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Analysis of tamoxifen and its metabolites in synthetic gastric fluid digests and urine samples using high-performance liquid chromatography with electrospray mass spectrometry

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Abstract

We report on the transformation of tamoxifen at 37°C in synthetic gastric fluid as studied by high-performance liquid chromatography with triple quadrupole mass spectrometry. The major transformation products detected were (*E*)-isomer of tamoxifen, metabolite D, and several unidentified components having *m*/*z* 404. Addition of pepsin to the gastric fluid inhibited formation of all of these products. We analyzed several urine samples from breast cancer patients undergoing tamoxifen treatment. Metabolite D was identified in the urine samples and in the gastric fluid digest at a retention time of 22.0 min eluting from a reversed-phase HPLC column. Although several metabolites were found in all the urine samples of patients, some metabolites were detected in one sample but not others, suggesting tamoxifen metabolism varies in patients.

Keywords: Tamoxifen

1. Introduction

Tamoxifen is a nonsteroidal antiestrogen and has become the agent of choice in the treatment and prevention of breast cancer [1–6]. Clinical trials have consistently shown that tamoxifen improves disease-free survival, and the overall survival rate of patients with early breast cancer [1–6]. The main concern regarding tamoxifen treatment in humans is that it may increase the risk of endometrial cancers and blood clots [6]. Animal studies indicate that the toxicity of tamoxifen is species-dependent [7–10]. This difference in toxicity between species is suspected to be due to different metabolic activities between animals. Due to the possible side effects of tamoxifen treatment, efforts have been made to study tamoxifen biotransformation in animals and humans [6–15].

Analytical techniques used to analyze tamoxifen and its metabolites include gas chromatography–mass spectrometry (GC–MS) [16,17], and high-performance liquid chromatography (HPLC)–UV absorbance or fluorescence detectors [18–23]. GC–MS is highly specific, but requires chemical derivatization of the sample. The most commonly used method is HPLC separation, with post-column UV irradiation to convert the compounds to corresponding

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phenanthrenes, followed by fluorescence detection. This method offers good sensitivity, but lacks molecular structural information. Recently, HPLC with electrospray mass spectrometry (ESI–MS) has shown promising performance for analyzing drugs and their metabolites [24–28]. HPLC analysis of isotopically labeled compounds has also been used, but without mass spectroscopic identification of metabolites [29,30].

A number of tamoxifen metabolites have been reported in various biological samples [13,17,22–27,31]. These metabolites [13] are listed in Table 1. However, identification of some of these metabolites lacks convincing evidence. The presence and distribution of tamoxifen and its metabolites are tissue-selective [8]. In human fluid samples, Lien et al. [31] found that tamoxifen and metabolites X and Z were the major species with small amounts of metabolites Y, B, and BX in serum. Bile and urine were rich in hydroxylated, conjugated metabolites Y, B, and BX. Tamoxifen and metabolite B were the predominant species in feces. The major components in breast tumor tissues were tamoxifen and metabolites B and X [22].

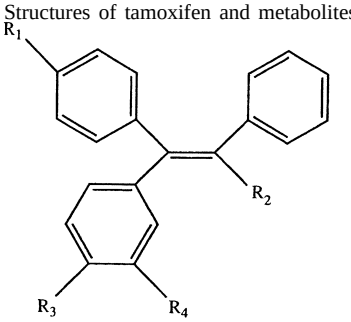
Tamoxifen is administered orally. The fate of tamoxifen in gastric fluid has not previously been reported. In this study, we present nonenzymatic transformation of tamoxifen in synthetic gastric fluid and the effects of pepsin (a digestive enzyme) on this process. We investigated tamoxifen transformation products in gastric fluid and possible metabolites in patient urine samples. We also demonstrate use of extracted ion chromatograms from HPLC–MS for analysis of tamoxifen and its metabolites in a complex matrix to minimize sample preparation procedures.

