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ORIGINAL PAPER

Urinary excretion profiles of toremifene metabolites by liquid chromatography-mass spectrometry. Towards targeted analysis to relevant metabolites in doping control

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Abstract In the present study, toremifene urinary excretion studies were evaluated in order to examine main metabolic reactions and to select target metabolites in doping control analysis. Urine samples from three female subjects were collected every 3 h for at least 15 days after the oral administration of a single dose of Fareston® (60 mg). The elemental compositions of the compounds detected were determined by liquid chromatography-mass spectrometry using a time-of-flight system with accurate mass measurement. More detailed structure elucidation was obtained by monitoring the presence or absence of structure-specific ions, using product ion scan and neutral loss acquisition modes, whereas the metabolites urinary profiles were evaluated in selected reaction monitoring acquisition mode. The results showed that the main routes of phase-I modifications involved carboxylation of the chlorinated side chain, *N*-demethylation and hydroxylation in different positions. Fifteen metabolites were found in all subjects studied, most of them were detected for more than 10 days in the free, glucuronide and sulphate fractions, with a maximum of excretion generally after 9–22 and 34–47 h from drug administration. These metabolites can be divided in two groups: metabolites with the characteristic chlorine isotope pattern and metabolites without the characteristic chlorine isotope pattern. The most abundant and long-term compounds were the carboxylated metabolites followed by the hydroxylated metabolites. Their product ions originating after collision-induced dissociation were observed to occur prevalently in the dimethylaminoethoxy and in the chlorinated side chains. These structure-specific ions were used to design screening and confirmation procedures to positively identify toremifene administration in doping control analysis.

Keywords Antidoping analysis · LC-MS · SERMs · Toremifene

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Introduction

Toremifene is a chlorinated derivative of tamoxifen with a similar binding affinity for the estrogenic receptor. According to previous pharmacological studies, toremifene is almost completely absorbed after oral administration. It reaches peak concentrations in plasma within 3 h and has an elimination half-life of 5 days. Elimination occurs mainly in the faeces with 10% excreted in the urine over 7 days [1–9]. Toremifene is extensively metabolized principally by the cytochrome P450 3A4 and 2D6 to *N*-demethylated and hydroxylated metabolites. Studies based on structure–function relationship demonstrated that the dimethylaminoethoxy side chain is necessary for anti-estrogenic action. The hydroxylation in position 4 leads to an increased anti-estrogenic activity, whereas the hydroxylation in other positions leads to compounds with weak anti-estrogenic activity [10, 11].

Clinically, toremifene is mainly used for the induction of ovulation in sub-fertile women attempting pregnancy, for

the treatment of breast cancer, for the treatment and prevention of post-menopausal osteoporosis in females and for the induction of spermatogenesis in males [1–9].

Athletes could illicitly use toremifene to increase endogenous testosterone, with the aim to by-pass the specific testing regimens for known synthetic androgens including exogenous testosterone and/or to balance the adverse effects of an extensive abuse of exogenous testosterone and/or synthetic anabolic androgenic steroids [12, 13]. Since 2005, toremifene is included with other similar drugs in the S4 class "agents with anti-estrogenic activity" of the World Anti-Doping Agency (WADA) list of prohibited substances and methods [14].

From the doping control point of view, the main interest of the metabolic studies is to characterize the most representative metabolites (long-term, most abundant and influenced in a small amount by ethnicity, pathologies, age and sex) and to know metabolites analytical behaviours in order to include the best target compound in the routine analytical procedures.

Very few studies were carried out to identify toremifene metabolites in human urine, and little is known about their profile in the free, glucuronide and sulphate fractions [15–26]. In a previous research carried out in our laboratory in pools of urine collected after toremifene or tamoxifen administration, several metabolites without the characteristic chlorine pattern were detected [26]. These metabolites may possess great potential as target analytes for doping control analysis.

In the present study, the toremifene urinary excretion profile in the free, glucuronide and sulphate fractions was evaluated using both time-of flight and triple quadrupole systems, in order to propose toremifene metabolic reactions, to find the most representative metabolites and to consider the best analytical approach for doping control purpose.

Materials and methods

Standards, chemicals and reagents

Toremifene (Fareston®) was purchased from Orion Pharma (Milano, Italy). The 17α-methyltestosterone (used as internal standard for the toremifene and its metabolites profile) and the toremifene (used as reference standard to estimate the toremifene metabolites urinary concentration) were supplied by Sigma-Aldrich (Milano, Italy).

All chemicals (sodium phosphate, sodium hydrogen phosphate, formic acid, *tert*-butylmethyl ether, methanol and acetonitrile) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). The ultrapure water used was of Milli-Q-grade (Millipore, Milano, Italy).

The enzymes β-glucuronidase (from *Escherichia coli*) and arylsulphatase/β-glucuronidase (from *Helix pomatia*) used for the enzymatic hydrolysis of glucuronide and of the sulphate conjugates, respectively, were purchased from Roche (Monza, Italy).

Administration study

Urine samples, obtained from a local medical practitioner by three female volunteers (38 years, 55 kg; 42 years, 65 kg and 35 years, 60 kg), were collected before and after (for at least 2 weeks) the administration of a single dose of Fareston® (60 mg toremifene per os). Samples were anonymized, and written consent was obtained from volunteers allowing the use of urine samples for research purposes.

All urine samples, collected in sterile containers, were stabilized with sodium azide (1 mg/mL) and stored at −20 °C until analysis.

All response values were adjusted for a value of specific gravity of 1.020, following the WADA guidelines for endogenous compounds. Samples with a pH value higher than 7 and/or a specific gravity value lower than 1.005 were not included in the study. The lack of certified reference materials did not allow an accurate determination of all metabolites urinary concentration; thus, the excretion profiles were obtained plotting the area ratio between the toremifene metabolites and the internal standard 17α-methyltestosterone.

Instrumental conditions

Liquid chromatography conditions

All LC experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies S.p.A, Cernusco sul Naviglio, Milano, Italy). Reversed-phase liquid chromatography was performed using a Zorbax Eclipse Plus C18 column (2.1×100 mm, 1.8 μm). The solvents used were water containing 0.1% (*v*/*v*) formic acid (eluent A) and acetonitrile containing 0.1% (*v*/*v*) formic acid (eluent B).

The gradient program started at 10% B and increasing to 30% B in 10 min, after 4 min, to 40% B, after 3 min, to 60% B in 5 min, and then after 4 min to 100% B. The column was flushed for 2 min at 100% B and finally re-equilibrated at 10% B for 4 min. The flow rate was set at 300 μL/min.

Triple-quadrupole system conditions

All LC-ESI-MS/MS experiments were performed using an Applied Biosystems (Applera Italia, Monza, Italy) API4000

triple-quadrupole instrument with positive electrospray ionization. The ion source was operated at 500 °C, whereas the applied capillary voltage was 5,500 V. Selected reaction monitoring (SRM), neutral loss, precursor ion scan and product ion scan experiments were performed employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8 mPa, obtained from a dedicated nitrogen generator system Parker-Balston model 75-A74, gas purity 99.5% (CPS analitica Milano, Italy). The collision energy and declastering potential values of each compounds were optimized analyzing the excretion studies samples. All aspects of instrument control, method setup parameters, sample injection and sequence operation were controlled by the Applied Biosystems Analyst software.

