive ion mode was expected for these compounds [13–15,18–25,27,42] (Table 1).

Unchanged toremifene and 20 metabolites were detected excreted free or conjugated with glucuronic acid and/or sulphate. Metabolic pathways detected for toremifene include N-desmethylation, hydroxylation, dihydroxylation, reduction, methylation and dehalogenation and combinations of them. Some of proposed metabolic pathways (N,N-didesmethylation, N-desmethylation + hydroxylation + hydroxymethylation, hydroxy-quinine and hydroxylation + oxidation) were not detected (Table 1) despite the fact that they were previously reported for tamoxifen, clomifene and toremifene [3,4,14,18–21,25,28–30]. As it can be observed in Table 1, N-desmethylated and dehalogenated metabolites were detected mainly in free form. Monohydroxylated metabolites were detected mainly as conjugated with glucuronic acid and/or sulphate and dihydroxylated metabolites were detected as free and/or conjugated with glucuronic acid.

3.3. Characterization of structures of the most abundant metabolites

The poor fragmentation observed for toremifene and metabolites (see above) causes serious difficulties to elucidate the structure of the metabolites detected. As stated previously, only one abundant product ion was obtained for each metabolite. The other product ions used for structural elucidation commonly exhibited abundances lower than 5%. Therefore, only the most abundant metabolites (**M2a**, **M3a**, **M4a**, **M5a**, **M7**, **M8** and **M9**) could be studied. Product ion mass spectra were obtained for each metabolite at 15 and 45 eV using accurate mass measurements, and they were compared with previously obtained for toremifene, **M3b** and **M1** (see supplementary information, Fig. 2 and Table 2). Proposed structures are shown in Fig. 1.

3.3.1. *M3a*

At low collision energy (15 eV), the most abundant ion of **M3a** (RT 10.81 min) was $[M+H]^+$ (accurate mass *m/z* 422.1900; $C_{26}H_{29}NO_2Cl$), i.e., one oxygen more than toremifene. Therefore, hydroxylation seemed to be the most feasible metabolic pathway. Product ion at *m/z* 72.0810, neutral losses of [M+H-35.9703 Da]$^+$, [M+H-45.0578 Da]$^+$ and [M+H-18.0106]$^+$, corresponding to losses of HCl, N-chain and H_2O, respectively, were observed (Fig. 5). The losses of HCl and N-chain together with the molecular formula and the ion at *m/z* 72.0810 confirmed that **M3a** is a chlorinated, N,N-dimethylated metabolite. Neutral losses containing water ($[M+H-H_2O]^+$ at *m/z* 404 and $[M+H-Nchain-H_2O]^+$ at *m/z* 359) suggested that the hydroxyl group was not in phenolic ring. This fact was supported by the results at high collision energy (45 eV), where characteristic ions of hydroxylation in C ring (Table 2) were not observed. For these reasons, hydroxylation in C3 was proposed (Fig. 1).

Other two late-eluting hydroxylated metabolites were detected as conjugated with glucuronic acid (**M3b** (p-hydroxytoremifene) and **M3c** in Table 1 and Fig. 4). These results are in agreement with those previously reported [17,26,29] where the first eluting monohydroxylated metabolite was described as α-hydroxytoremifene and can correspond to **M3a**.