2. Experimental

2.1. Material and reagents

Tamoxifen, pepsin, and synthetic gastric fluid were purchased from Sigma (St. Louis, MO, USA). 4-Hydroxy-tamoxifen (*Z*- and *E*-isomers) was obtained from Research Biochemical International (Natik, MA, USA). HPLC-grade acetonitrile (ACN) and methanol were obtained from EM Science

Table 1
Structures of tamoxifen and metabolites



Compound	R_1	R_2	R_3	R_4	Formula	Mass [MH^+]
Tamoxifen	$(CH_3)_2N(CH_2)_2O$	C_2H_5	H	H	$C_{26}H_{29}NO$	372.23
Metabolite A[a]	$(CH_3)_2N(CH_2)_2O$	C_2H_5	H	H	$C_{26}H_{31}NO_2$	390.24
Metabolite B	$(CH_3)_2N(CH_2)_2O$	C_2H_5	OH	H	$C_{26}H_{29}NO_2$	388.22
Metabolite BX	$CH_3NH(CH_2)_2O$	C_2H_5	OH	H	$C_{25}H_{27}NO_2$	374.21
Metabolite C	$(CH_3)_2N(CH_2)_2O$	C_2H_5	OH	OCH_3	$C_{27}H_{31}NO_3$	418.23
Metabolite D	$(CH_3)_2N(CH_2)_2O$	C_2H_5	OH	OH	$C_{26}H_{29}NO_3$	404.22
Metabolite E	HO	C_2H_5	H	H	$C_{22}H_{20}O$	301.15
Metabolite X	$(CH_3)HN(CH_2)_2O$	C_2H_5	H	H	$C_{25}H_{27}NO$	358.21
Metabolite Y	$HO(CH_2)_2O$	C_2H_5	H	H	$C_{24}H_{24}O_2$	345.18
Metabolite Z	$H_2N(CH_2)_2O$	C_2H_5	H	H	$C_{24}H_{25}NO$	344.20
Tamoxifen *N*-oxide	$(CH_3)_2N0(CH_2)_2$-O	C_2H_5	H	H	$C_{26}H_{29}NO_2$	388.22

[a] With water added across the ethylenic double bond.

(Gibbstown, NJ, USA). Formic acid (analytical grade) was purchased from BDH (Toronto, Canada).

2.2. Instrumentation

The HPLC system used in our studies consisted of a pair of pumps (Perkin–Elmer Series 200 Micro, Norwalk, CT, USA) with a 75-μl dynamic mixer, a Keystone (Bellefonte, PA, USA) EDS Hypersil C_{18}, 3 μm, 120 Å, 50 mm×1 mm column, and an autosampler (Perkin–Elmer Series 200). The mobile phase was composed of solvent A (0.1% formic acid in deionized water) and solvent B (0.1% formic acid in acetonitrile). Separation was carried out by gradient elution from 5 to 95% solvent B in 50 min at a flow-rate of 60 μl min^{-1}. The injection volume of samples was 3 μl.

The mass spectrometers used were an API 3000 triple quadrupole system or a tandem quadrupole time-of-flight system (Model Qstar, PE-SCIEX Instruments, Concord, Canada) connected to the HPLC system using an ion spray source. Electrospray ionization conditions were ion spray voltage 5400 V, orifice 65 V, ring voltage 220 V, curtain gas (CUR) 12, and nebulizer gas (NEB)12. The full scan mass range was 250 to 810 u with a step size of 0.25 u and dwell time of 0.8 ms.

2.3. Tamoxifen digestion in synthetic gastric fluid

Gastric fluid-mediated digestion was conducted to simulate tamoxifen metabolism in the stomach. Tamoxifen at concentrations of 0, 1, 5 and 10 μg ml^{-1} was incubated with synthetic gastric fluid (mainly consisting of NaCl and HCl at pH 1.4) at 37°C for 1 week. Pepsin, a major digestive enzyme in the stomach, was added to a second set of samples to examine its effect on tamoxifen metabolism.

