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Mass spectrometric characterization of tamoxifene metabolites in human urine utilizing different scan parameters on liquid chromatography/tandem mass spectrometry

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Different liquid chromatographic/tandem mass spectrometric (LC/MS/MS) scanning techniques were considered for the characterization of tamoxifene metabolites in human urine for anti-doping purpose. Five different LC/MS/MS scanning methods based on precursor ion scan (precursor ion scan of *m/z* 166, 152 and 129) and neutral loss scan (neutral loss of 72 Da and 58 Da) in positive ion mode were assessed to recognize common ions or common losses of tamoxifene metabolites. The applicability of these methods was checked first by infusion and then by the injection of solution of a mixture of reference standards of four tamoxifene metabolites available in our laboratory. The data obtained by the analyses of the mixture of the reference standards showed that the five methods used exhibited satisfactory results for all tamoxifene metabolites considered at a concentration level of 100 ng/mL, whereas the analysis of blank urine samples spiked with the same tamoxifene metabolites at the same concentration showed that the neutral loss scan of 58 Da lacked sufficient specificity and sensitivity. The limit of detection in urine of the compounds studied was in the concentration range 10–100 ng/mL, depending on the compound structure and on the selected product ion. The suitability of these approaches was checked by the analysis of urine samples collected after the administration of a single dose of 20 mg of tamoxifene. Six metabolites were detected: 4-hydroxytamoxifene, 3,4-dihydroxytamoxifene, 3-hydroxy-4-methoxytamoxifene, *N*-demethyl-4-hydroxytamoxifene, tamoxifene-*N*-oxide and *N*-demethyl-3-hydroxy-4-methoxytamoxifene, which is in conformity to our previous work using a time-of-flight (TOF) mass spectrometer in full scan acquisition mode.

For many years, all compounds that interact directly with intracellular oestrogen receptors were classified simply as oestrogen agonists or antagonists. More recently, the pharmacological properties of a new class of compounds, referred to as selective oestrogen receptor modulators (SERMs),[1–5] were investigated. Compounds currently approved for clinical use that display SERM-like activity fall into two main chemical families: the triphenylethylenes and the benzothiophenes.[1–5] Tamoxifene, together with clomiphene and toremifene, belongs to the class of triphenylethylenes (Fig. 1).

SERMs are used clinically 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.[1–5]

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The use of SERMs has been banned in sports by the World Anti Doping Agency (WADA) since 2005 as these are included in the S4 class 'agents with anti-oestrogenic activity'.[6] Athletes could use selective oestrogen receptor modulators to increase endogenous testosterone, with the aim of by-passing 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.[7,8]

The metabolism of the SERMs is complex and our knowledge of it is still incomplete. SERMs are generally metabolized by CYP3A4 and CYP2D6 in the liver to *N*-demethyl and hydroxyl compounds and eliminated mainly in the faeces, with 10% excreted as free or glucuronate metabolites in the urine.[9–16] However, some unchanged drug and metabolites are excreted in the urine. Thus, in order to positively identify SERMs a procedure based on the detection of their most representative metabolites is essential.

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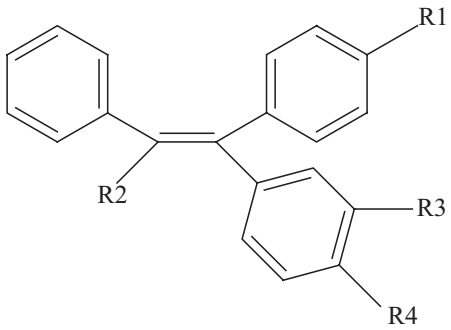

Compound	R1	R2	R3	R4
Tamoxifene	$OCH_2CH_2N(CH_3)_2$	CH_2CH_3	H	H
Toremifene	$OCH_2CH_2N(CH_3)_2$	CH_2CH_2Cl	H	H
Clomiphene	$OCH_2CH_2N(CH_2CH_3)_2$	Cl	H	H
Droloxiphene	$OCH_2CH_2N(CH_3)_2$	CH_2CH_3	H	OH
Idoxifene	$OCH_2N(CH_2)_4$	CH_2CH_3	I	H
Ospemiphene	OCH_2CH_2OH	CH_2CH_2Cl	H	H

Figure 1. General molecular structure of the triphenylethylene SERMs.

The goal of this study was to compare different approaches based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the comprehensive detection and structural elucidation of tamoxifene metabolites, and to design a screening procedure to positively identify administration of SERMs. The applicability of these approaches for the detection of tamoxifene metabolites was tested by the analysis of spiked urines and positive urine samples collected after the administration of a single dose of 20 mg of tamoxifene.

EXPERIMENTAL

Standards, chemicals and reagents

Purified standards of 3-hydroxy-4-methoxytamoxifene were supplied by NMI (National Measurement Institute, Pymble, Australia); 4-hydroxytamoxifene and *N*-demethyl-4-hydroxytamoxifene were purchased from Toronto Research Chemicals Inc. (Toronto, Canada); 17α-methyltestostrone (used as internal standard) was supplied by Sigma-Aldrich (Milano, Italy). Tamoxifene (Nolvadex®) was purchased from Novartis Farma S.p.A. (Origgio Varese, Italy). 3,4-Dihydroxytamoxifene was synthesized in-house following the procedure described by Zhang *et al.*[17]

All chemicals (potassium carbonate, sodium phosphate, formic acid, *tert*-butyl methyl ether, methanol, acetonitrile, ethyl acetate and sodium hydroxide) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). The enzyme β-glucuronidase (from *E. coli*), used for the enzymatic hydrolysis of glucuronide conjugates, was purchased from Roche (Monza, Italy). The ultrapure water used was of Milli-Q-grade (Waters, Milano, Italy).

Administration study

An excretion study was performed on a male subject (age: 42 years; weight: 78 kg) and on a female subject (age: 38, weight 45 kg). Written consent was obtained from both subjects allowing the use of urine samples for research purposes. Urine samples were collected before drug administration and for ten days after treatment with a single tablet of 20 mg of tamoxifene (Nolvadex®).