Time-of-flight (TOF) system conditions

High-resolution/high-accuracy measurements were performed on an Agilent Technologies 6210 orthogonal acceleration time-of-flight mass spectrometer, equipped with an electrospray ionization (ESI) source operated in positive ion mode. Nitrogen was used as the drying and nebulising gas. The drying gas flow rate and temperature were 10 L/min and 350 °C, respectively. The nebulizer gas

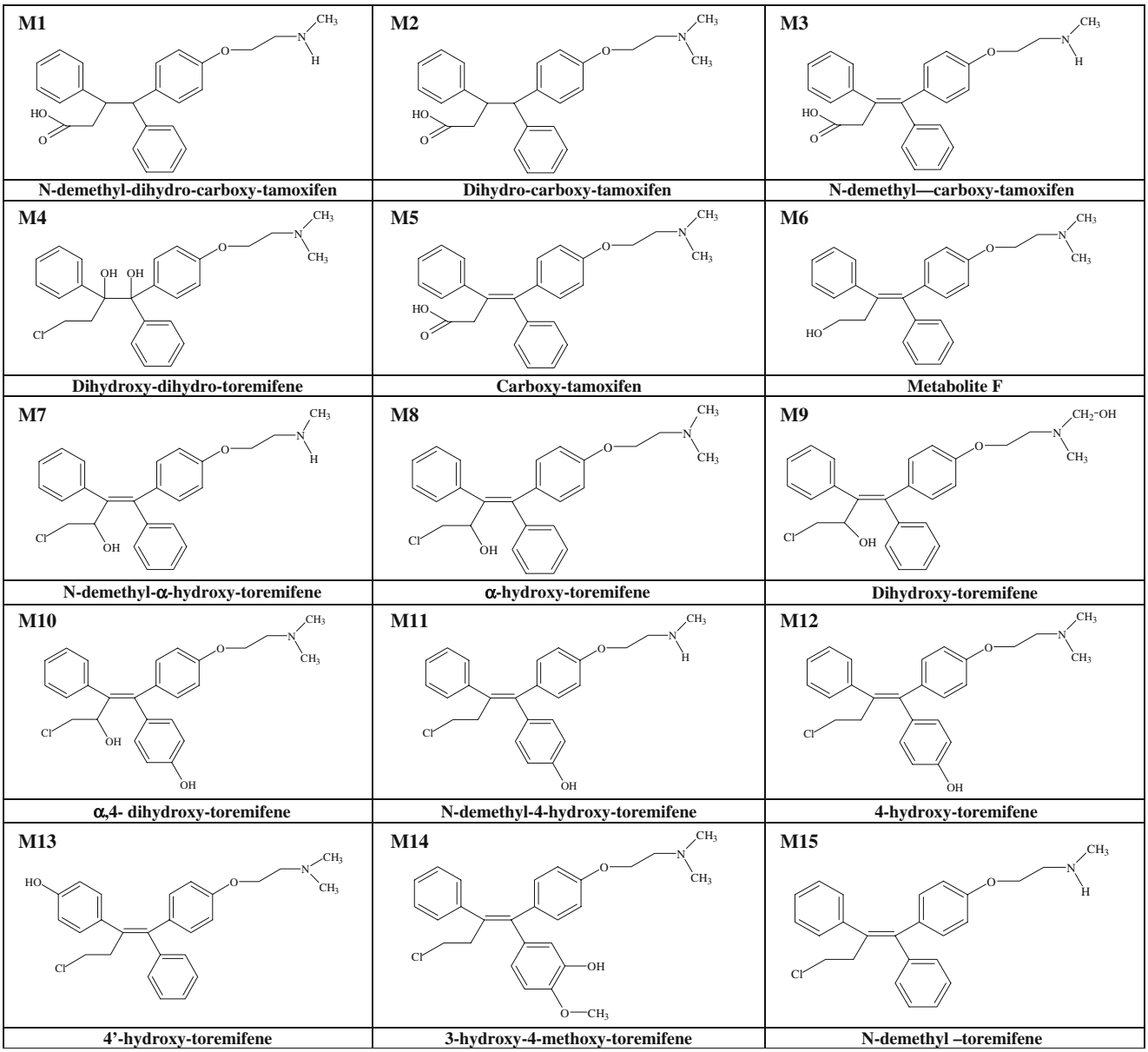


Fig. 1 Suggested chemical structure of the toremifene metabolites

pressure was 45 psi. The applied capillary voltage optimized by direct infusion of toremifene was set at 4,000 V. Different fragmentor voltage condition were tested (150, 175 and 200 V). Mass spectra data were collected from *m/z* 100 to 1,100 at 9,300 transients per second. All other MS parameters (transfer optic voltage, voltage of the ion focus and octapole lens for optimizing the beam shape as it enters the TOF analyzer, TOF voltages and detector voltage) were automatically optimized by the instrument autotuning procedure, performed daily. The mass calibration was also performed daily before starting the analysis using a calibration solution provided by the manufacturer.

Purine with an $[M+H]^+$ ion at *m/z* 121.0509 and an Agilent proprietary compound (HP0921) yielding an ion at *m/z* 922.0098 were simultaneously introduced via a second orthogonal sprayer, and these ions were used as internal calibrants along all the analysis. All aspects of instrument control, tuning, method setup and parameters, sample injection and sequence operation were controlled by the Agilent Technologies Mass Hunter software.

Sample preparation

The sample preparation was based on a previously described procedure [26, 27]. Briefly, to 3 mL of urine, 1.5 mL of phosphate buffer (1 M, pH 7.4) and 50 μL of the internal standard (ISTD: solution of 17α-methyltestosterone 12 μg/mL) were added, and the liquid/liquid extraction was carried out with 10 mL *tert*-butylmethyl ether for 6 min on a mechanical shaker. After centrifugation, the organic layer was evaporated to dryness. The residue was reconstituted in 50 μL of mobile phase, and an aliquot of 10 μL was injected on the liquid chromatography-mass spectrometry systems (for the determination of free metabolites).

To the aqueous layer, 50 μL of β-glucuronidase from *E. coli* and 50 μL of the internal standard (ISTD: solution of 17α-methyltestosterone 12 μg/mL) were added, and the sample was incubated for 1 h at 55 °C. After hydrolysis, 10 mL of *tert*-butylmethyl ether was added, and the liquid/liquid extraction was carried out for 6 min on a mechanical shaker. After centrifugation, the organic layer was evaporated to dryness. The residue was reconstituted in 50 μL of mobile phase, and an aliquot of 10 μL was injected on the liquid chromatography-mass spectrometry systems (for the detection of the glucurono-conjugated metabolites).

To the aqueous layer, 1 mL of acetate buffer (0.8 M, pH 5.2), 50 μL of β-glucuronidase/arylsulphatase from *H. pomatia* and 50 μL of the internal standard (ISTD: solution of 17α-methyltestosterone 12 μg/mL) were added, and the sample was incubated for 2 h at 55 °C. After hydrolysis, 2 mL of phosphate buffer (1 M, pH 7.4) was added, and the liquid/liquid extraction was carried out with 10 mL of *tert*-butylmethyl ether for 6 min on a mechanical shaker. After centrifugation, the organic layer was evaporated to dryness. The residue was reconstituted in 50 μL of mobile phase, and an aliquot of 10 μL was injected on the liquid chromatography-mass spectrometry systems (for the detection of the sulpho-conjugated metabolites).