2.4. Extraction of urine samples

Six urine samples were obtained from breast cancer patients currently being treated with tamoxifen. Extraction of urine samples was modified based on what was reported by Poon et al. [25]. Briefly, an aliquot of 10 ml urine sample was extracted twice with 5 ml of 98% hexane and 2% butanol, and the organic layers combined. The volume of organic solvent was reduced by roto-evaporation and then dried under an argon stream. The sample residues were re-constituted with 100 μl of methanol prior to MS analysis.

3. Results and discussion

3.1. Method and calibration

Three commercially available standards including (*Z*)-tamoxifen, (*Z*)-4-hydroxytamoxifen and (*E*)-4-hydroxytamoxifen were separated and detected using HPLC–ESI–MS. Despite the structural similarity of these compounds, they were completely resolved as shown in Fig. 1. In this reversed-phase separation, (*E*)-4-hydroxytamoxifen eluted before its (*Z*)-isomer. Based on this information, we predict that the (*E*)-isomer of tamoxifen should elute before (*Z*)-tamoxifen. We also studied the reproducibility of retention times and detection using this method. The relative standard deviations (RSD, $n=5$) of retention times were 0.2% for (*E*)-4-hydroxytamoxifen (25.76 min), 0.2% for (*Z*)-4-hydroxytamoxifen (26.36 min), and 0.5% for (*Z*)-tamoxifen (34.69 min). The calibration curves (based on peak heights versus concentrations)

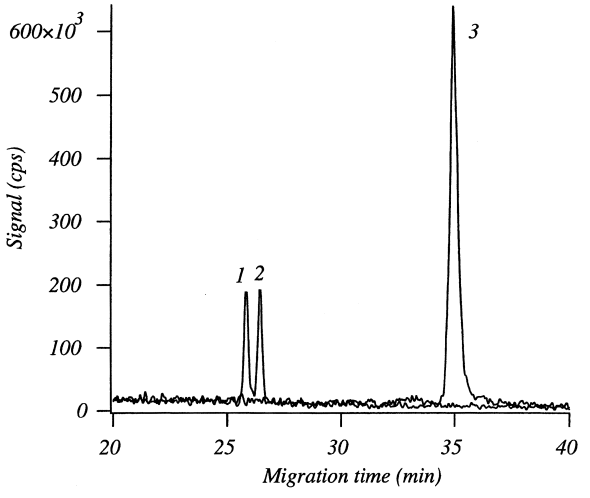


Fig. 1. Superimposed extracted ion chromatograms of m/z 388 and 372. Peak 1: (*E*)-4-hydroxytamoxifen (m/z 388); Peak 2: (*Z*)-4-hydroxytamoxifen (m/z 388); Peak 3: (*Z*)-tamoxifen (m/z 372).

of the three components from 50, 100, 500, and 1000 ng ml^{-1} samples were linear with $r^2>0.97$.

3.2. HPLC–MS analysis of tamoxifen and its transformation products in gastric fluid without extraction

The HPLC–ESI–MS method was applied to study nonenzymatic transformation of tamoxifen in a synthetic gastric fluid to mimic human stomach conditions. A one-week incubation was used to increase the concentration of minor transformation products. Four samples, a tamoxifen digest in gastric fluid without, a similar digest with pepsin (tamoxifen in gastric fluid with pepsin), a control (gastric fluid and pepsin without tamoxifen), and a standard (tamoxifen in methanol, data not shown), were sequentially injected into the HPLC–ESI–MS without extraction. The total ion chromatograms from the samples are compared in Fig. 2a. A few product peaks are difficult to observe. However, the product peaks are clearly observed when extracted ion chromatograms are compared. The extracted ion chromatograms (XIC) for tamoxifen (*m*/*z* 372.0, Fig. 2b) from the digest without pepsin (A), the digest with pepsin (B), and the control (C) show two chromatographic peaks in the digests and no peak in the control, respectively. We detected only one peak from the (*Z*)-tamoxifen standard solution (data not shown). These results suggest that the small peak eluting before (*Z*)-tamoxifen was produced in the gastric fluid. The two chromatographic peaks detected in the digests are probably due to acid-catalyzed isomerization of tamoxifen. Comparing the retention times of these two peaks with that of the (*Z*)-tamoxifen standard, the second peak was identified as (*Z*)-tamoxifen. We tentatively identified the product peak as (*E*)-tamoxifen, based on its mass spectrum, and comparison with the elution order of (*E*)- and (*Z*)-4-hydroxytamoxifen (Fig. 1).