The urine samples, collected in sterile containers, were stabilized with sodium azide (1 mg/mL) and stored at $-20^{\circ}C$.[18,19]

LC/MS/MS conditions

All LC/MS/MS experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump with a binary gradient system and an 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; Agilent Technologies). 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 rapid gradient program started at 15% B, increased to 60% B in 7 min, and then after 5 min to 100% B. The column was flushed for 1 min at 100% B and finally re-equilibrated at 10% B for 4 min. The flow rate was set at 300 μL/min.[20]

The slow gradient program started at 10% B, increased 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.[20]

Mass spectrometry was performed using an API4000 triple quadrupole instrument (Applied Biosystems Italia, Monza, Italy) with positive electrospray ionization. The ion source was operated at 550°C, the applied capillary voltage was 5500 V and precursor ion scan and neutral loss experiments were performed employing collision-induced dissociation (CID) using nitrogen as the collision gas at a pressure of 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

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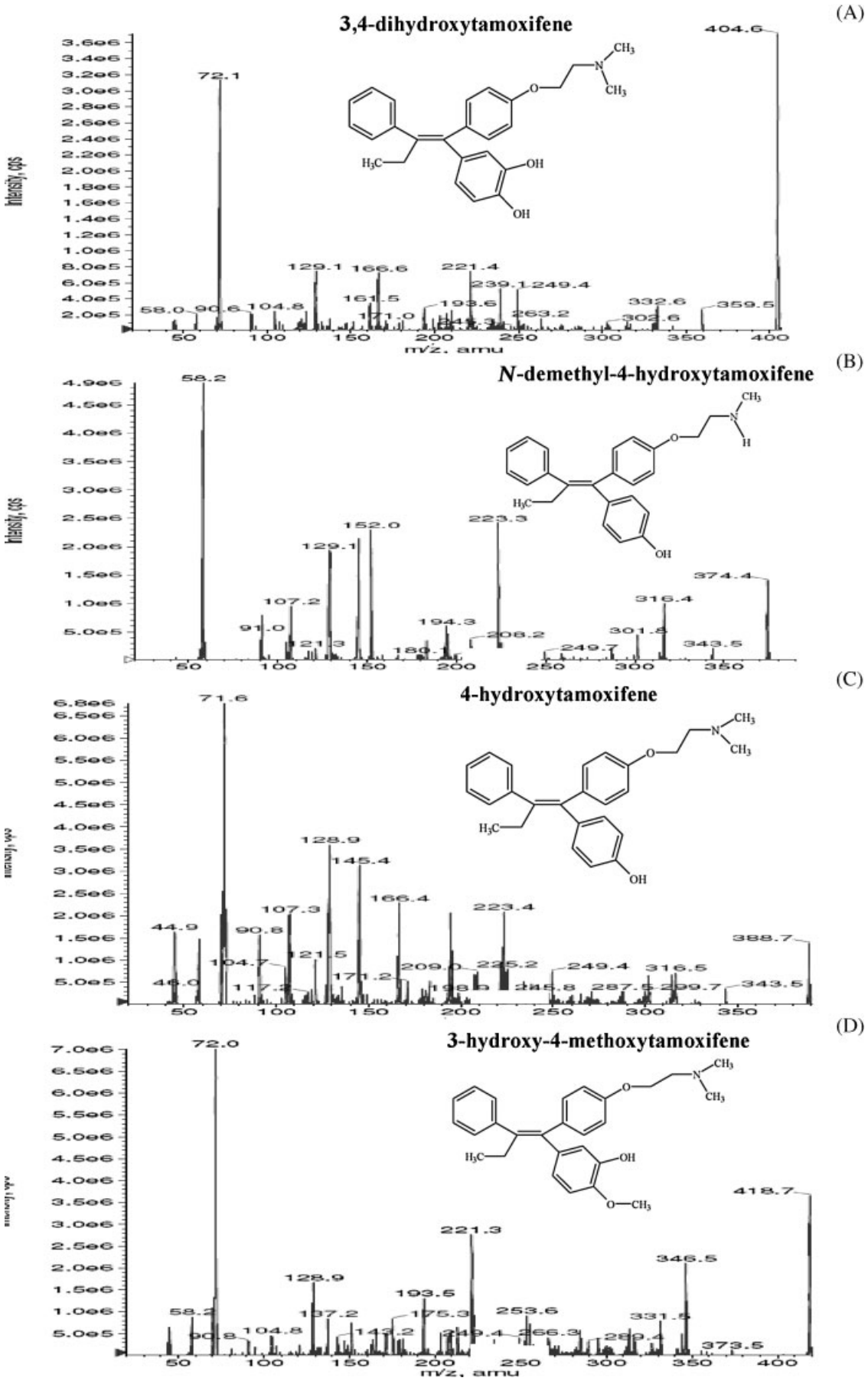

Figure 2. Product ion scan in positive polarity and chemical structures of 3,4-dihydroxytamoxifene (A), *N*-demethyl-4-hydroxytamoxifene (B), 4-hydroxytamoxifene (C), and 3-hydroxy-4-methoxytamoxifene (D).

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mass parameters values used were optimized by the direct infusion of four tamoxifene metabolite standards available in our laboratory.

Sample preparation

To 3 mL of urine 1.5 mL of phosphate buffer (1 M, pH 7.4), 50 μL of β-glucuronidase from *E. coli* and 50 μL of the internal standard (ISTD: 17α-methyltestosterone: 12 μg/mL) were added and incubated for 1 h at 55°C. After hydrolysis 1 mL of either a phosphate buffer (1 M, pH 7.4), a carbonate-bicarbonate buffer (0.8 M, pH 9) or a formate buffer (2.7 M, pH 3.8) was added to check the optimal extraction pH. The extraction was then carried out with 10 mL of ethyl acetate or *tert*-butyl methyl 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 onto the LC/MS/MS system.

