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Identification and quantification of tamoxifen and four metabolites in serum by liquid chromatography–tandem mass spectrometry

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Abstract

We have developed a method for the determination of tamoxifen (tam) and its metabolites 4-hydroxytamoxifen (4OHtam), *N*-demethyltamoxifen (NDtam), *N*-dedimethyltamoxifen (NDDtam), tamoxifen-*N*-oxide (tamNox), and 4-hydroxy-*N*-demethyltamoxifen (4OHNDtam) in 50 μl human serum. Serum proteins were precipitated with acetonitrile. Deuterated-tamoxifen (D_5tam) was added as internal standard. Sample supernatant was injected into an on-line reversed-phase extraction column coupled with a C_{18} analytical column and analytes were detected by tandem mass spectrometry. The lower limits of quantification were 0.25 ng/mL for 4OHtam, NDtam and tam, 1.0 ng/mL for NDDtam and tamNox. Ranges of within- and between-day variation were 2.9–15.4% and 4.4–12.9%, respectively.
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Keywords: Mass spectrometry; Tamoxifen; 4-Hydroxytamoxifen; Tamoxifen-*N*-oxide; 4-Hydroxy-*N*-demethyltamoxifen; Breast cancer

1. Introduction

Tamoxifen (tam), a first generation selective estrogen receptor modulator (SERM), was introduced as a treatment option for breast cancer more than three decades ago. It is now used among women of all ages for the treatment of breast cancer, irrespective of cancer stage. Tam is also approved by the U.S. Food and Drug Administration as a chemopreventive agent against breast cancer, and is now prescribed to women at high risk of this disease [1]. However, the drug has serious side effects, like endometrial cancer and venous thrombotic events [1–3] which may be dose-dependent or related to the accumulated dose [4].

Tam metabolism is complex and involve *N*-demethylation, aromatic hydroxylation, side chain α-hydroxylation and *N*-oxidation (Fig. 1). The cytochrome P450 enzymes (CYPs) CYP 3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6, CYP1A2 [5–11] and a flavin-containing monooxygenase [12] are involved in the metabolism of tam. CYP2D6 has a polymorphic distribution of activity that divides populations into “slow” or “rapid” metabolizers [13], whereas the CYP3A4 is inducible [14].

Although tamoxifen is the most used anticancer drug, its optimal dose is currently unknown. Effects and side effects of tamoxifen may partly be due to its active metabolites. Due to their high affinity to the estrogen receptor [15–17], the hydroxylated metabolites 4OHtam and 4OHNDtam are believed to give a major contribution to the effects of tam. Side effects, however, may be caused by tam and its demethylated metabolites NDtam and NDDtam that are present in serum in concentrations 50–135 times that of 4OHtam and 4OHNDtam [18–20].

Recently we demonstrated retained effect on the proliferation marker Ki-67 even after decreasing tam doses from the conventional 20 to 1 mg per day [21,22]. The serum concentrations of tam and its metabolites varied by a factor of more than 10 in each dose regimen. Furthermore, the concentrations in serum of tam and its metabolites were related to those observed in breast cancer tissues. These findings sug-

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gest that low dose regimens combined with therapeutic drug monitoring may result in reduction of side effects without loss of effects. However, during low dose treatment, serum levels of the potent hydroxylated metabolites were below detection levels of the LC-fluorescence method used in a major portion of the patients [21–23]. A more sensitive method for quantification of tam and its metabolites was therefore warranted.

Methods using high pressure liquid chromatography (HPLC) for the study of tam metabolism have been reported [23–27]. These often need a large sample volume, complex extraction procedures, or have insufficient sensitivity or selectivity. The development of mass spectrometry has made it possible to improve analytical sensitivity and selectivity. Based on this technology, gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry methods have been developed for the analyses of tam [28–30]. However, they require sample derivatization, extraction or evaporation. In a recently developed liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for simultaneous estimation of several selective estrogen receptor modulators, the results for 4OHtam did not meet the acceptance criteria, especially with regard to precision [31].

Here we describe a sensitive method based on LC–MS–MS for the simultaneous determination of tam, 4OHtam, NDtam, NDDtam, tamNox, and 4OHNDtam. We used a simple protein precipitation step, followed by an on-line extraction column coupled with a C_{18} analytical column.