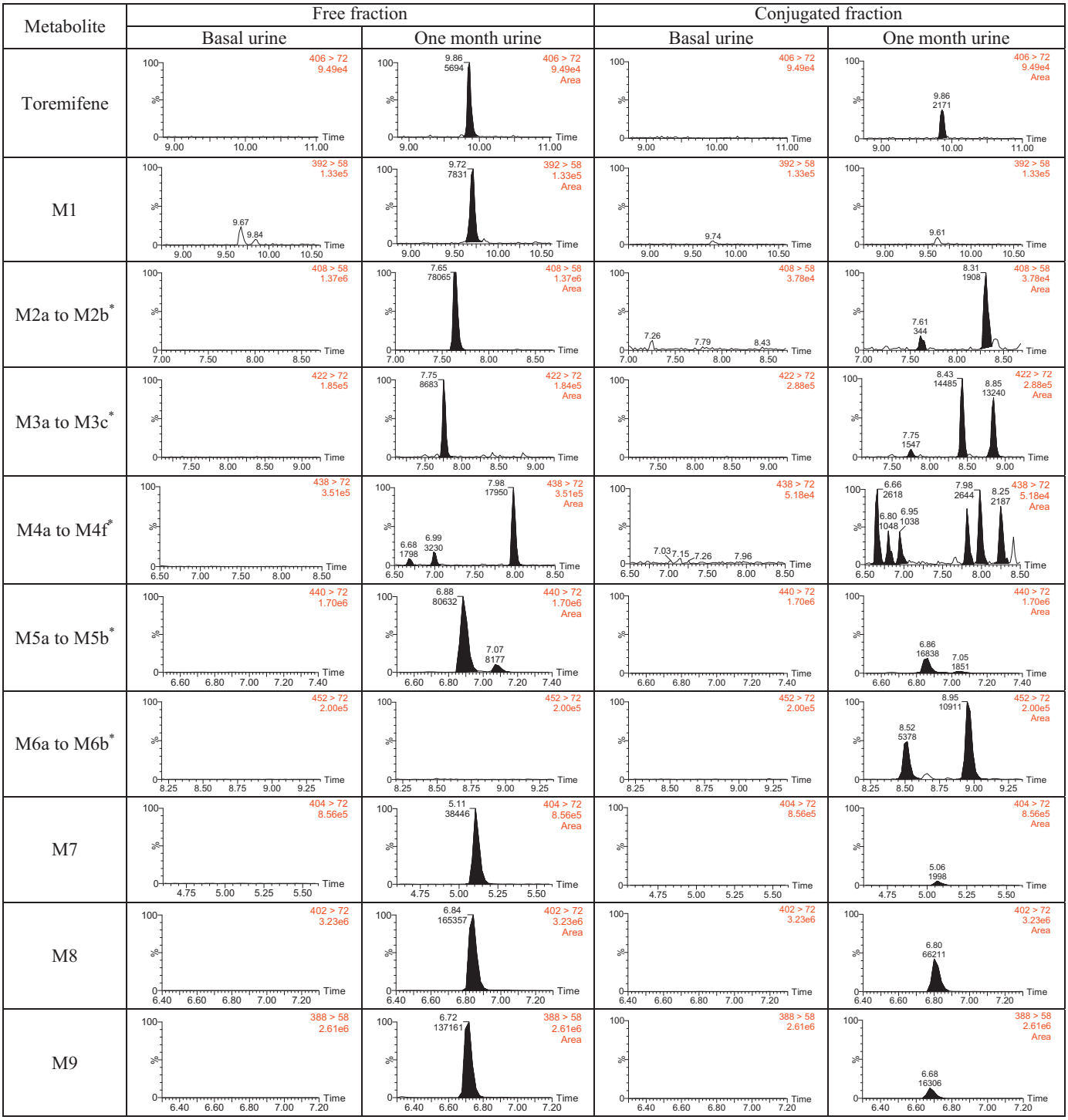


Fig. 4. SRM chromatograms of toremifene metabolites. Basal urine and urine obtained one month after toremifene administration (60 mg, orally). Free (left) and conjugated fraction (right). (*) For identification of the different metabolites, see retention time in Table 1.