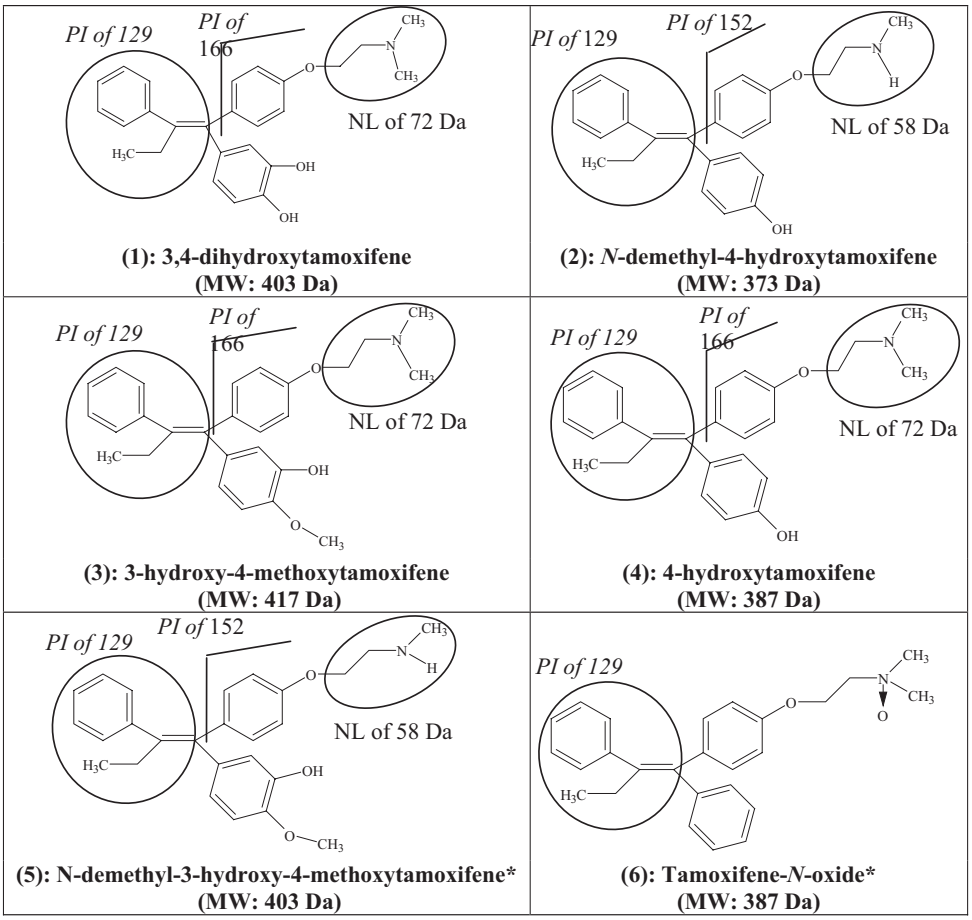
Validation parameters

Experiments were performed using human urine to determine the limit of detection (LOD), specificity, recovery, and repeatability of recovery and retention time required for the validation of a qualitative screening procedure. Each of the target analytes was added to 20 different blank urine samples (10 males, 10 females) at a concentration of 200 ng/mL. Serial 1:2 dilutions were made and the LOD was reported as the lowest concentration at which a compound could be identified in all twenty urines tested, with the diagnostic product ion observed with a signal-to-noise (S/N) ratio greater than 3.

To determine the specificity, the same 20 urines were extracted and analyzed without spiking.

The ion suppression due to the matrix components was calculated by comparing the responses between the spiked extracts and a standard at the same concentration prepared in the mobile phase. As for the extraction recovery, the ion suppression in real urine samples was calculated by means of internal standards. Ten different urines were extracted and the extracts were then spiked with the internal standards. The ion suppression was calculated by comparison between the areas obtained for each extract and those for the corresponding standard. The standard deviation of the ion suppressions was calculated in order to evaluate the variation between different urine matrices.

The recovery of all the tested compounds from urine by liquid/liquid extraction was determined at a concentration of 100 ng/mL. Ten different blank urine samples were fortified with all the compounds, and another ten blank urine



* proposed structures of tamoxifene metabolites for which certified reference standards are not commercially available

Figure 3. Common structures and common losses selected for the precursor ion scan and neutral loss methods used for the detection of tamoxifene metabolites.

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specimens were extracted according to the described protocol, followed by the addition of all the compounds into the organic layer before the evaporation. To both sets of samples, 50 μL of ISTD were added into the organic layer before the evaporation. The recovery was then calculated by comparison of mean peak area ratios of the analyte and the ISTD of samples fortified prior to and after liquid/liquid extraction (see Table 2).

The retention time repeatability was evaluated from the intra-assay variations of the relative retention times (RRTs) of the analytes at a concentration of 100 ng/mL.

RESULTS

Several common ions and neutral losses were observed by the study of the product ion spectra, in positive polarity, of the three tamoxifene metabolites commercially available as certified standards (4-hydroxytamoxifene, 3-hydroxy-4-methoxytamoxifene and *N*-demethyl-4-hydroxytamoxifene) and of a tamoxifene metabolite (3,4-dihydroxytamoxifene) synthesized in-house following a procedure already described[17] (see Figs. 2(A)–2(D) for the product ion spectra and Fig. 3 for the common product ions and neutral losses).