2. Experimental

2.1. Reagents

Tam citrate and 4OHtam were purchased from Sigma–Aldrich (Steinheim, Germany), the internal standard deuterated$_5$-tamoxifen (D_5tam) from BioChem (US), and tamNox from Sintef Materials and Chemistry (Oslo, Norway). NDtam and NDDtam were gifts from Imperial Chemical Industries, PLC Pharmaceutical divisions (Macclesfield, UK). Acetonitrile (HPLC-grade) and formic acid (analytical grade) were obtained from E. Merck (Darmstadt, Germany).

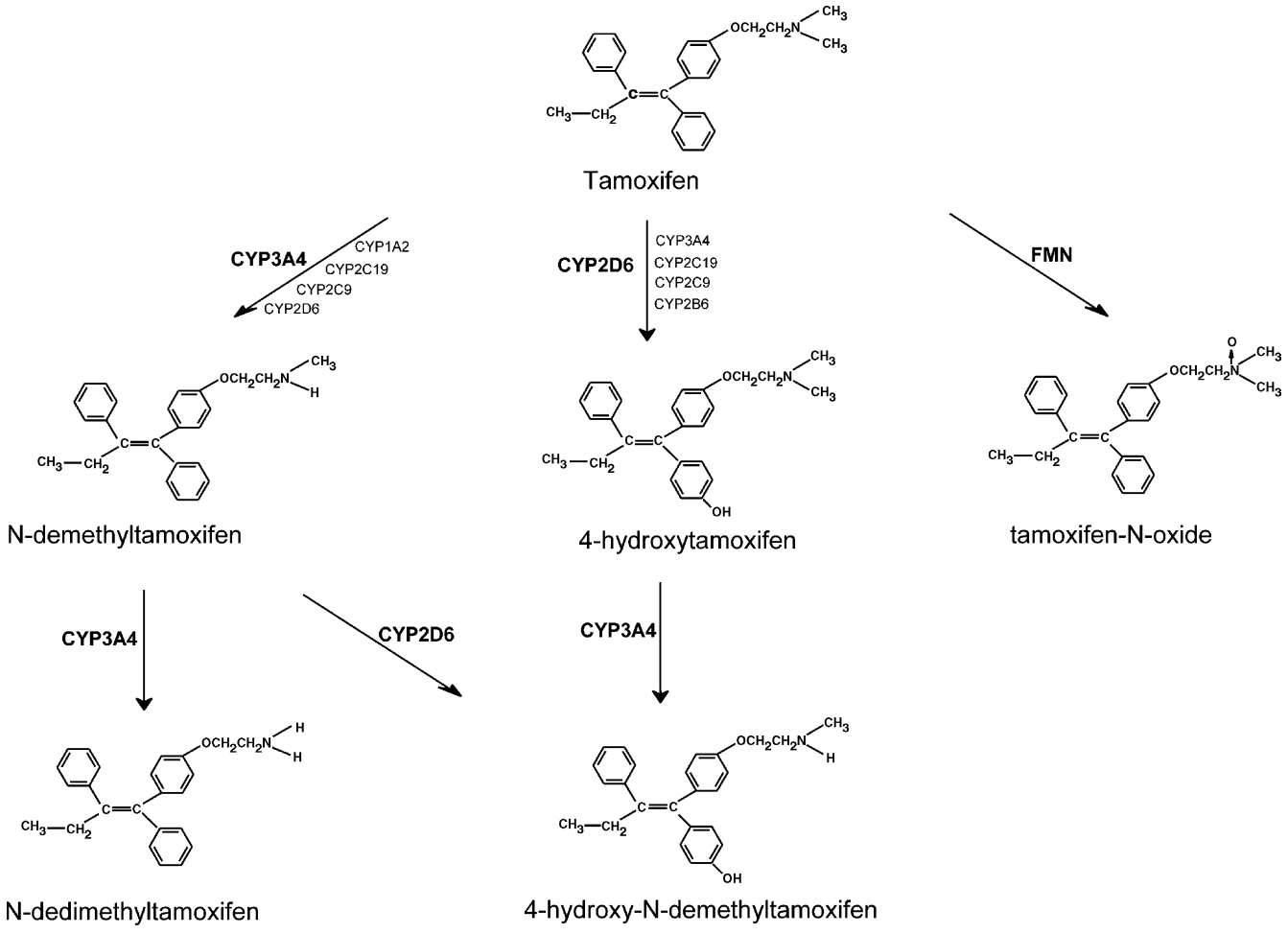

Fig. 1. Proposed metabolic pathways of tamoxifen in human. The principal cytochrome P450 isoforms responsible are highlighted in larger fonts and in bold.