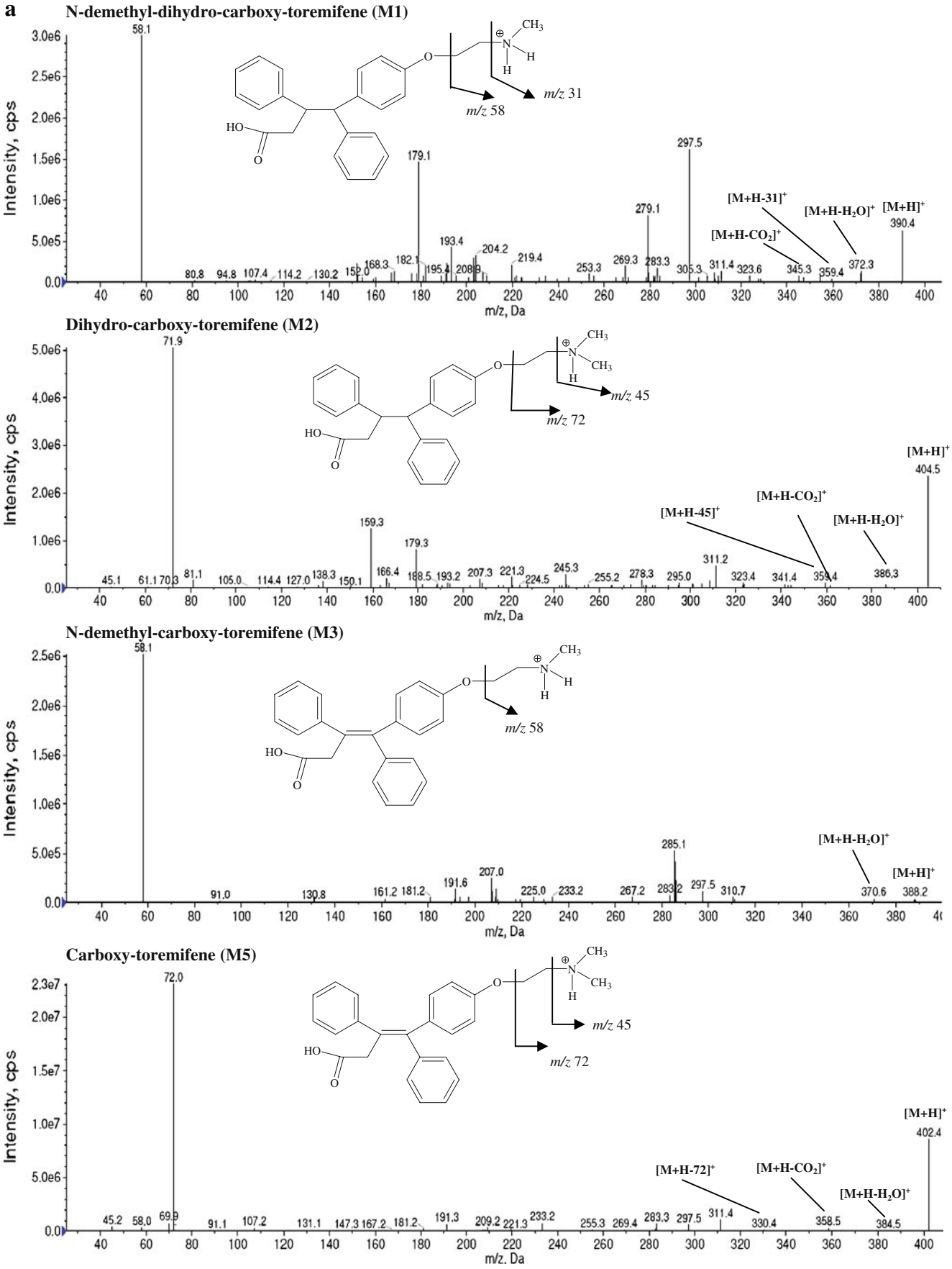
Fig. 2 Product ion scan of the most abundant toremifene metabolites: *N*-demethyl-dihydro-carboxy-tamoxifen (**M1**), dihydro-carboxy-tamoxifen (**M2**), *N*-demethyl-carboxy-tamoxifen (**M3**) and carboxy-toremifene (**M5**) (**a**); *N*-demethyl-α-hydroxy-toremifene (**M7**), α-hydroxy-toremifene (**M8**), 4-hydroxy-toremifene (**M11**), 4′-hydroxy-toremifene (**M12**) and dihydroxy-toremifene (**M9**) (**b**)

Results

LC-MS/(MS) analysis

The excretion studies samples obtained after oral administration of a single dose of toremifene were analyzed in positive and negative ESI in order to study the toremifene metabolites ionization behaviour. The elemental composition was obtained using time-of flight system with accurate mass measurement in the MS full scan mode. Different fragmentor (150, 175 and 200 V) conditions were tested to achieve an increase in the sensitivity and to eliminate compounds source fragmentation. Twelve toremifene metabolites were detected all as protonated species $[M+H]^+$ with a fragmentor voltage value of 175 V. These metabolites can be divided in two groups: metabolites with the characteristic chlorine isotope pattern and metabolites without the characteristic chlorine isotope pattern. More detailed structures elucidation was obtained by monitoring the presence or absence of structure-specific ions using product ion scan and neutral loss acquisition modes and different collision energies (10, 20, 25, 30, 35 and 40 eV) (Fig. 1).

Figure 2a, b shows the product ion spectra (CE 35 eV) of the most abundant compounds found in the urines collected after toremifene administration. The product ions originating after collision-induced dissociation occurred prevalently in the dimethylaminoethoxy and in the chlorinated side chains. More specifically, neutral losses of 31 Da (CH_5N) and 58 Da (C_3H_8N), found in the spectra of the compounds with elemental composition $C_{25}H_{27}NO_3$ (**M1**; *m/z* 390), $C_{25}H_{25}NO_3$ (**M3**; *m/z* 388) and $C_{25}H_{26}ClNO_2$ (**M7** and **M11**; *m/z* 408), could be considered specific for the detection of *N*-demethylated metabolites; neutral losses of 45 Da (C_2H_7N) and 72 Da ($C_4H_{10}N$), found in the spectra of the compounds with elemental composition $C_{26}H_{29}NO_3$ (**M2**; *m/z* 404), $C_{26}H_{30}ClNO_3$ (**M4**; *m/z* 440), $C_{26}H_{27}NO_3$ (**M5**; *m/z* 402), $C_{26}H_{29}NO_2$ (**M6**; *m/z* 388), $C_{26}H_{28}ClNO_3$


a
N-demethyl-dihydro-carboxy-toremifene (M1)
m/z 58
m/z 31
[M+H-31]+
[M+H-H2O]+
[M+H]+
[M+H-CO2]+
Dihydro-carboxy-toremifene (M2)
m/z 45
m/z 72
[M+H]+
[M+H-CO2]+
[M+H-45]+
[M+H-H2O]+
N-demethyl-carboxy-toremifene (M3)
m/z 58
[M+H-H2O]+
[M+H]+
Carboxy-toremifene (M5)
m/z 45
m/z 72
[M+H]+
[M+H-72]+
[M+H-CO2]+
[M+H-H2O]+
Intensity, cps
m/z, Da