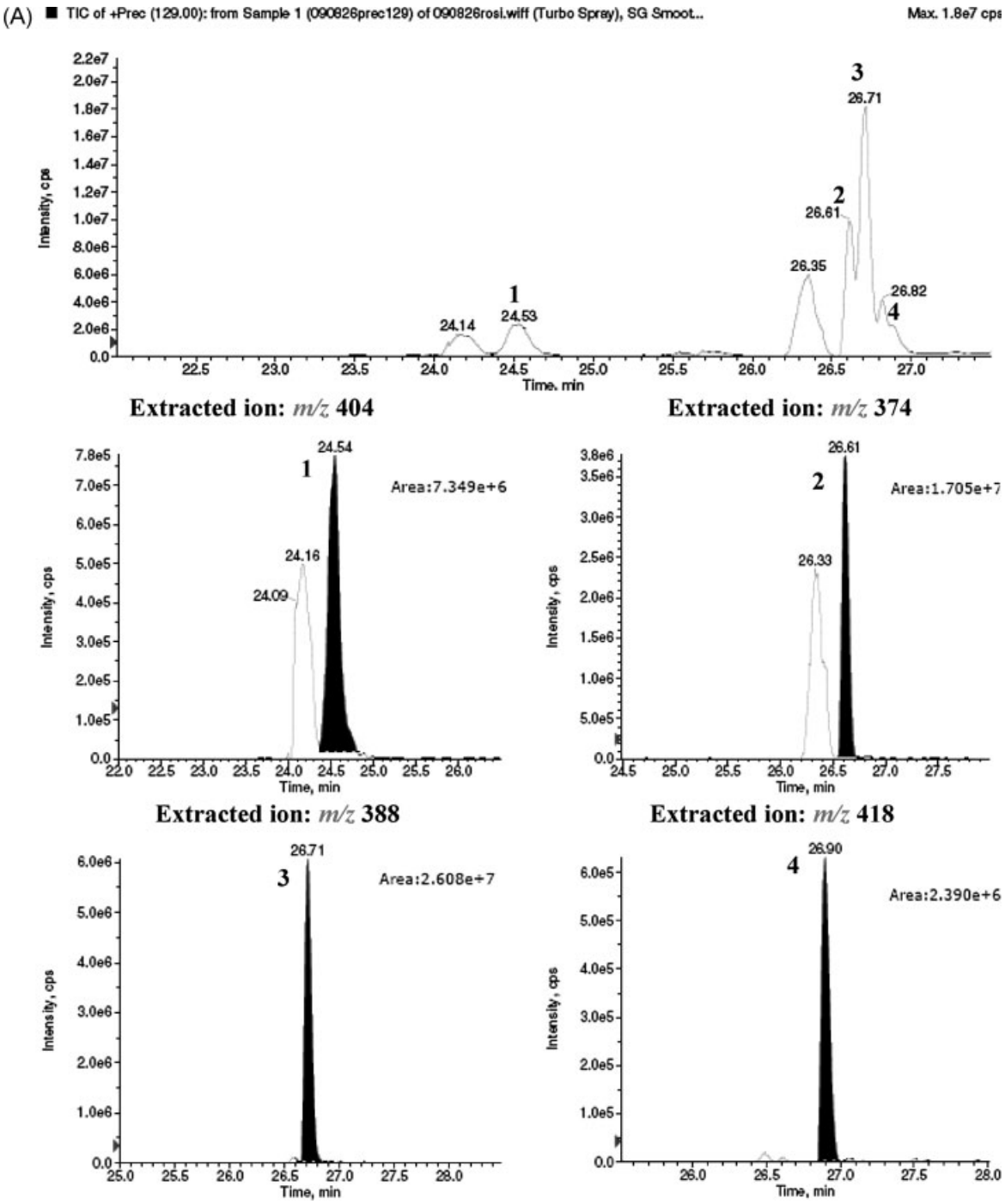

Figure 4. Extracted chromatograms of the precursor ion scan of *m/z* 129 (A), 166 (B) and 152 (C1) and the neutral loss of 58 Da (C2) and 72 Da (D) analyses of a standard mixture of the four tamoxifene metabolites standards available in our laboratory. Peak identification: 1. 3,4-dihydroxytamoxifene, 2. *N*-demethyl-4-hydroxytamoxifene, 3. 4-hydroxytamoxifene and 4. 3-hydroxy-4-methoxytamoxifene.

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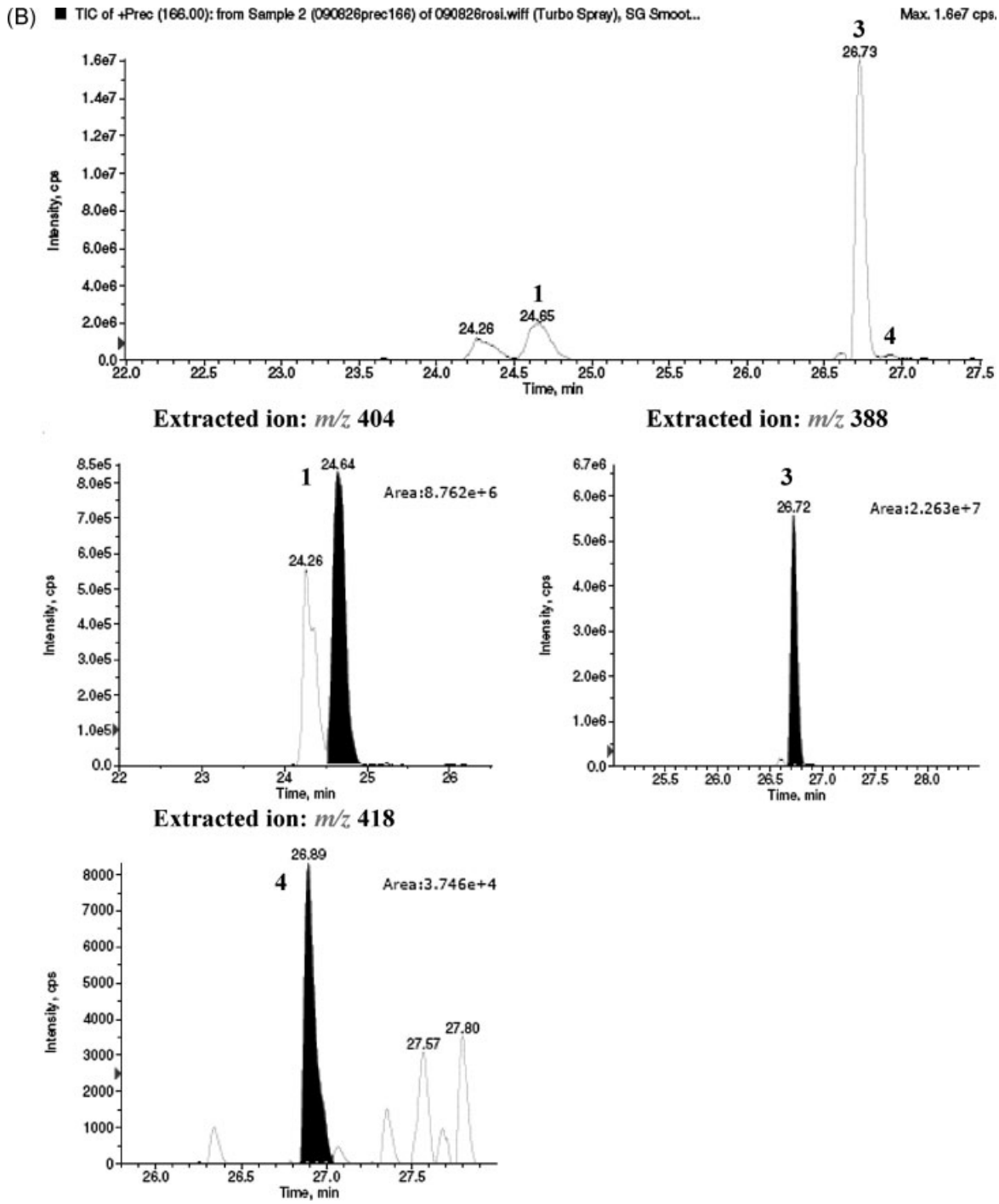

Figure 4. (Continued).