2.2. Patient samples

Serum samples from 21 patients on tamoxifen therapy of 20 mg per day were obtained by collecting blood into Vacutainer Tubes with no additive. Blood was allowed to clot at room temperature for 30 min before centrifugation, and the serum fraction was transferred to empty plastic vials. Aliquots of 75 μl serum were precipitated with equal volume of 25 ng/mL D_5tam in acetonitrile. Samples were vortexed and centrifuged at 15,000 × *g* for 15 min. The supernatant was collected, and 100 μl was injected in the HPLC system.

2.3. Liquid chromatography–mass spectrometry

Chromatographic condition: an Agilent 1100 HPLC system pump was used to equilibrate the reversed-phase extraction column (Oasis HLB, 50 mm × 1 mm, Waters, Milford, MA, USA) with 100% solution A (100% distilled water) prior to 100 μl sample injection. The same solvent delivery system equipped with a degasser, a thermostated autosampler and column oven (Agilent) was used to elute the analytes from the extraction column to the analytical column (Chromolith Performance, RP-18e, 100 mm × 4.6 mm, Merck) [32]. We used the solutions A, B (0.05% formic acid, pH 2.8) and C (100% acetonitrile). The Agilent 1100 pump was programmed as follows: 0–0.7 min, 100% A; 0.8–1.0 min, 75% B and 25% C; 1.4 min, 30% B and 70% C; 2.5 min, 15% B and 85% C; 2.7–4.0 min, 5% B and 95% C and 4.1–5.0 min, 100% A. All gradient steps were linear.

We used a triple-quadrupole mass spectrometry system from Applied Biosystems (AB MDS Sciex, Concord, Canada) Q-Trap 2000 with a built-in switching valve controlled by Analyst 1.3 e-pack software. The valve was programmed as follows: 0–1.0 min (A) extraction mode, from 1.1 to 4.0 (B) separation mode and 4.1–6 min (A) extraction mode (Fig. 2). At the extraction mode, the extraction column was equilibrated with 100% distilled water prior to 100 μl sample injection. At the same time an external HP1000 pump (Waldbronn, Germany) was used to precondition the analytical column with 100% Solution D (10% acetonitrile and 90%, 0.05% formic acid). In the separation mode, the same solvent delivery system from Agilent 1100 pump was used to elute the analytes from the extraction column to the analytical column. The flow rate from the separation column was split to 0.4 mL/min before introduction into the mass spectrometer to avoid overload of sample.

The triple-quadrupole mass spectrometry was equipped with TurboIonSpray (AB MDS Sciex, Concord, Canada). Parent and fragment ions were detected in multiple-reaction monitoring (MRM) mode. The electrospray needle voltage was set at +4500 V. Dwell times were 100 ms per channel with a unit mass resolution on both mass analyzers. All spectra were acquired in the positive ion mode over a mass range of *m*/*z* 40–500. Nitrogen was used as nebulizer, turbo (heater)