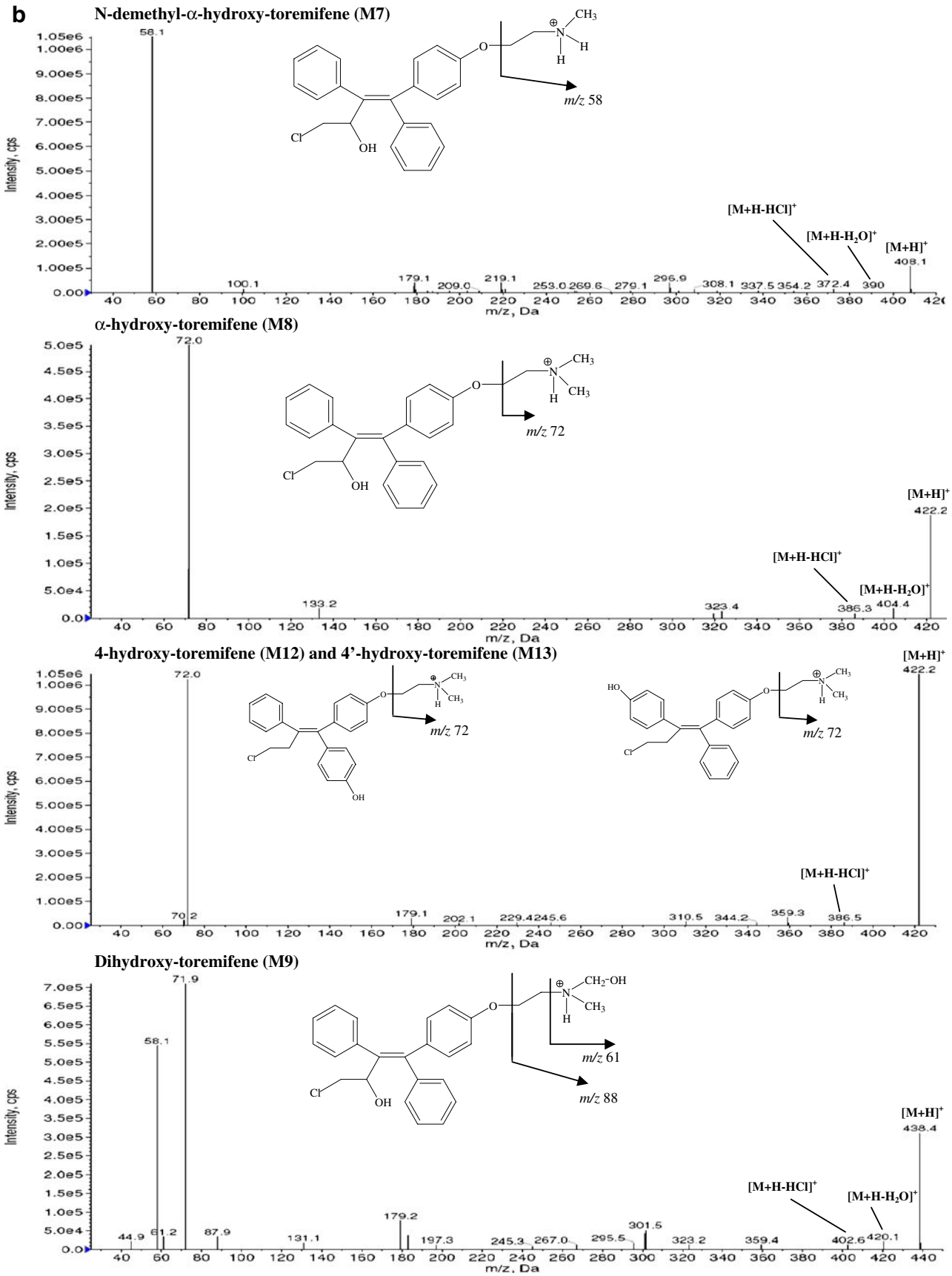


Fig. 2 (continued)

(**M9**; *m/z* 438), $C_{26}H_{28}ClNO_2$ (**M8**, **M12** and **M13**; *m/z* 422) and $C_{27}H_{30}ClNO_3$ (**M14**; *m/z* 452), could be considered specific for the *N*,*N*-dimethylated metabolites. Neutral losses of 61 Da (C_2H_7NO) and 88 Da ($C_4H_{10}NO$), found in the spectrum of the metabolite with elemental composition $C_{26}H_{28}ClNO_3$ (**M9**; *m/z* 438), could be considered specific for the hydroxylation in the N-chain. Chlorine loss was detected for all compounds with the characteristic chlorine isotope pattern, whereas neutral losses of 44 Da (CO_2) and 46 Da (HCOOH), found in the spectra of the metabolites with elemental composition $C_{25}H_{27}NO_3$ (**M1**; *m/z* 390), $C_{26}H_{29}NO_3$ (**M2**; *m/z* 404), $C_{25}H_{25}NO_3$ (**M3**; *m/z* 388) and $C_{26}H_{27}NO_3$ (**M5**; *m/z* 402), could be considered specific for the detection of carboxylated metabolites. At the end, water loss was observed in the spectra of the compounds with elemental composition $C_{25}H_{27}NO_3$ (**M1**; *m/z* 390), $C_{26}H_{29}NO_3$ (**M2**; *m/z* 404), $C_{25}H_{25}NO_3$ (**M3**; *m/z* 388), $C_{26}H_{30}ClNO3$ (**M4**; *m/z* 440), $C_{26}H_{27}NO_3$ (**M5**; *m/z* 402), $C_{25}H_{26}ClNO_2$ (**M7**; *m/z* 408), $C_{26}H_{28}ClNO_2$ (**M8**; *m/z* 422) and $C_{26}H_{28}ClNO_3$ (**M9**; *m/z* 438). The relatively stable toremifene core structure did not allow to obtain more informative product ions. Thus, the proposed structures reported in Fig. 1 were based taking into account, also, the relative elution on the chromatographic system, the data obtained by previous researches [15–26] and the fragmentation pattern observed in a previous study carried out in our laboratory for tamoxifen metabolites [27].

The structure-specific ions obtained by the MS/MS experiments described above were used to set up a specific SRM method useful to check if other toremifene metabolites, reported or unreported by previous investigators and not detected with the full scan experiments, could be detected. For this purpose, the precursor ions were obtained by calculating the protonated molecular ion $[M+H]^+$ of the potential toremifene metabolite, whereas the product ions selected were the neutral losses of 58 and 31 Da for *N*-demethylated metabolites, the neutral losses of 72 and 45 Da for *N*,*N*-dimethylated metabolites, the neutral losses of 88 and 61 Da for the *N*-hydroxylated metabolites and the neutral loss of 44 Da for the *N*,*N*-didemethylated metabolites.