However, insufficient structural information was obtained from the product ion spectra in negative ion mode (data not shown).

Five different LC/MS/MS scan methods, based on precursor ion scans of *m*/*z* 166, 152 and 129 and neutral loss scans of 72 Da and 58 Da in positive ion mode, were selected for the detection of tamoxifene metabolites. The precursor ion scan of *m*/*z* 129 was specific for tamoxifene metabolites whereas other methods (precursor ion scan of *m*/*z* 166 and 152, and neutral losses of 72 Da and 58 Da) could be applied to detect other structurally related triphenylethylene SERMs, such as for example toremifene, a chlorinated derivative of tamoxifene (data not shown). In addition to the above, as shown by Figs. 4(A)–4(C) the precursor ion scan of *m*/*z* 152 and the neutral loss of 58 Da could be considered specific for the detection of *N*-demethylated metabolites, whereas the precursor ion scan of *m*/*z* 166 was specific for non-*N*-demethylated metabolites. Thus, a combination of these methods was proposed as the best strategy to detect tamoxifene metabolites and to obtain metabolite structural elucidation.

The suitability of these approaches was also checked by the analysis of urine samples collected after the administration of a single dose of 20 mg of tamoxifene. Figures 5(A) and 5(B) show the extracted chromatograms obtained by the analysis of the excretion study sample collected after 10 h from the

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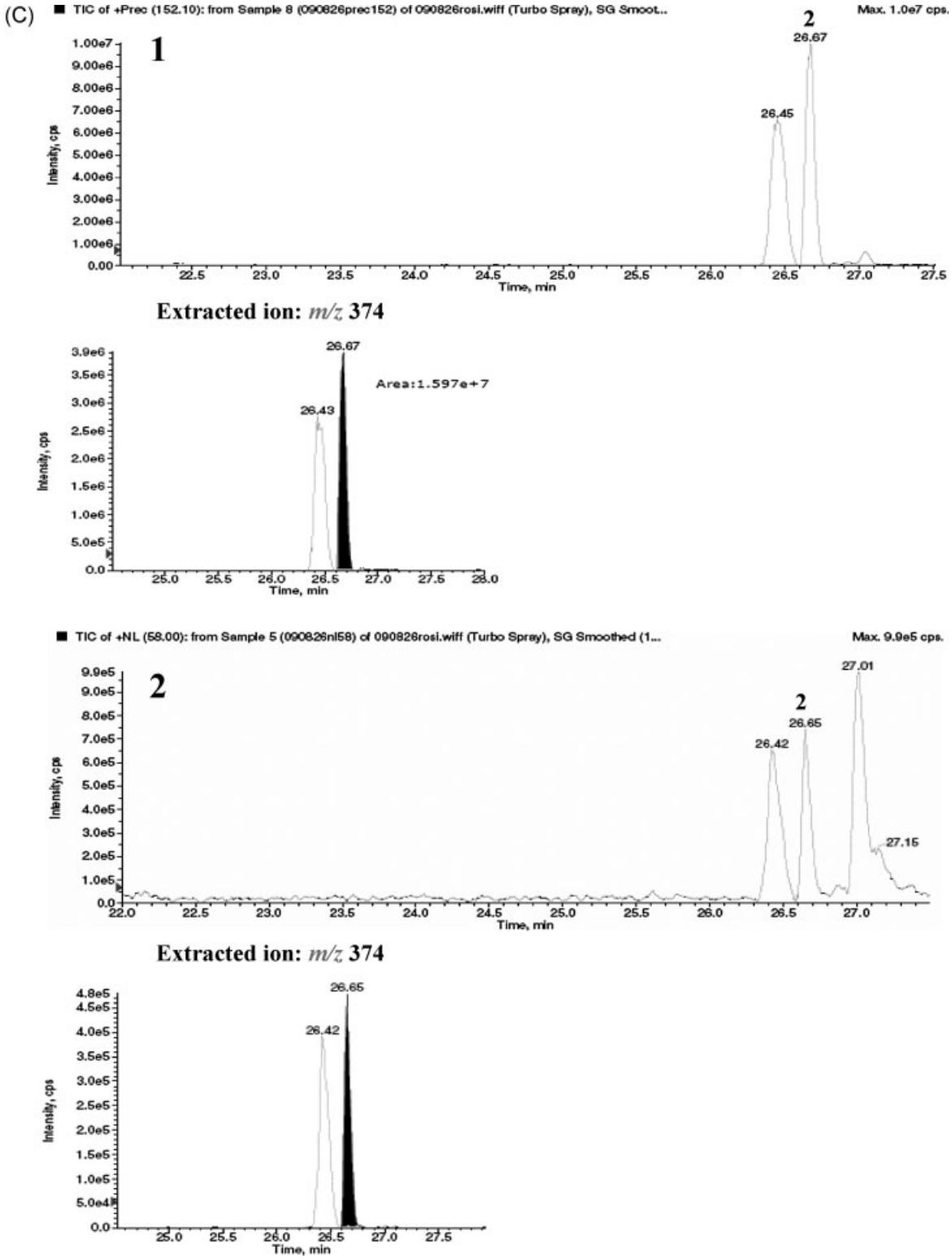

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tamoxifene administration using the methods described before. Using the precursor ion scan methods six metabolites were detected: 4-hydroxytamoxifene, 3,4-dihydroxytamoxifene, 3-hydroxy-4-methoxytamoxifene, *N*-demethyl-4-hydroxytamoxifene, tamoxifene-*N*-oxide and *N*-demethyl-3-hydroxy-4-methoxytamoxifene (see Fig. 3 for the structures of the proposed tamoxifene metabolites), which is in agreement with our previous work using time-of-flight (TOF) mass spectrometry in full scan acquisition mode.[21] Using the neutral loss methods, however, only two tamoxifene metabolites were detected (see Table 1).