The ratios of peak intensity (E/*Z* isomers of tamoxifen) were 0.4 from the digests without pepsin (Fig. 2b, trace A) and 0.1 with pepsin (Fig. 2b, trace B). This difference could result from preferential binding of the *E* isomer to pepsin, which would result in loss of this isomer during extraction. Alternatively, hydrolysis of the *Z* isomer may be reduced by binding to pepsin. Additional work is

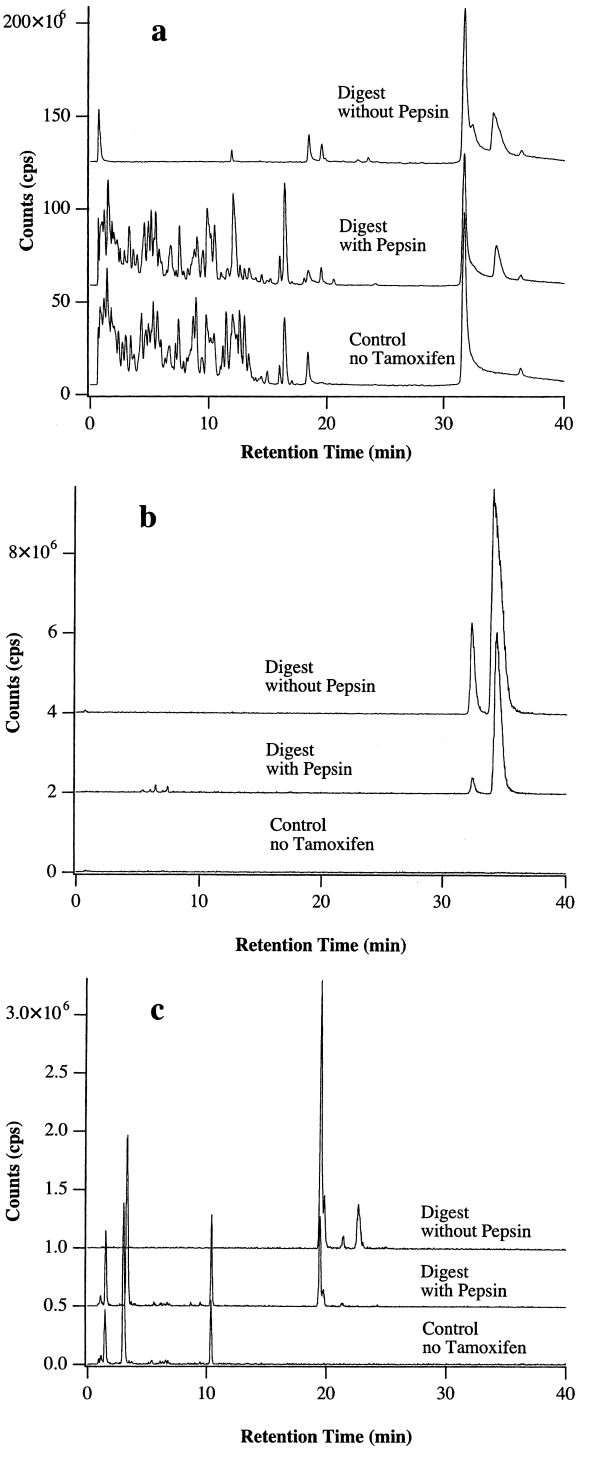


Fig. 2. Total ion chromatograms (a), extracted ion chromatograms of *m*/*z* 372 (b) and of *m*/*z* 404 (c) from tamoxifen digest without pepsin, a digest with pepsin, and control with gastric fluid, pepsin, but not tamoxifen.

required to identify the mechanism by which pepsin affects the analysis of these compounds.