3.3.2. ***M2a***

M2a elutes at 9.93 min. The accurate mass of $[M+H]^+$ (m/z 408.1735, elemental composition $C_{25}H_{27}NO_2Cl$) can be assigned to a N-desmethylated and hydroxylated metabolite. At 15 eV, the product ion at m/z 58.0662, characteristic of N-demethylated metabolites, was observed (Fig. 2). Similarly to **M3a**, neutral losses of HCl $[M+H-35.9703\,Da]^+$, N-chain $[M+H-31.0422\,Da]^+$ and N-chain + H_2O $[M+H-31.0422-18.0106]^+$, were observed for **M2a** at low collision energy. These losses suggested the presence of a hydroxyl group in an alkylic chain of a N-desmethylated metabolite. At 45 eV, several ions with high relative abundances were obtained as described in N-desmethylated standard. Characteristic ions of

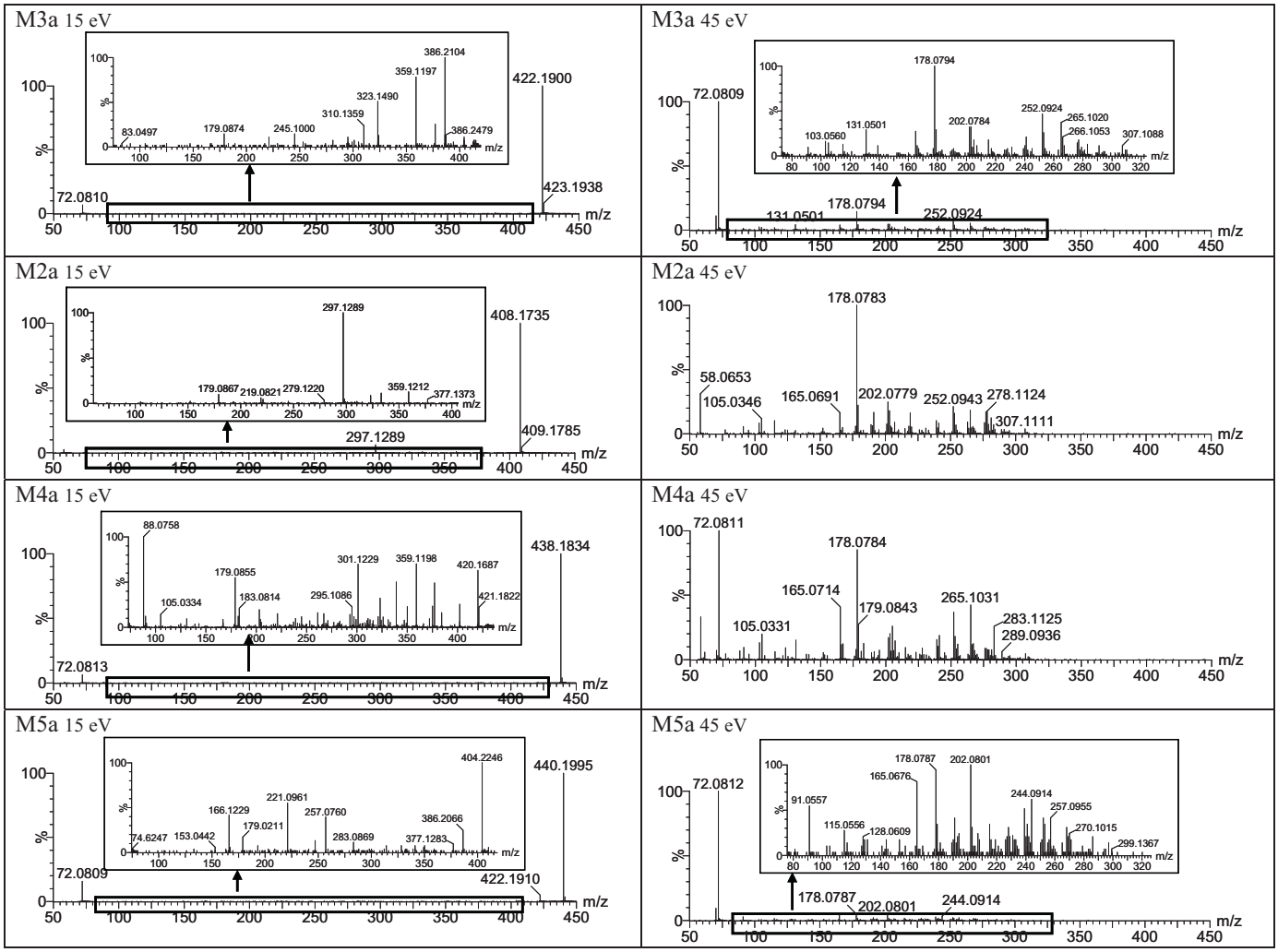


Fig. 5. Product ion mass spectra of $[M+H]^+$ of most abundant toremifene metabolites, at 15 eV (left) and 45 eV (right).

para-hydroxylation of C ring were not observed confirming that C3 is the most feasible place to locate the hydroxyl group. Proposed structure is shown in Fig. 1.