Parallel to the above, in order to optimize the resolution in distinguishing the known peak from other background peaks, different chromatographic gradients (run time 15 and 28 min) and columns (particle size: 5, 3 and 1.8 μm) were tested. Using a 28 min gradient, baseline

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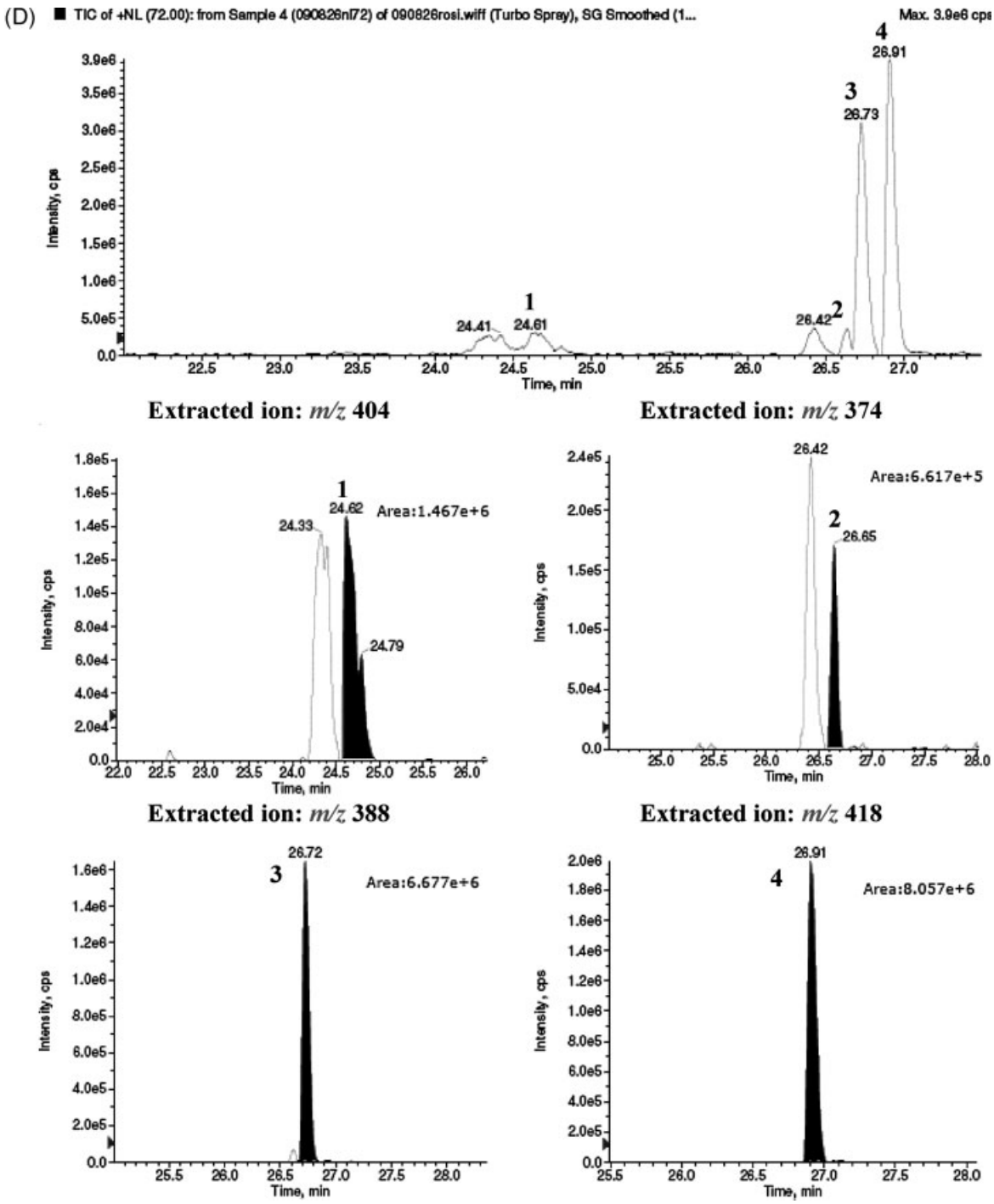

Figure 4. (Continued).

separation was obtained for all tamoxifene metabolites available in our laboratory. Moreover, no co-eluting matrix components were observed. Interferences by the urinary matrix were studied by the analysis of 20 different blank urine samples. When neutral loss and precursor ion scanning methods were used for the analysis of urine samples, the background increased with a consequent decrease in the S/N ratio, leading to LODs 2–5 times higher (depending on the compound structure and on the selected product ion) than those obtained in reference standards. The LODs in urine were found to be between 10 and 100 ng/mL for all compounds considered (see Table 2). The ion suppression was less than 15% for all scan methods tested and for all compounds considered in the study. No carryover signal was detected in the blank urine samples that were injected in sequence after the analysis of the fortified urine samples at the highest concentration (200 ng/mL). The repeatability of extraction recovery (coefficient of variance (CV%) <10 for all tamoxifene metabolites tested) and the repeatability of relative retention times (CV% <1 for all tamoxifene metabolites tested), obtained by analyzing 20 different urine samples spiked with the tamoxifene metabolites available in our laboratory, were satisfactory for all compounds.