Figure 3 shows the extracted chromatograms obtained analyzing the free, glucuronide and sulphate fraction of the excretion study sample (from subject 1) collected after 9 h from the toremifene administration. All 12 toremifene metabolites found using the full scan acquisition mode were clearly detected. Other four compounds were found: the toremifene (**P**), the *N*-demethyl-toremifene (**M15**), the *N*-demethyl-4-hydroxy-toremifene (**M11**) and the α,4-dihydroxy-toremifene metabolite (**M10**), whereas the *N*-demethylated-hydroxy-methoxy, the hydroxy-quinine, the *N*-oxide and the *N*,*N*-

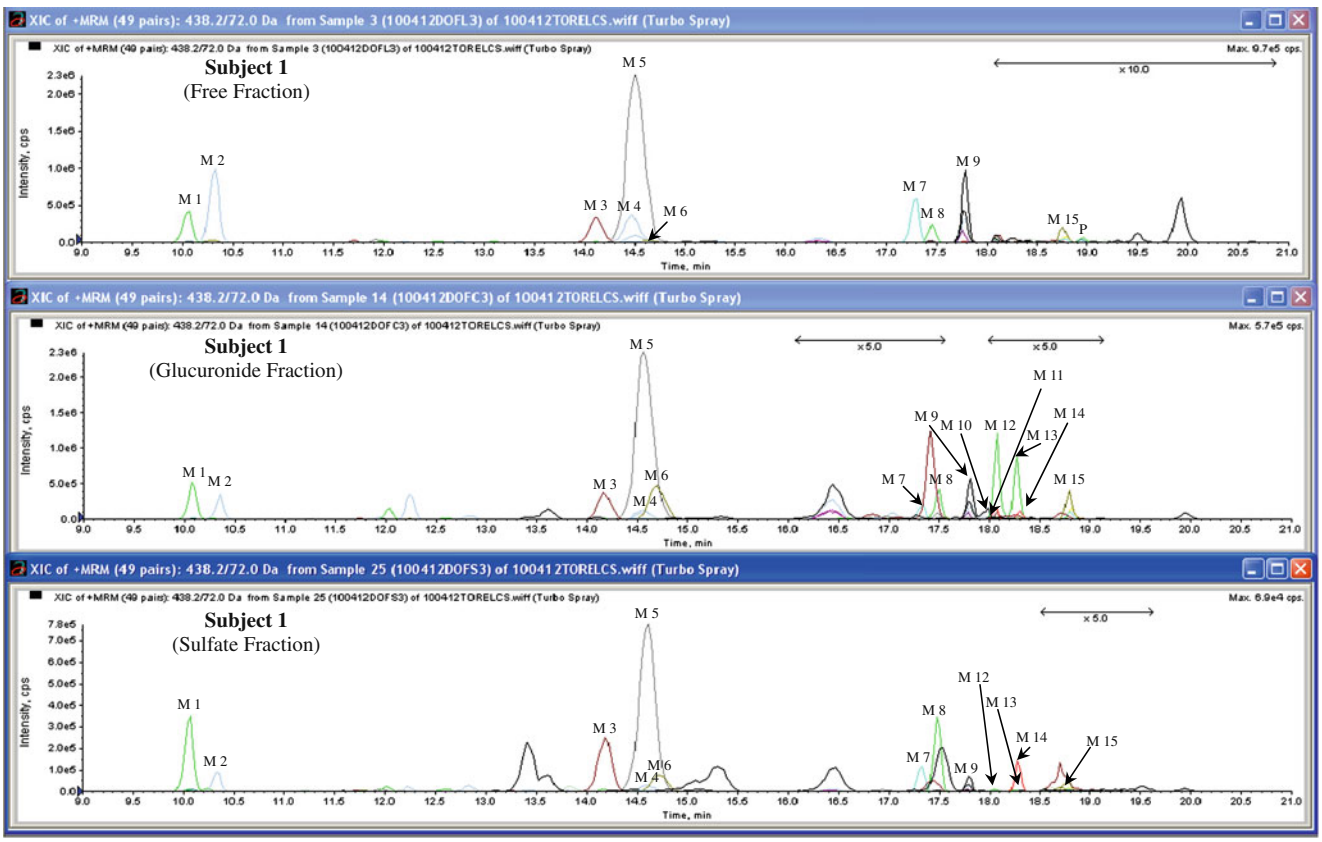


Fig. 3 Extracted chromatogram of subject 1 excretion study sample collected after 9 h from the toremifene administration

Table 1 Summary of the results obtained using LC-MS/MS and LC-QTOF

ID	Compound	Elemental composition	Molecular weight (Da) (calc)	Molecular weight (Da) (exp)	Error (ppm)	Characteristic transitions (*m/z*)	RT (min)	Free fraction	Glucuronide fraction	Sulphate fraction
M1	*N*-demethyl-dihydro-carboxy-tamoxifen	$C_{25}H_{27}NO_3$	389.1991	389.1978	−3.22	*390/58*; 390/372	10.1	Detected	Detected	Detected
M2	Dihydro-carboxy-tamoxifen	$C_{26}H_{29}NO_3$	403.2147	403.2139	−2.06	404/45; *404/72*; 404/386	10.3	Detected	Detected	Detected
M3	*N*-demethyl-carboxy-tamoxifen	$C_{25}H_{25}NO_3$	387.1834	387.1835	−0.21	*388/58*; 388/370	14.1	Detected	Detected	Detected
M4	Dihydroxy-dihydro-toremifene	$C_{26}H_{30}ClNO_3$	439.1914	439.1913	0.38	440/45; *440/72*; 440/404	14.5	Detected	Detected	Detected
M5	Carboxy-tamoxifen	$C_{26}H_{27}NO_3$	401.1991	401.1994	0.22	402/45; *402/72*; 402/384	14.5	Detected	Detected	Detected
M6	Metabolite F	$C_{26}H_{29}NO_2$	387.2198	387.2192	−1.74	388/45, *388/72*	14.6	Detected	Detected	Detected
M7	*N*-demethyl-α-hydroxy-toremifene	$C_{25}H_{26}ClNO_2$	407.1652	407.1656	−0.99	*408/58*; 408/372; 408/390	17.3	Detected	Detected	Detected
M8	α-hydroxy-toremifene	$C_{26}H_{28}ClNO_2$	421.1809	421.1803	1.24	*422/72*; 422/386; 422/404	17.4	Detected	Detected	Detected
M9	Dihydroxy-toremifene	$C_{26}H_{28}ClNO_3$	437.1758	437.1760	−0.50	*438/72*; 438/61; 438/88; 438/402	17.8	Detected	Detected	Detected
M10	α,4-dihydroxy-toremifene	$C_{26}H_{28}ClNO_3$	437.1758	N.D	–	438/45; *438/72*; 438/402	17.9	Not detected	Detected	Not detected
M11	*N*-demethyl-4-hydroxy-toremifene	$C_{25}H_{26}ClNO_2$	407.1652	N.D	–	*408/58*; 408/372	18.0	Not detected	Detected	Not detected
M12	4-hydroxy-toremifene	$C_{26}H_{28}ClNO_2$	421.1809	421.1803	1.24	*422/72*; 422/386	18.1	Not detected	Detected	Detected
M13	4′-hydroxy-toremifene	$C_{26}H_{28}ClNO_2$	421.1809	421.1803	1.24	*422/72*; 422/386	18.3	Not detected	Detected	Detected
M14	3-hydroxy-4-methoxy-toremifene	$C_{27}H_{30}ClNO_3$	451.1914	451.1911	0.75	452/45, *452/72*; 452/416	18.3	Not detected	Detected	Detected
M15	*N*-demethyl-toremifene	$C_{25}H_{26}ClNO$	391.1703	N.D	–	*392/58*; 392/356	18.7	Detected	Detected	Detected
P	Toremifene	$C_{26}H_{28}ClNO$	405.1859	N.D	–	406/45; *406/72*; 406/370	18.8	Detected	Not detected	Not detected
ISTD	17α-methyltestosterone	–	–	–	–	*303/97*	19.3	–	–	–

Ions in italics were used for quantification

didemethylated metabolites described by previous studies were not detected [16–26].