An other metabolite with $[M+H]^+$ at *m/z* 408 (**M2b**) was also detected mainly as conjugated with glucuronic acid and sulphate (Table 1), showing a similar behaviour as results described for **M2a** metabolites.

A metabolite with the same characteristics (N-desmethylated and hydroxylated) was previously reported as N-desmethyl-p-hydroxytoremifene [3,13,14,18–20,24,29,31]. The position of the hydroxyl group in the aromatic ring was already questioned by Watanabe et al. [28]. Results obtained in this study support the alkylic nature of the hydroxyl group for **M2a** and this is the first time that this structure is proposed.

3.3.3. *M4a*

At 15 eV, the most abundant ion of **M4a** (RT: 12.32 min) was also $[M+H]^+$ (*m/z* 438.1834, $C_{26}H_{29}NO_3Cl$), i.e., two oxygen atoms more than toremifene. Therefore dihydroxylation could be the most feasible metabolic pathway. Neutral loss of N-chain was not observed as the common losses previously reported ($[M+H-45.0578\ Da]^+$ for N,N-dimethylated and $[M+H-31.0422\ Da]^+$ for N-desmethylated metabolites). However, it presented an ion at $[M+H-61.0540\ Da]^+$ corresponding to a loss of C_2H_7NO (toremifene N-chain + O). Ions at *m/z* 88.0759 and 58.0656 were also observed. A neutral loss of 30 Da $[M+H-30.0106\ Da]^+$ was described as characteristic for compounds having a CH_2OH group [48,49]. For all of these facts (losses of 61 Da and 30 Da and the presence of an ion at *m/z* 88), hydroxylation in C7 is suggested (Fig. 1). In addition, at 45 eV, several ions presented greater abundances than 30%, contrarily to the common behaviour observed for N,N-dimethylated metabolites and also confirmed that C7 is the most probable location for hydroxyl group.

Similarly to **M3a** and **M2a**, neutral losses of HCl and H_2O were observed for **M4a** suggesting the presence of at least one hydroxyl group in an alkylic chain. Besides, characteristic ions of para hydroxylation in C ring were not observed. Due to these facts, the second hydroxyl group was proposed in C3 (Fig. 1).

In contrast with previously dihydroxylated metabolites described where hydroxylation is mainly reported in aromatic rings [3,18,19,24,25,28,29], results for **M4a** suggested that the two hydroxylations occur in alkylic moieties. Up to our knowledge, a metabolite with this structure has not been previously reported.

3.3.4. *M5a*

At low collision energy, an abundant ion at $[M+H]^+$ (*m/z* 440.1995; $C_{26}H_{31}NO_3Cl$) was shown for **M5a** (RT 6.95 min). The formula corresponds to two oxygen atoms more and a double bond less than toremifene. Besides the typical ion at *m/z* 72.0809, an ion at *m/z* 422.1910, corresponding to $[M+H-18.0106]^+$, was also significantly abundant (>5%). The relatively abundant neutral loss of water and the fact that the presence of two hydrogen atoms more