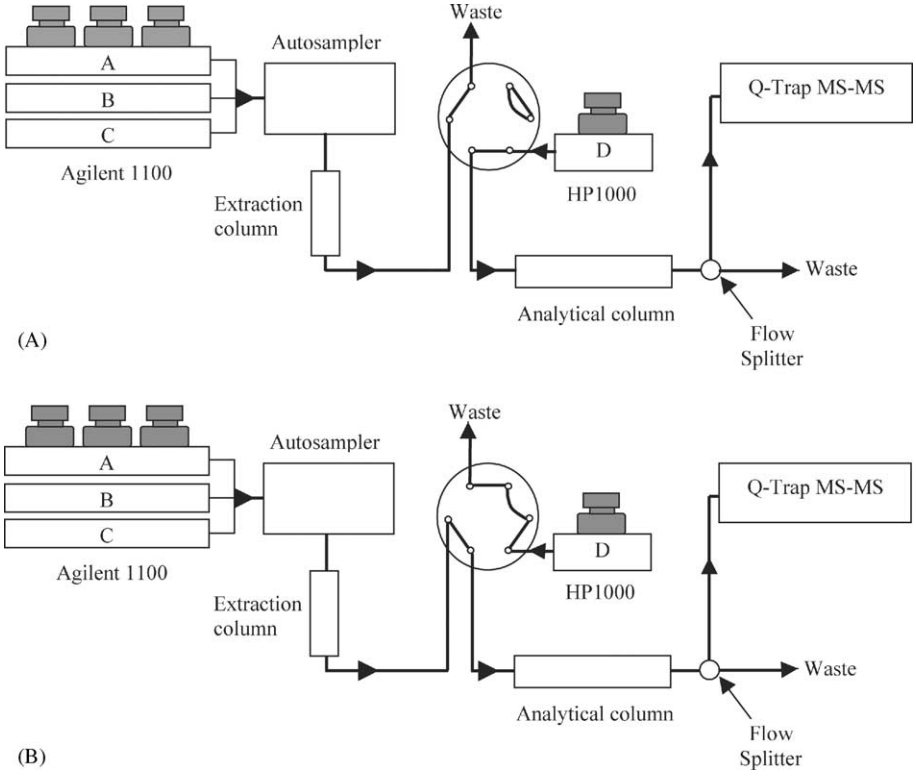

Fig. 2. Flow chart of the LC–MS–MS system: (A) during extraction mode and (B) during separation mode.

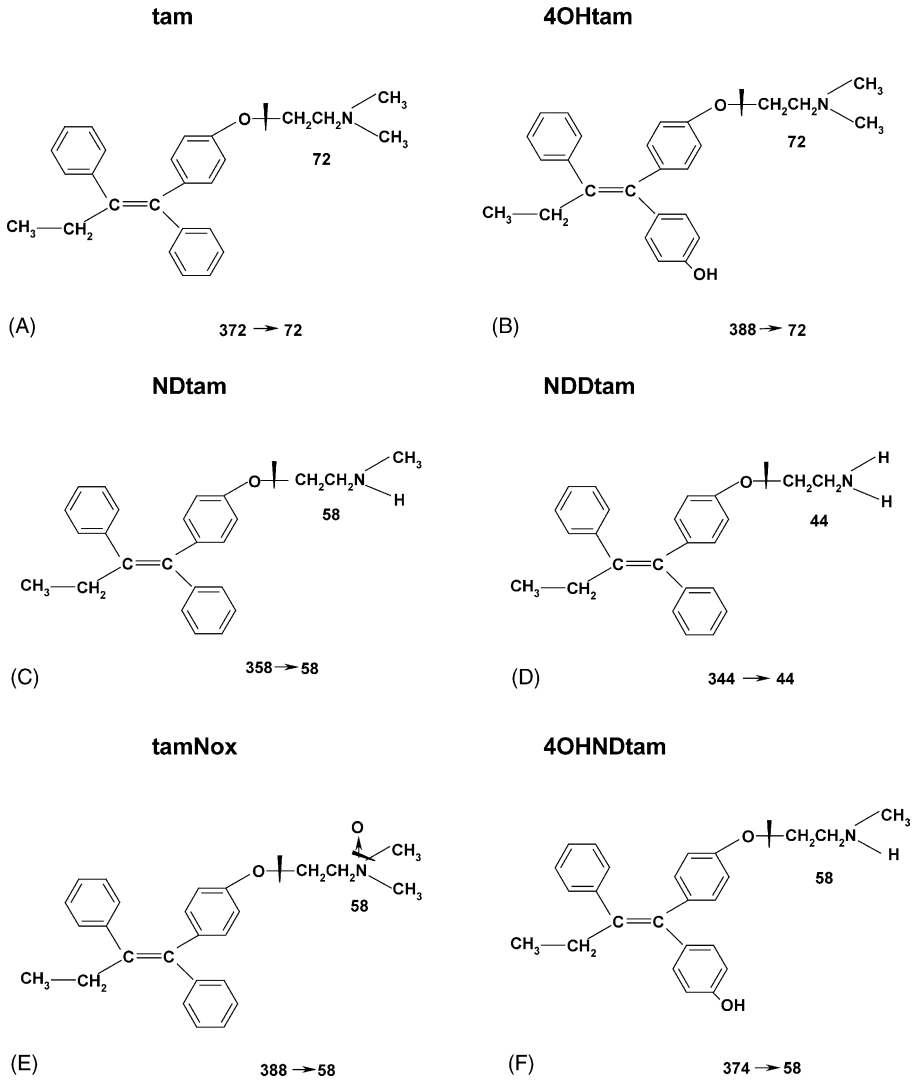

Fig. 3. The pattern of fragmentation for the positive ions detected by tandem mass spectroscopy in multiple-reaction monitoring mode, using the molecular transitions *m/z*, parent ion → fragment ion for tam (A), 4OHtam (B), NDtam (C), NDDtam (D), tamNox (E) and 4OHNDtam (F).

gas, curtain and collision activated dissociation gas and was set at 1.4, 3.5, 2.1 and 0.2 bar, respectively. The temperature of the interface probe heater gas in the TurboIonSpray source was 500 °C.