Urinary profile and metabolic pathways

The toremifene metabolites urinary profiles were obtained analyzing the samples collected after oral administration of a single dose of the commercial drug Fareston® (60 mg of toremifene) using the SRM method described before (see Table 1 for the transitions selected). The results obtained, confirming the data of a previous study carried out in our laboratory [26], showed that toremifene was extensively metabolized in particular via hydroxylation in different positions, *N*-dealkylation and carboxylation in the chlorinated side chain (see Fig. 4 for the suggested main metabolic routes of toremifene). Most of the toremifene metabolites were excreted mainly as free and glucurono-conjugated, whereas only small amounts were excreted as sulphate (see Fig. 5 for the relative abundance of each toremifene metabolites in the three fractions). The 3-hydroxy-4-methoxy-toremifene was an exception; in fact, it was mainly detected in the sulphate fraction and not detected in the free fraction. In all three fractions, the most abundant compounds were the carboxylated metabolites (**M1**, **M2**, **M3** and **M5**) followed by the dihydroxylated (**M9**) and the mono-hydroxylated (**M7**, **M8**, **M12** and **M13**) metabolites (see Fig. 6a–c). Most of toremifene metabolites were detected for more than 10 days with a maximum of excretion generally after 9–22 and 35–47 h from drug(s) administration (see Fig. 7a–c for the urinary excretion profiles). The lack of certified reference materials for most of the toremifene metabolites did not allow an accurate determination of their urinary concentration; however, an estimation taking into account the response factor of toremifene allowed to estimate that all metabolites were present in a range of concentration comprised between 30 and 500 ng/mL.

The metabolites selected as target compounds to design a screening procedure to positively identify toremifene administration in the routine doping control analysis were the *N*-demethyl-dihydro-carboxy-tamoxifen (**M1**), the dihydro-carboxy-tamoxifen (**M2**), the *N*-demethyl-carboxy-tamoxifen (**M3**), the carboxy-tamoxifen (**M5**), the *N*-demethyl-α-hydroxy-toremifene (**M7**), the α-hydroxy-toremifene (**M8**), the dihydroxy-toremifene (**M9**), the 4-hydroxy-toremifene (**M12**) and the 4′-hydroxy-toremifene (**M13**), whereas for confirmation purpose, the most useful compound is the *N*-hydroxymethylated metabolite (**M9**) because its spectrum showed more than one product ion higher than 10% (see Fig. 2a, b).

Discussion and conclusions

In the present study, samples collected after toremifene administration were analyzed in order to examine toremifene metabolic reactions, main metabolites and liquid chromatography-tandem mass spectrometry behaviours for doping control purpose. From a pharmacological point of view, the metabolic reactions of major interest are those producing active metabolites, whereas in anti-doping field, the main interest of the metabolic studies is to check for the most representative metabolites and to know their analytical behaviours in order to include them in the routine screening procedures. Targeted analysis to relevant metabolites of compounds included in the WADA list of prohibited substances and methods exhibits a challenge in the anti-