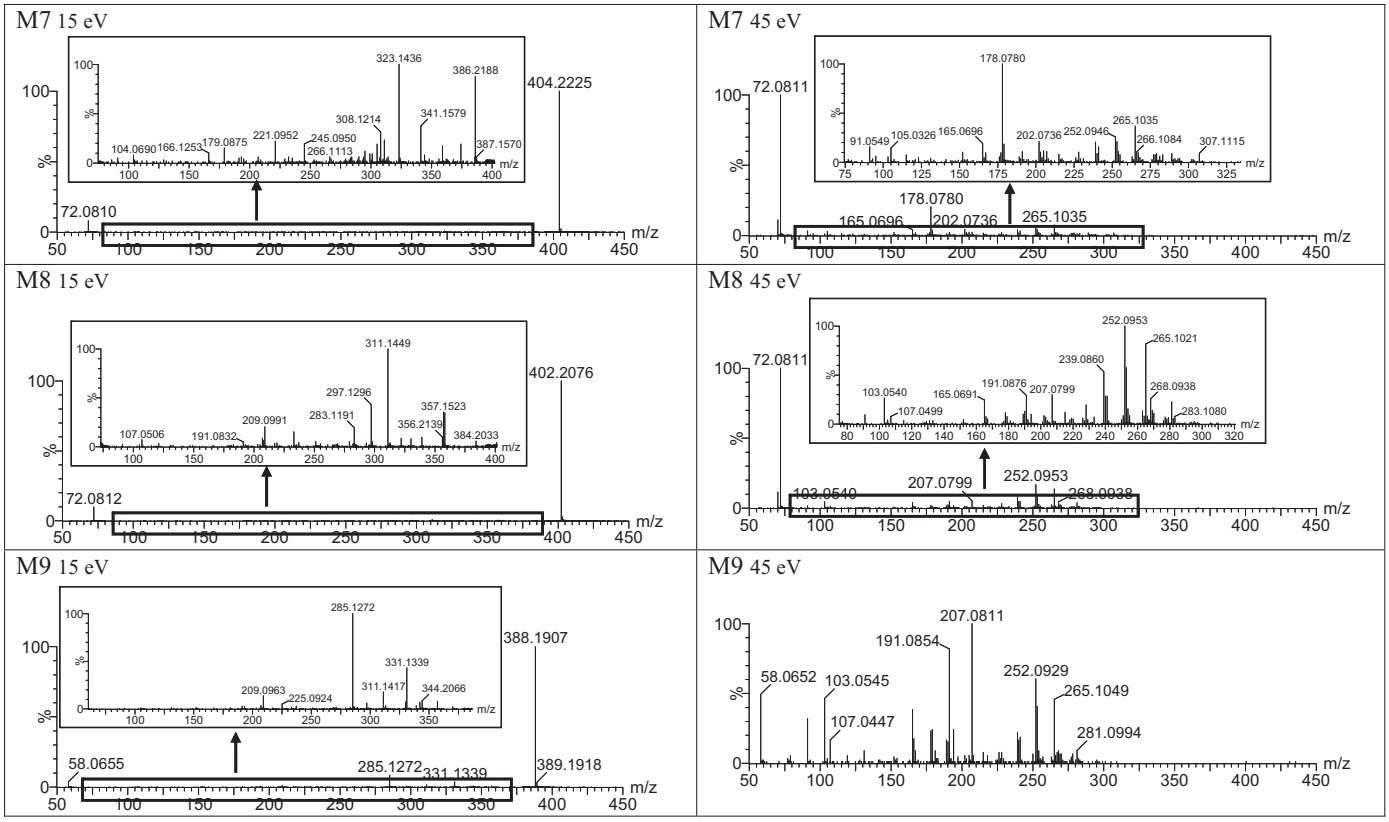


Fig. 5. (*Continued*)

than toremifene seems to indicate that a hydroxyl group is situated in C1 or C2. Neutral loss of HCl was also observed.

Ions at *m*/*z* 166.1229, 179.0211, 221.0961 and 257.0760, obtained at 15 eV, described previously in p-hydroxytoremifene, were observed (see Fig. 5). Mass spectra obtained for **M5a** is very similar than those obtained for **M3b** (p-hydroxytoremifene) in fact, ions described as a marker of C ring hydroxylation (e.g. ions at *m*/*z* 244.0914 and 257.0955) were also observed at 45 eV. These facts suggested that the other hydroxyl group could be located in *para*-position of C ring. Proposed structure of **M5a** (A or B) is shown in Fig. 1.