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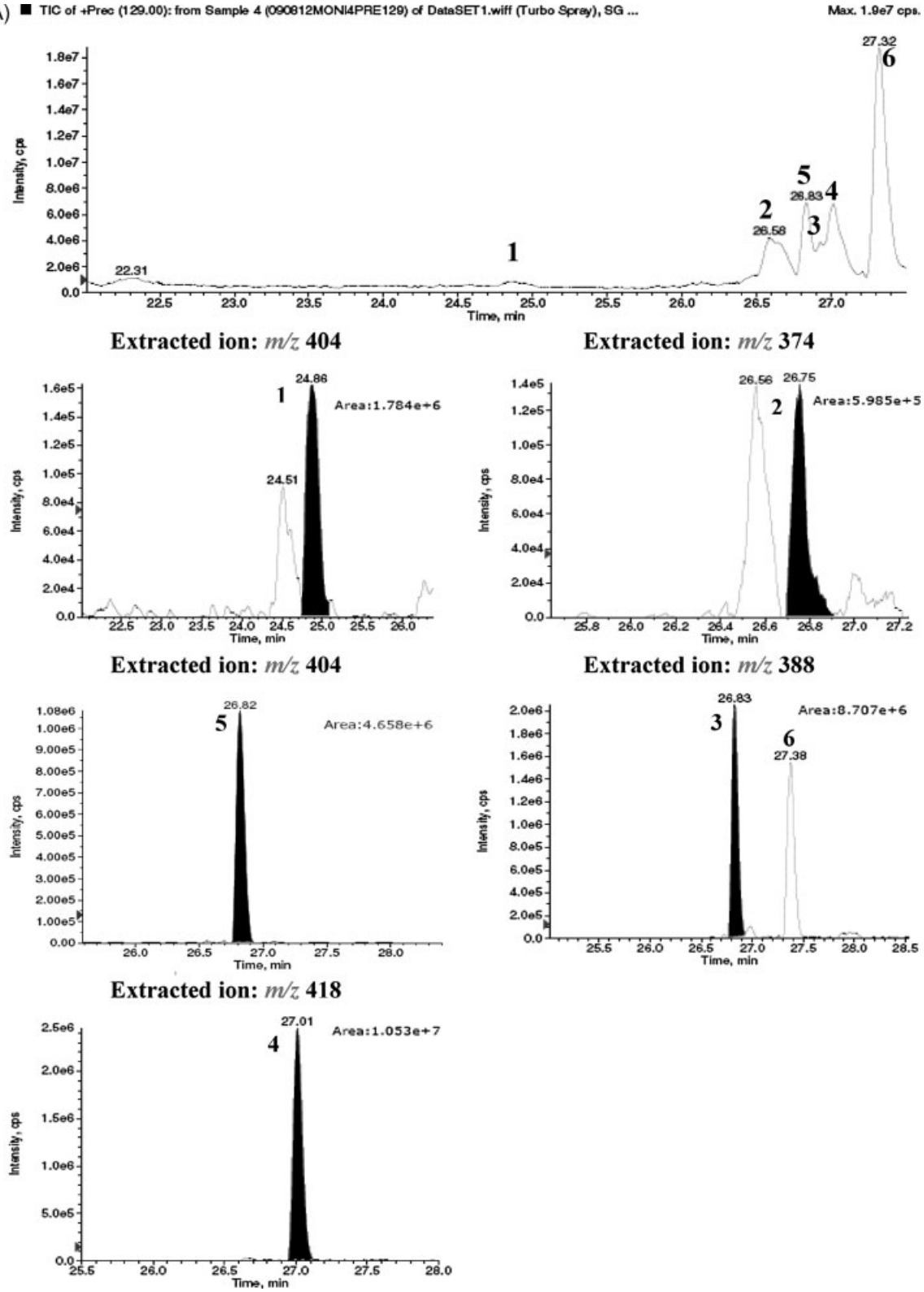

Figure 5. Extracted chromatograms of the precursor ion scan of *m/z* 129 (A) and 166 (B). Peak identification: 1. 3,4-dihydroxytamoxifene, 2. *N*-demethyl-4-hydroxytamoxifene, 3. 4-hydroxytamoxifene, 4. 3-hydroxy-4-methoxytamoxifene, 5. *N*-demethyl-3-hydroxy-4-methoxytamoxifene, and 6. tamoxifene-*N*-oxide.

DISCUSSION AND CONCLUSIONS

Since the introduction of the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques, LC/MS/MS has become a widely used method for the analysis of drug metabolites, especially for thermal unstable compounds which are difficult to study by gas chromatography. In particular, most metabolite analyses are carried out using triple quadrupole mass spectrometers. The main advantage of triple quadrupole mass spectrometers is

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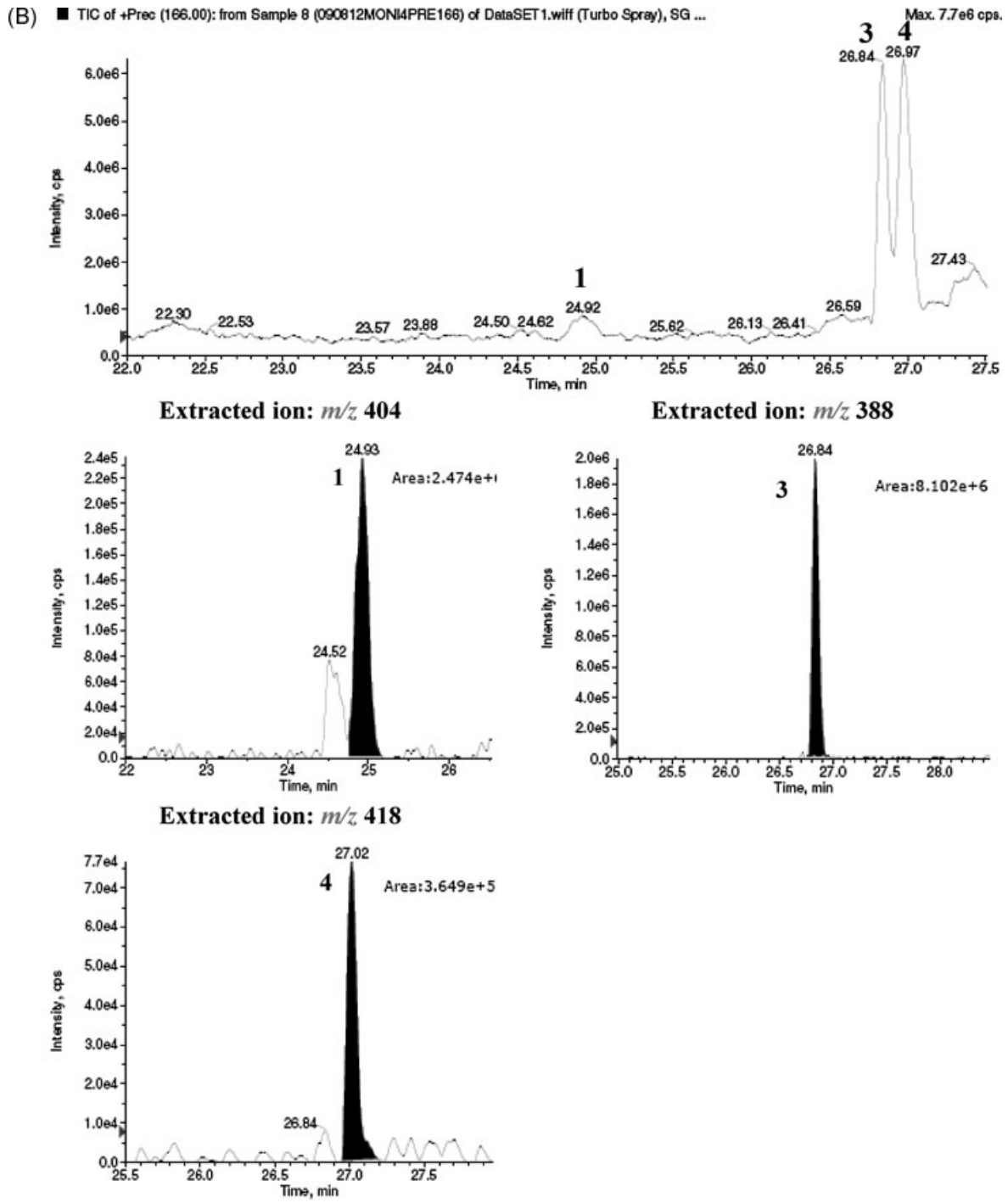