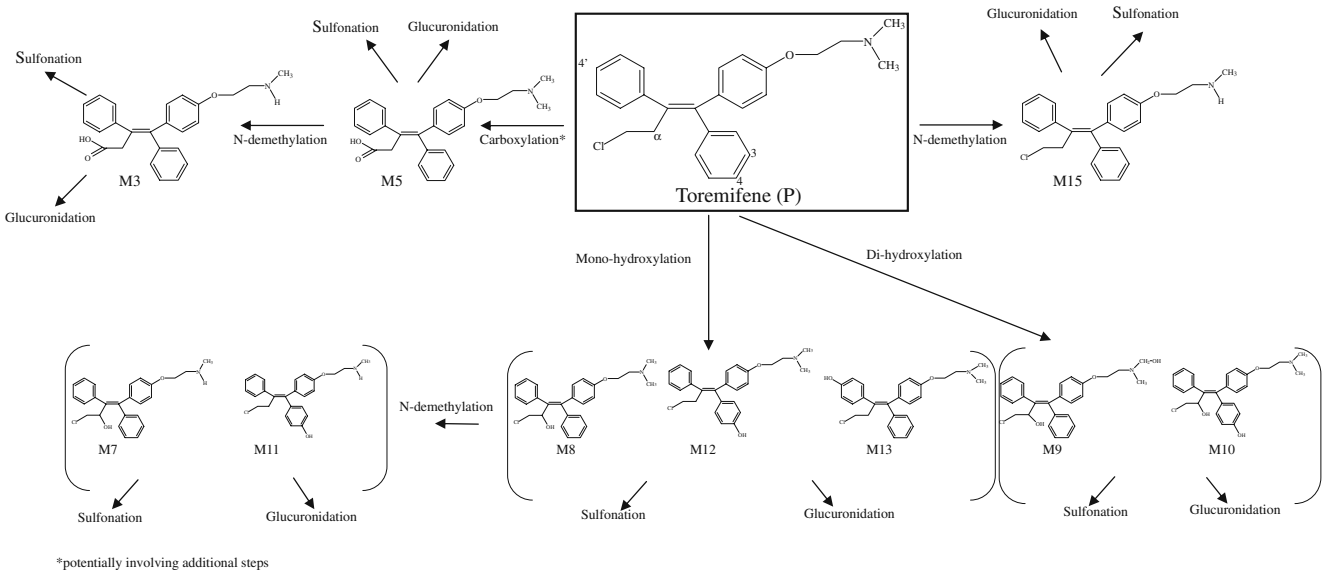


Fig. 4 Suggested main metabolic routes of toremifene

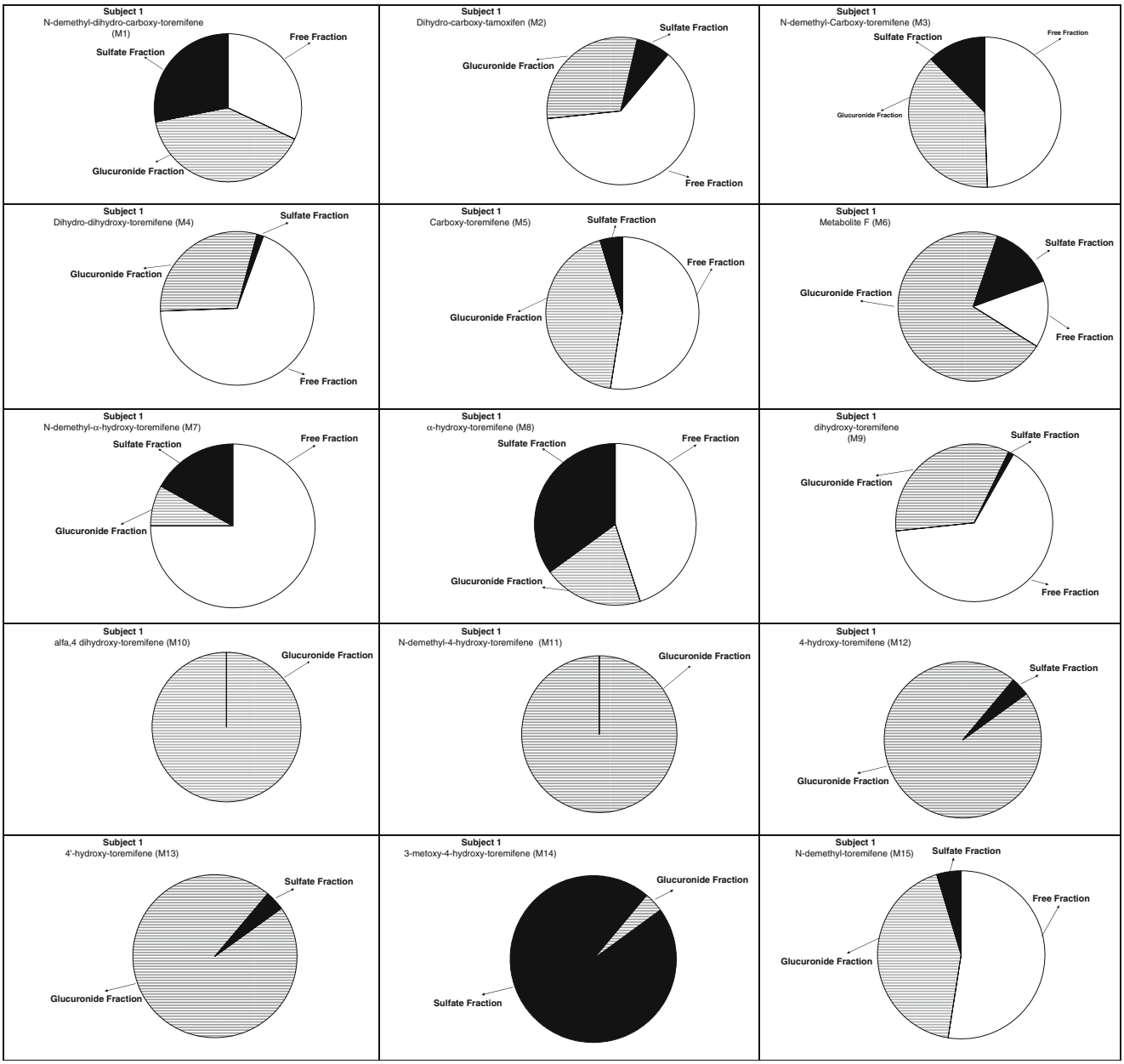


Fig. 5 Relative abundance of each toremifene metabolites in the three fractions (free, glucuronide and sulphate) of the excretion study obtained from subject 1

doping field. Many factors can alter hepatic and intestinal drug metabolism, including the presence or absence of disease and/or concomitant medications; for this reason, the most representative compound has to be not only the long-term and most abundant metabolite but also the compound that is influenced in a little amount by ethnicity, pathologies, age and sex. In this regard, the most significant differences between the results obtained by other investigators and those obtained by our group are (1) the detection of metabolites without the characteristic chlorine isotope pattern. These metabolites received little attention in the past maybe because they are not active and thus not interesting for clinical purpose and showed a great potential as target analytes for doping control purpose. This is because they are present in all three excretion studies in a very high amount and for a long period of time. In addition to this, their relative abundance between the different subjects studied was very stable; on the contrary, the relative abundance of the other metabolites was highly variable; (2) the presence in very low amount of metabolites, such as the toremifene *N*-demethylated metabolite, described by other researchers as the most abundant metabolite [16–24], and (3) the absence of the *N*-oxide, of the hydroxy-quinine and of the *N*,*N*-didemethylated metab-