A metabolite with the same molecular weight and similar characteristics (two hydroxyl groups and the reduction of a double bond) than **M5a** was previously reported as dihydroxydehydrotoremifene, with two hydroxyl groups in C1 and C2 [3,28,29]. However, according to the mass spectrometric data obtained in our study, one of the hydroxyl group is located in para position in phenolic ring, and the second in C1 or C2. For this reason, the structure proposed for **M5a** is that shown in Fig. 1. Up to our knowledge, a metabolite with this structure has not been previously reported.

3.3.5. *M7*

M7 elutes at 2.75 min. The accurate mass of $[M+H]^+$ (*m*/*z* 404.2225; $C_{26}H_{30}NO_3$) and the absence of characteristic chlorine isotopic pattern indicated the elimination of the chlorine. Molecular formula showed that the addition of two hydroxyl groups was the most feasible metabolic pathway. At low collision energy, neutral loss of N-chain $[M+H-45.0578]^+$ and ion at *m*/*z* 72.0810 were observed, indicating that no presence of hydroxyl group in C5, C6 or C7. Neutral losses of H_2O ($[M+H-18.0106\,Da]^+$ and $[M+H-45.0578-2H_2O]^+$) could indicate that both hydroxyl groups were in alkylic chain. This fact was supported by the results obtained at 45 eV, where no characteristic ions of hydroxylation of C ring were observed. Therefore one hydroxyl group could be located in C3.

The neutral loss of 30 Da $[M+H-30.0106\,Da]^+$ observed, can be considered as characteristic for compounds having a CH_2OH group as described for **M4a**. Therefore one hydroxyl group could be located in C4.

Mazzarino et al. [3] and Lu et al. [29], described a metabolite with the same characteristics (absence of chlorine atom, $[M+H]^+$ at *m*/*z* 404 and product ion at *m*/*z* 72) as common metabolite with tamoxifen. Analyses of a urine sample obtained after administration of tamoxifen, showed a metabolite with the same transition that **M7** but with a retention time differing 6.4%. This fact, suggested that metabolites of tamoxifen and toremifene are structurally related but they are not the same compound.

3.3.6. *M8*

M8 elutes at 6.64 min. The accurate mass of $[M+H]^+$ (*m*/*z* 402.2073; $C_{26}H_{28}NO_3$) and the absence of characteristic chlorine isotopic pattern, indicated the absence of chlorine atom. Two oxygen atoms more than toremifene were obtained, therefore, dihydroxylation and oxidation was considered as the most feasible metabolic pathway. At high collision energy, ions at *m*/*z* 191.0876, 207.0799, 252.0953 and 265.1021, previously described for toremifene (Table 2) were observed, indicating that there were no changes in aromatic rings. At low collision energy, product ion at *m*/*z* 72.0812 and neutral loss of N-chain $[M+H-45.0578\,Da]^+$ were indicative that C5, C6 and C7 were unaltered. However, at 15 eV fragmentation observed exhibited several differences when comparing with the other described metabolites. Neutral losses of CO_2 $[M+H-43.9898\,Da]^+$, CO $[M+H-27.9949\,Da]^+$ and HCOOH $[M+H-46.0055\,Da]^+$ were observed (see Fig. 5), suggesting the presence of a carbonyl group. These fragments observed could be in agreement with previously described metabolite with a carboxylic acid in C4

[3,29] (**M8**B in Fig. 2). Contrarily to the expected behaviour for acidic compounds, no signal in negative ionization mode was obtained. Other alternative structure is proposed (**M8**A in Fig. 2), involving an oxidation of **M7** and cyclization. This structure could also give the neutral losses of CO_2, CO and HCOOH. Ion at *m/z* 384.2033 corresponding to a loss of water [M+H-18.0106 Da]$^+$ was more difficult to be explained from the structure **M8**A. Synthesis and comparison with reference material would be needed to confirm the structure.