Figure 5. (Continued).

that families of metabolites can easily be identified using neutral loss and precursor ion scans.[22–24] A limitation of the use of neutral loss scans and precursor ion scans is that any change in the structure which results in a change in the mass of the neutral loss or the *m/z* value of the product ion being monitored may result in major metabolites not being detected. In this study we have investigated the capabilities of different LC/MS/MS scan modes for the detection of tamoxifene metabolites in human urine, especially to characterize their metabolites, sometimes described only *in vitro*, for which there is little information of human *in vivo* metabolism. More specifically, some considerations arise from the data presented here that are relevant both to tamoxifene metabolism and to the possibility of detecting administration of SERMs in anti-doping tests: (1) the precursor ion scan of *m/z* 129 was found to be indicative of the tamoxifene structure without any restriction, whereas the other methods considered in this study (precursor ion scan of *m/z* 166, 152; neutral loss of 72 Da and 58 Da) could in principle also be applied to detect other structurally related triphenylethylene SERMs, such as for example toremifene, a chlorinated derivative of tamoxifene; (2) the complementary use of a different precursor ion scanning method seems to be the best option for the detection of the greatest number of metabolites that are unknown and/or not commercially available. The male and female excretion studies both

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Table 1. Summary of LC/MS/MS results for tamoxifene metabolites

		Precursor ion scan (standards mixture/excretion study) (*m*/*z*)			Neutral loss (standards mixture/excretion study) (Da)	
	Compound	129	166	152	58	72
1	3,4-dihydroxytamoxifene	detected/detected	detected/detected	—	—	detected/not detected
2	*N*-demethyl-4-hydroxytamoxifene	detected/detected	—	detected/detected	detected/not detected	detected/not detected-
3	4-hydroxytamoxifene	detected/detected	detected/detected	—	—	detected/detected
4	3-hydroxy-4-methoxytamoxifene	detected/detected	detected/detected	—	—	detected/detected
5	N-demethyl-3-hydroxy-4-methoxytamoxifene	—/detected	—	—/detected	—/not detected	—
6	tamoxifene-*N*-oxide	—/detected	—	—	—	—

Table 2. Extraction recovery and limits of detection (LODs)

Compound	Recovery pH: 7/9/5 (ethyl acetate) (%)	Recovery pH: 7/9/5 (*tert*-butyl methyl ether) (%)	LOD (PI of 129.1) (ng/mL)	LOD (PI of 166.4) (ng/mL)	LOD (PI of 152) (ng/mL)	LOD (NL of 72 Da) (ng/mL)	LOD (NL of 58 Da) (ng/mL)
3,4-dihydroxytamoxifene	97/79/69	82/69/59	10	10	—	50	—
N-demethyl-4-hydroxytamoxifene	93/85/66	86/75/56	20	—	20	70	100
4-hydroxytamoxifene	94/82/69	83/77/67	10	10	—	70	—
3-hydroxy-4-methoxytamoxifene	98/81/65	78/72/55	20	70	—	50	—

PI = precursor ion scan of

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showed that: (i) using the precursor ion scan of *m/z* 129, six tamoxifene metabolites can be detected; (ii) using the precursor ion scan of *m/z* 166, the non-*N*-demethylated tamoxifene metabolites were detected; (iii) using the neutral loss of 72 Da, three tamoxifene metabolites were detected; and (iv) using the precursor ion scan of *m/z* 152 and the neutral loss of 58 Da, *N*-demethylated tamoxifene metabolites were detected; (3) different liquid/liquid extraction conditions were tested (pH 7, 9, 5 using either ethyl acetate or *tert*-butyl methyl ether). The highest recoveries, for all compounds studied, were obtained using ethyl acetate at pH 7 (Table 2). Nevertheless, when using ethyl acetate the interferences were higher than when using *tert*-butyl methyl ether (data not shown). Thus an extraction at pH 7 with *tert*-butyl methyl ether, a gradient of 28 min and a column with particle size of 1.8 μm were chosen for two main reasons: (i) co-eluting matrix components may decrease the signal several-fold by ion suppression, and (ii) the metabolism of a drug may lead to the formation of several isobaric compounds that should be separated. In these conditions, no significant interferences were found at the expected retention time of the metabolites identified, thus excluding incorrect mass assignment for a co-eluting peak; (4) the LODs of the selected precursor ion scan methods were in the range of 10–70 ng/mL, whereas with the neutral loss scans the LODs were in the range of 50–100 ng/mL, thus limiting its applicability. In conclusion, the use of a combination of different precursor ion scanning methods could be an interesting strategy for metabolism studies, especially when the metabolites to be monitored are not commercially available and/or are unknown.

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