Metabolite D (m/z 404) is another potential product. The MC of m/z 404 (Fig. 2c) showed three chromatographic peaks in the digests. The digest with pepsin produced only a small quantity of the products corresponding to m/z 404, whereas the non-pepsin digest produced approximately six times more product based on the intensity of the main peak. This further supports the hypothesis that pepsin reduces the degradation rate of tamoxifen.

Metabolite B (m/z 388) is commonly found in the blood samples from patients on tamoxifen treatment but at low concentrations [13]. The presence of metabolite B in the gastric fluid digests was investigated but not detected in the samples described above. This suggests that metabolite B is primarily produced by enzymatic transformation in humans.

3.3. HPLC–MS–MS analysis of the major products at m/z 372 and 404

We further analyzed the major transformation products at m/z 372 and 404 using HPLC–MS–MS. The two species at m/z 372 described above produced very similar product ion spectra consisting of major fragment ions of m/z 372 and 72. These results support the identification of the two chromatographic peaks at m/z 372 as (*E*)- (early eluting) and (*Z*)isomers (late eluting) of tamoxifen.

Fig. 3 shows the chromatogram of m/z 404 obtained using HPLC–MS–MS and the product ion spectra of the corresponding peaks. Four chromatographic peaks corresponding to m/z 404 were detected at retention times of 18.4, 19.6, 20.6 and 22.0 min. Peaks 1 to 3 produced similar product ion spectra consisting of major fragments at m/z 360 and 72. The fragment ion of m/z 360 is probable due to loss of CO_2 (44=404−360) or an uncommon fragmentation pathway with an even-electron precursor ion. A product ion spectrum of the tamoxifen standard did not produce any intense peak at 328.2 u (372.2−44), suggesting that new tamoxifen metabolites may be produced in acidic conditions. Further experiments are required to identify these products.

Unlike peaks 1 to 3, Peak 4 at 22.0 min showed a different MS–MS spectrum. This spectrum consisted of major ions at m/z 404, 105, and 72, but the fragment m/z 360 was absent. Mass ions of m/z 404 and 72 corresponds to MH^+ of metabolite D and $(CH_3)_2N(CH_2)_2^+$, respectively, indicate that this peak is likely to be metabolite D. The identification of this peak as metabolite D was further confirmed by the results obtained using hybrid quadrupole time-of-flight mass spectrometry, which will be described later.

We carefully examined total ion chromatograms and extracted ion chromatograms. The major digest products were (*E*)-tamoxifen (m/z 372), metabolite D (m/z 404), and the other products (m/z 404 and 360) described above. No other metabolites listed in Table 1 were detected.

3.4. Analysis of urine samples from breast cancer patients on tamoxifen treatment

The HPLC–MS method described above was used to analyze six extracts of urine samples from patients who are on tamoxifen treatment for more than two years. Total ion chromatograms were obtained. The structures, molecular formulae and accurate masses of protonated ions of tamoxifen and several reported metabolites [5] are shown in Table 1. Mass ions of these metabolites listed in Table 1 were extracted from the total ion chromatograms. Retention times and intensities of these peaks from two patient urine samples are summarized in Table 2. As shown in Table 2 tamoxifen was detected in both urine samples, and identified by matching the retention time with that of the standard in addition to comparing the electrospray mass spectra.

We particularly investigated the presence of (*E*)-tamoxifen and metabolite D (m/z 404) in the urine samples because these were the major products in gastric fluid digestion. (*E*)-tamoxifen was not detected. Low concentrations of metabolite D (m/z 404) were detected at a retention time of 22.0 min in both urine samples, shown in Fig. 4. This figure presents the extracted ion chromatograms of m/z 404 from (A) gastric fluid digest, (B) patient-1, and (C) patient-2 urine samples. A peak at 22.0 min was detected in all three samples. This peak was further analyzed using hybrid quadrupole-time-of-flight MS to obtain the accurate mass of MH^+. The measured mass was 404.2178, a 12 ppm deviation from the theoretical mass (MH^+) of metabolite D (404.2226).