3.3.7. *M9*

M9 elutes at 6.25 min. The accurate mass of [M+H]$^+$ (*m/z* 388.1909; $C_{25}H_{26}NO_3$) and the absence of characteristic chlorine isotopic pattern, as described in previous metabolites (**M7** and **M8**), indicated the absence of chlorine atom. At 15 eV, product ion at *m/z* 58.0656 and neutral loss of N-chain [M+H-31.0422 Da]$^+$, described as characteristic of N-demethylated metabolites, were observed (Fig. 5). In a similar way than **M8**, neutral loss of HCOOH [M+H-46.0055 Da]$^+$ was observed. A structure with a carboxylic acid in C4, as described previously [3] was proposed (**M9**B in Fig. 2) but no signal in negative ionization mode was obtained. This metabolite seem to be the N-desmethylated **M8**, and as the same way, an oxidation of **M7** and cyclization was also proposed (**M9**A in Fig. 2). Synthesis and comparison with reference material would be needed to confirm the structure.

3.4. Relevant target analytes for doping control assays.

The analysis of urine samples of the whole excretion study revealed that all of metabolites were detected up to 10 days after toremifene administration (Fig. 3). In the first hours after administration, the relative response increased for all metabolites detected with maximum concentrations for most of them between 12 and 48 h after administration. The most abundant response was observed for metabolites excreted in free form, dehalogenated (**M7**, **M8**, **M9**) and dihydroxylated (**M4a**) metabolites. Metabolites with most abundant relative response in conjugated fraction were hydroxylated metabolites **M3b** and **M3c**, and hydroxylhydroxymethyl metabolite **M6a**. Similar results were obtained for both excretion studies analyzed.

A spot urine sample was additionally collected one month after administration, and all free and conjugated metabolites could be detected with satisfactory sensitivity (Fig. 4). The most important metabolites detected one month after administration were dehalogenated metabolites (**M7**, **M8**, and **M9**) and metabolites resulting from hydroxylation **M3b**, **M4a** and **M5a**, all of them detected in free fraction.

4. Conclusions

A metabolic study of toremifene was performed. The SRM method created based on the study of the CID for available metabolites, allowed for the detection of unchanged toremifene and 20 metabolites in post administration studies. Different metabolic pathways were proposed, including mono- and di-hydroxylation, N-desmethylation, methylation, reduction, dehalogenation and combinations of them. Hydroxylation in the alkylic chains has been confirmed as an important metabolic pathway for toremifene. Most of the detected metabolites were excreted free (mainly N-desmethylated, dihidroxylated and dehalogenated metabolites) and/or conjugated with glucuronic acid (mono and dihydroxylated metabolites). Some metabolites involving hydroxylation (**M2b**, **M3b** and **M6c**) were also detected as conjugated with sulphate.

The most abundant metabolites were detected in the free fraction. Among them, some metabolites do not have the chlorine atom (**M7**, **M8** and **M9**), some were monohydroxylated (**M2a** and **M3a**) and some dihydroxylated metabolites (**M4a** and **M5a**). The structures of these abundant metabolites have been proposed based on fragmentation pattern with mass accurate measurements. Structures of metabolites **M2a**, **M4a**, **M5a**, **M7** and **M9** have not been previously reported.

All metabolites could be detected up to one month after administration of toremifene. According to results obtained, high sensitivity and less chemical background were obtained using a free faction extraction procedure although the conjugated fraction could be also used in order to detect toremifene misuse. The most abundant conjugated metabolites were: monohydroxylated (**M3b** and **M3c**), and hydroxylated and hydroxymethylated metabolite (**M6a**). Therefore, these metabolites can be considered as the most adequate target analytes for doping control purposes.

The poor fragmentation observed in toremifene and metabolites could be a problem in confirmation purposes. N-desmethylated metabolites (**M2a** and **M9**) and the N-methyl-N-hydroxymethyl metabolite (**M4a**), at high collision energy (45 eV), presented more than one product ion with a relative abundance higher than 10%. Therefore, these metabolites would be the most useful compounds for confirmation purposes in doping control analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.073.

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