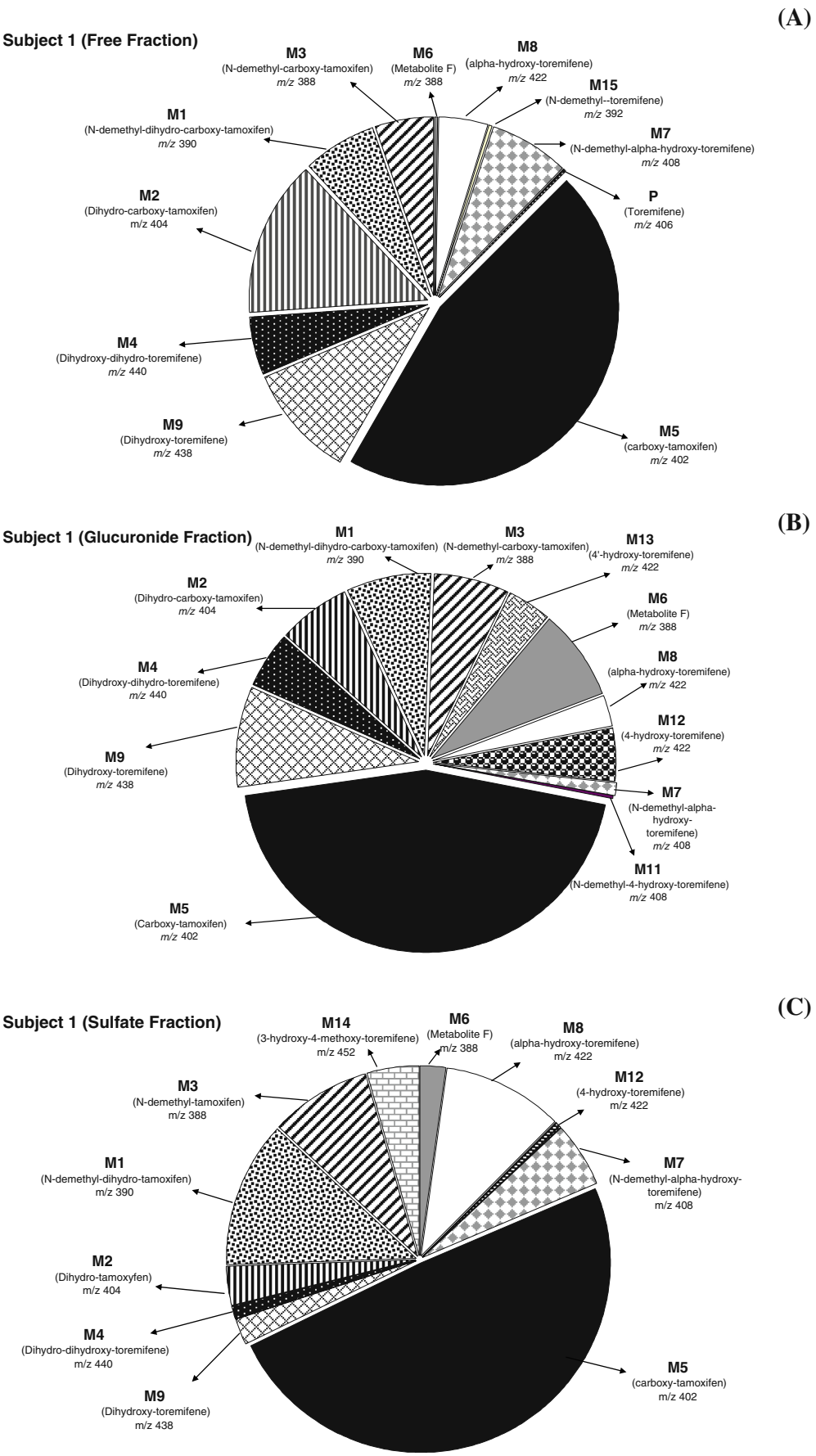


Fig. 6 **a** Relative abundance of the detected compounds in the free fraction of the excretion study obtained from subject 1. **b** Relative abundance of the detected compounds in the glucuronide fraction of the excretion study obtained from subject 1. **c** Relative abundance of the detected compounds in the sulphate fraction of the excretion study obtained from subject 1

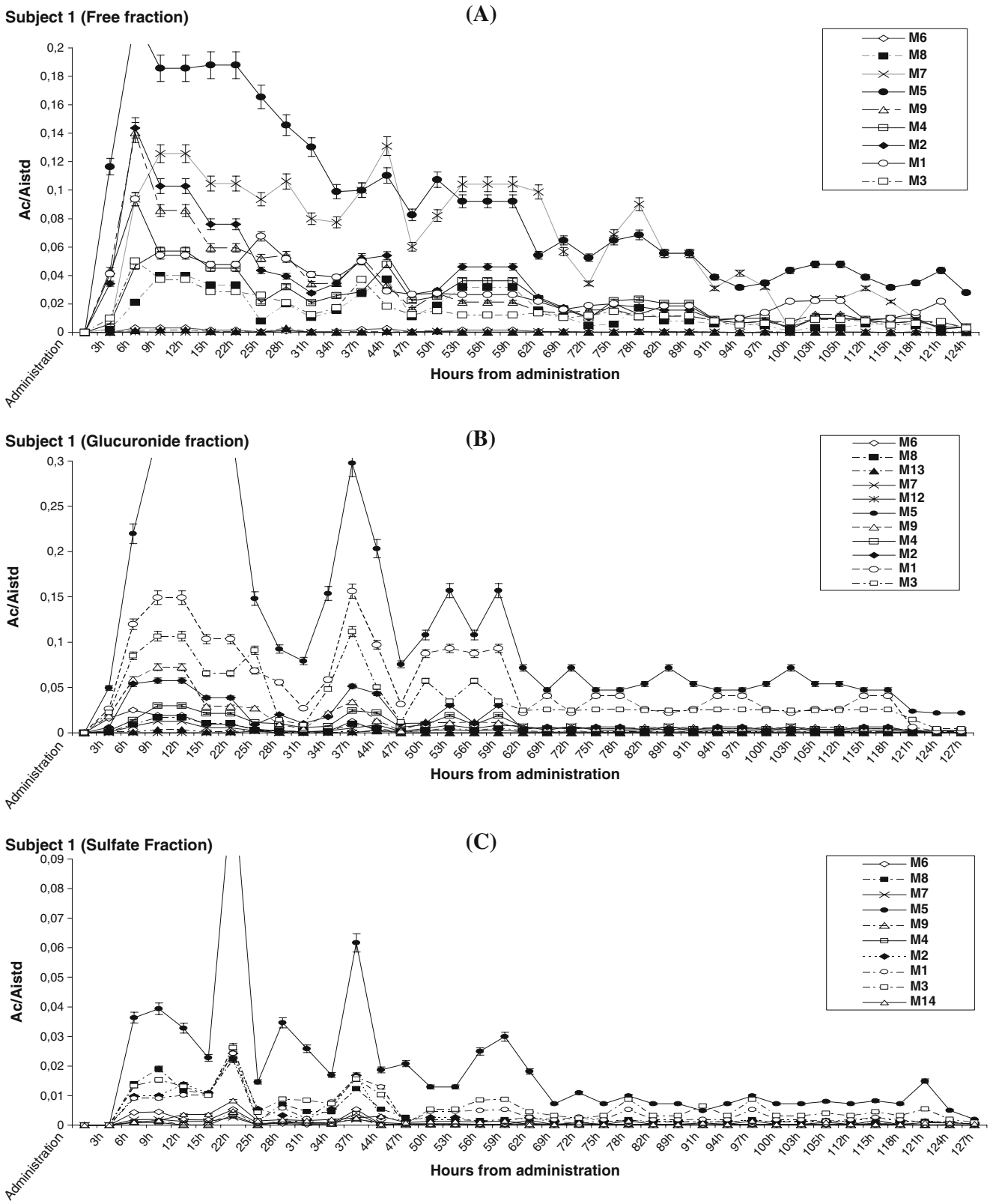


Fig. 7 Urinary profile of the most abundant toremifene metabolites in subject 1: (**a**) free fraction, (**b**) glucuronide fraction and (**c**) sulphate fraction

olites [24, 25]. Other considerations that arise from the data presented here that are relevant to the possibility of detecting toremifene administration in anti-doping test are reported: (a) the most abundant and long-term toremifene metabolites were the carboxylated (**M1**, **M2**, **M3** and **M5**), the *N*-demethylated-mono-hydroxylated (**M7**), the mono-hydroxylated (**M8**, **M12** and **M13**) and the dihydroxylated (**M4** and **M9**) metabolites. These compounds were mainly excreted in the free and glucuronide fractions; thus, the analytical procedure currently used by our laboratory for the analysis of other anti-estrogenic agents [28] could be easily applied also for the detection of toremifene administration; (b) most of the toremifene metabolites found in this study showed the highest recovery using ethylacetate as extraction solvent. Nevertheless, as already described for tamoxifen [27], using *tert*-butylmethyl ether as cleaner extract was obtained with a recovery higher than 60%; (c) the carboxylated metabolites, as described previously [26, 27], are also tamoxifen metabolites; thus, these compounds could be used as markers for both toremifene and tamoxifen intake. Nevertheless, the chemical structures proposed for these metabolites have to be confirmed by synthesis because contrary to the normal behaviour for acidic compounds, no signal in negative ionization mode was observed; (d) the estimated limits of detection for all toremifene metabolites were in the range of 30–50 ng/mL, thus satisfying the minimum required performance levels (MRPL) set by the WADA for the WADA anti-doping accredited laboratories, that for anti-estrogenic drugs correspond to 50 ng/mL [29].

Additional experiments are currently in progress to synthesize the toremifene metabolites in order to confirm the structures proposed in this paper.

In vitro studies will also be performed to better examine the metabolic pathways leading to the formation of the toremifene metabolites with special emphasis to the carboxylated metabolites. These studies could be useful also to explain the presence of common metabolites in the excretion studies of tamoxifen and toremifene.

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