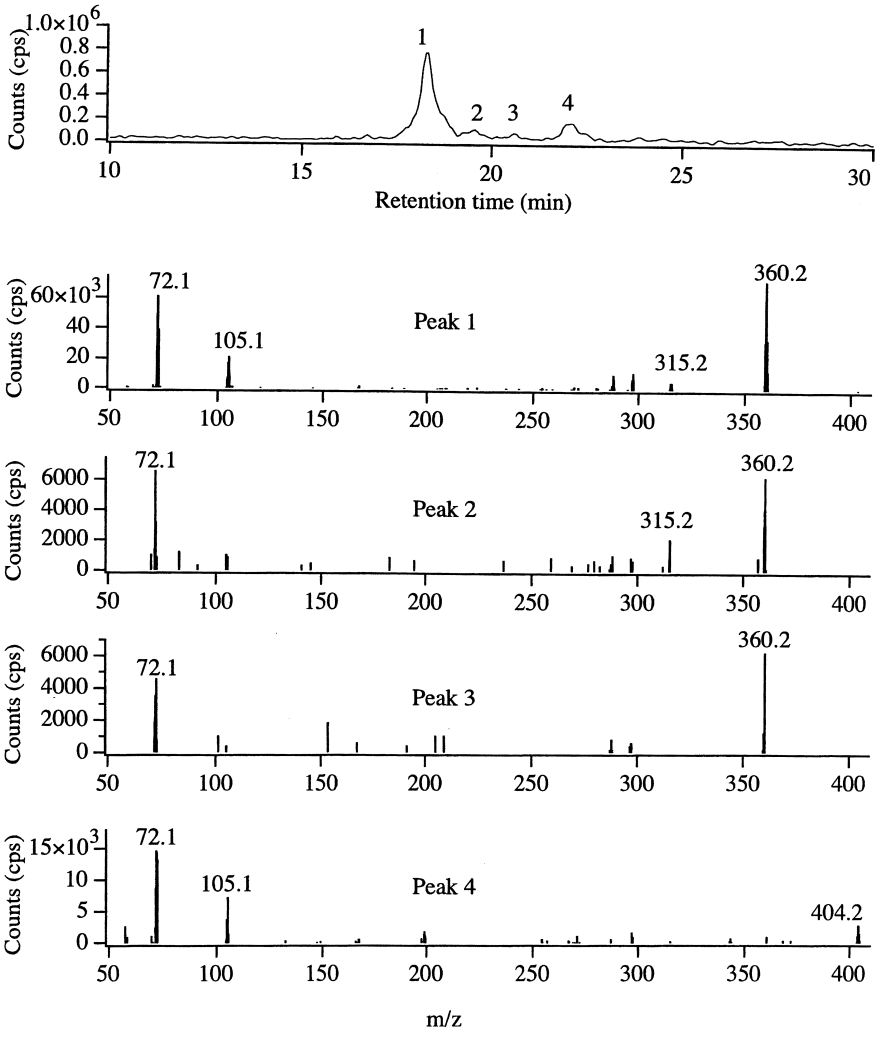


Fig. 3. HPLC–MS–MS analysis of tamoxifen digest in gastric fluid without pepsin and MS–MS spectra of the four peaks corresponding to m/z 404.

This confirms the identification of metabolite D in the gastric fluid digest described above.

Although a few peaks corresponded to m/z 388, no metabolite B was detected based on retention time matching. The peak at 20.7 min is possibly tamoxifen *N*-oxide. Other peaks corresponding to m/z 388 were only present in patient-2 urine.

Other metabolites were also detected and are listed in Table 2. Since no standards for these metabolites were available, we tentatively identified these metabolites based on their mass spectra and elution order. None of these peaks were detected in a blank urine sample from a healthy subject, not undergoing tamoxifen treatment. The relative retention times of the metabolites detected in the two urine samples were identical or very similar, partially supporting the metabolite identification.