2.4. Assay validation

2.4.1. Linearity of assay calibration

Serum samples for the standard curves were prepared by adding to human serum increasing amounts of tam, 4OHtam, NDtam, NDDtam and tamNox. The standard curves were obtained in the concentration range of 0.25–1000 ng/mL for tam, 4OHtam, NDtam and tamNox while for NDDtam 1–1000 ng/mL. Five replicates of each concentration of the spiked serum specimens were analyzed in the same run.

2.4.2. Precision and accuracy

Assay precision was calculated by measurements in blank serum samples added with low (10 ng/mL tam, 0.5 ng/mL 4OHtam, 10 ng/mL NDtam, 5 ng/mL NDDtam and 5 ng/mL tamNox), medium (50 ng/mL tam, 2.5 ng/mL 4OHtam, 50 ng/mL NDtam, 25 ng/mL NDDtam and 25 ng/mL tamNox) and high (1000 ng/mL tam, 50 ng/mL 4OHtam, 1000 ng/mL NDtam, 500 ng/mL NDDtam and 500 ng/mL tamNox) analytes concentrations. To determine within-day variation, we used the data from the recovery experiments. Between-day precision was determined by assaying the same samples on each level on 10 different days over a period of 3 weeks. For apparatus precision, samples were analyzed within a single sequence order of low/medium/high and this injection sequence was repeated ten times.

2.4.3. Extraction efficiency and stability

The recovery was evaluated at the low and high concentration levels. Identical amounts of analytes were added to a mixture of acetonitrile and distilled water (1:1, v/v), called standard, and to blank serum. Serum samples were precipi-

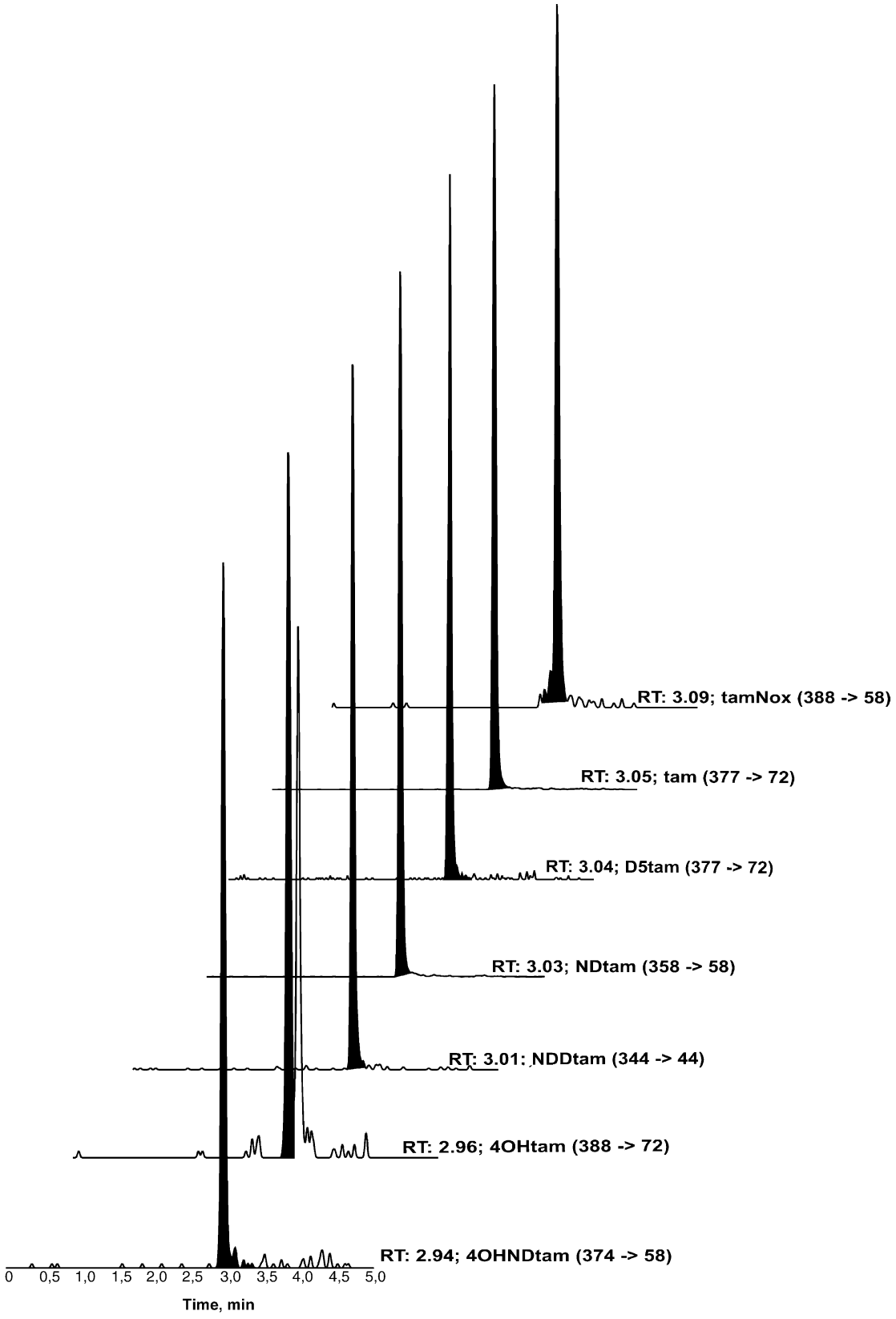

Fig. 4. LC–MS–MS multiple reaction monitoring chromatograms of serum sample from a patient using 20 mg per day for breast cancer treatment. The serum sample contained 8.0 ng/mL 4OHNDtam, 5.8 ng/mL 4OHtam, 34.5 ng/mL NDDtam, 172.9 ng/mL NDtam, 25 ng/mL D_5tam, 67.0 ng/mL tam and 7.9 ng/mL tamNox. The second peak of the 4OHtam trace represents tamNox, which could be identified using another fragment ion of tamNox (*m*/*z* 58, upper trace).

tated with an equal volume of acetonitrile added with internal standard D_5tam before analysis on LC–MS–MS while standard samples were directly analyzed on LC–MS–MS. The added concentration on serum and standard samples prior to injection was the same. Recovery was calculated as:

$$\left(\frac{\text{measured serum concentration}}{\text{measured standard concentration}}\right) \times 100$$

For freeze-thaw stability studies, patient samples were subjected to four freeze-thaw cycles. Samples were prepared and analyzed immediately after thawing. The results were compared to those obtained on the day of initial sample preparation.

2.4.4. Statistics

The results are described as percentages, mean and standard deviations. Two-tailed Spearman Correlation rank tests were used to examine the correlation between LC–MS–MS and our standard LC-fluorescence method [23] of measured tam, 4OHtam and NDtam. A *p*-value of less than 0.05 was regarded as significant. A statistical package (SPSS 12.0, SPSS Inc. Chicago, IL) was used for this analysis.

3. Results

3.1. LC–MS–MS screening, identification and quantification

The protonated molecular $[M+1]^+$ ions for tam (*m/z* 372), 4OHtam (*m/z* 388), NDtam (*m/z* 358), NDDtam (*m/z* 344), tamNox (*m/z* 388) and D_5tam (*m/z* 377) were the predominant ions obtained by Q1 scan (*m/z* 40–500) mass spectra (data not shown). The parent and fragment ions of the analytes and the proposed pattern of fragmentation for each metabolite are depicted in Fig. 3. By using MRM mode and the molecular transitions *m/z* 374 → 58 (4OHNDtam), 388 → 72 (4OHtam), *m/z* 344 → 44 (NDDtam), *m/z* 358 → 58 (NDtam), *m/z* 377 → 72 (D_5tam), *m/z* 372 → 72 (tam) and *m/z* 388 → 58 (tamNox), we obtained a sensitive method of quantification (Fig. 4). The second peak observed in the trace of 4OHtam could be identified as tamNox using a second fragment ion with an *m/z* of 58 (upper trace, Fig. 4). Signal suppressions were avoided because the high level metabolites like tam and NDtam are separated from low levels metabolites like 4OHtam, 4OHNDtam and tamNox. We have standards for tam, 4OHtam, NDtam, NDDtam, tamNox and D_5tam but not 4OHNDtam. When we quantified 4OHNDtam with the use of either 4OHtam or NDtam as an external standard, both standards gave the same measured concentration.