Different metabolites were found in the two urine samples. Interestingly, metabolite BX resulting from demethylation and hydroxylation of tamoxifen was detected in patient-1 urine, but not in patient-2 urine. Demethylation products, metabolites X and Z, were detected in both samples, however, a primary alcohol of tamoxifen, metabolite Y, was absent. The different

Table 2
Retention times and peak intensity of tamoxifen and metabolites detected in two urine samples

Tamoxifen and metabolites[a] MH^+ (m/z)	Patient-1			Patient-2		
	Retention time (min)	Relative retention time[b]	Peak height (cps)	Retention time (min)	Relative retention time[b]	Peak height (cps)
Tam/372	34.91	0	5.31e^5	34.73	0	2.02 e^6
BX/374	22.22	−0.36	1.86 e^6	–[c]	–[c]	–[c]
C/418	39.94	0.14	1.29 e^5	39.85	0.15	7.62 e^4
D/404	21.96	−0.37	1.70 e^5	21.96	−0.37	1.91 e^5
E/301	33.35	−0.045	3.04 e^6	33.33	−0.04	2.49 e^6
X/358	33.71	−0.03	3.29 e^5	33.59	−0.03	3.65 e^5
Y/345	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]
Z/344	23.09	−0.338	6.37 e^4	23.09	−0.335	3.37 e^4
Tam. *N*-oxide/388	20.73	−0.41	6.55 e^5	20.76	−0.40	1.77 e^6
Other unknown metabolite/388	34.91		4.22 e^5	18.40		1.77e^5
				18.46		1.71 e^5
				22.05		6.87 e^4
				34.79		2.07 e^5

[a] Tam.=Tamoxifen, the metabolites are represented by one letter.
[b] Relative retention time is calculated by $[t_1 - t$ (tamoxifen)], (tamoxifen) where t_1 is the retention time of a metabolite and t is the retention time of tamoxifen.
[c] – not detected.

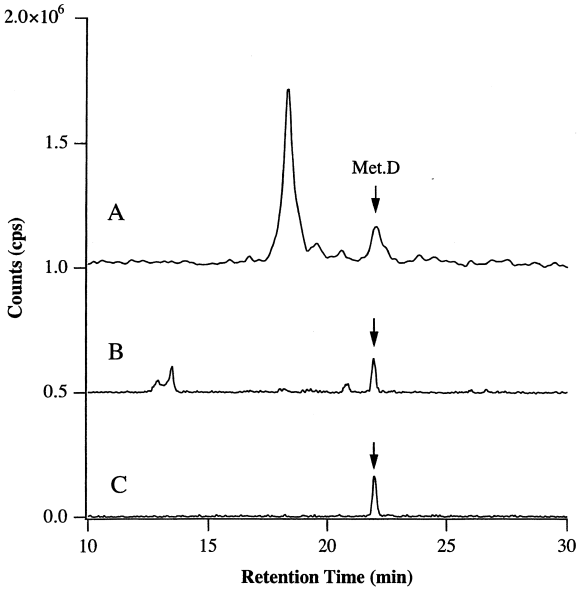


Fig. 4. Extracted ion chromatograms of m/z 404 from (A) tamoxifen digest in gastric fluid without pepsin, (B) patient-1 urine extract and (C) patient-2 urine extract.

metabolites found in the two urine samples indicate possible variation in tamoxifen metabolism in different patients. Analysis of more urine samples would confirm this finding.

4. Conclusions

We demonstrated that use of extracted ion chromatograms from HPLC–MS can provide sensitive detection for complex samples with minimal sample preparation. Tamoxifen is relatively stable and can be further stabilized by adding proteins in an acidic environment. The major transformation products of tamoxifen in synthetic gastric fluid are the (*E*)-isomer of tamoxifen and metabolite D (m/z 404), however, only small amounts of metabolite D and no (*E*)-tamoxifen were detected in urine samples. This suggests that nonenzymatic transformation of tamoxifen in the human stomach may not be significant to the overall metabolism of tamoxifen in humans. Analysis of metabolites in urine samples may provide information about differences in metabolic activity among patients. These findings may warrant further studies on a larger number of patients [32].

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