Table 2
Analytical recovery of the assay[a]

Analyte	Added (ng/mL)	Recovery (%)
tam	10	110
	1000	100
4OHtam	0.50	95
	100	78
NDtam	10	119
	1000	95
NDDtam	5	130
	500	86
tamNox	5	65
	500	56

[a] $n = 10$ for all concentrations.

3.2. Calibration

The resulting calibration curves were divided into two concentration ranges (high and low). All coefficients of correlation were higher than 0.994 (Table 1). The method showed linearity over the concentration ranges used (Table 1).

3.3. Assay validation

Columns with smaller diameters such as 2.1 and 1.0 mm I.D., gave better peak concentration. However, these columns caused high back-pressure. Better column stability was obtained by using the 4.6 mm I.D. Chromolith Performance column.

The lower limits of quantification were 0.25 ng/mL for 4OHtam, NDtam and tam, 1.0 ng/mL for NDDtam and tamNox. Analyte levels in low, medium and high concentration samples were determined within the sequence and during the course of ten consecutive sequences. The analytical recovery was 78–130% for tam, 4OHtam, NDtam and NDDtam for both low and high concentrations, whereas the recovery for tamNox was 56–65% (Table 2). The within- and between-day variations were 2.9–15.4% and 4.4–12.9%, respectively, while apparatus variations were 3.7–13.8% for all concentrations examined, as shown in Table 3. The results from

Table 1
Linear regression data at low and high concentration ranges

	Low concentration				High concentration			
	Linear range (ng/ml)	Coefficient of correlation	Slope	Intercept	Linear range (ng/ml)	Coefficient of correlation	Slope	Intercept
tam	0.25–5.0	0.996	0.044	0.005	25–1000	0.999	0.040	0.111
4OHtam	0.25–5.0	0.995	0.015	−0.002	25–1000	1.000	0.017	−0.032
NDtam	0.25–5.0	0.995	0.022	0.020	25–1000	1.000	0.021	0.035
NDDtam	1.0–5.0	0.993	0.007	−0.002	25–1000	1.000	0.006	−0.005
tamNox	1.0–5.0	0.998	0.020	0.001	25–1000	0.999	0.020	0.033

Table 3
Within-, between-day and apparatus precision data of the LC–MS–MS assay[a]

Analyte	Within-day		Between-day		Apparatus precision	
	ng/mL	RSD, %	ng/ml	RSD, %	ng/mL	RSD, %
tam	8.8 (0.3)	3	8.9 (0.6)	7	9.0 (0.4)	5
	42 (1.3)	3	42 (2.2)	5	44 (1.7)	4
	810 (24)	3	800 (62)	8	670 (12)	2
4OHtam	0.5 (0.1)	15	0.5 (0.1)	13	0.5 (0.05)	11
	2.1 (0.2)	12	2.4 (0.3)	12	2.1 (0.2)	8
	71 (4)	6	69 (7)	10	65 (4)	6
NDtam	8.6 (0.6)	7	9.7 (0.6)	6	9.5 (0.7)	8
	39 (1.4)	4	44 (1.9)	4	45 (1.9)	4
	680 (31)	4	810 (84)	10	660 (24)	4
NDDtam	5.8 (0.7)	12	5.1 (0.4)	9	4.2 (0.6)	14
	27 (1.2)	4	25 (2.1)	9	19 (1.1)	6
	460 (23)	5	480 (33)	7	440 (29)	4
tamNox	2.6 (0.2)	9	3.2 (0.3)	10	3.2 (0.3)	9
	15 (0.8)	6	16 (0.9)	6	16 (1.3)	8
	370 (57)	15	420 (21)	5	400 (16)	4

Concentrations are given as the mean (SD).
[a] $n = 10$ for all concentrations.

the freeze-thaw experiments verified that tam and metabolites were stable. The concentration ranged between −5% and +8% after four freeze-thaw cycles.

3.4. Peak purity, selectivity and method comparisons

Data from six blank samples taken from different patients before tam treatment revealed no interfering peaks of the analytes. Two high peaks (*m*/*z* 58 and 72) were observed in the product ion spectrum of tamNox. When we monitored the second fragment of tamNox in 388 → 72 and compared it with 388 → 58, the intensity ratios were identical in patient samples examined. These values were also identical to the values observed in the tamNox standard. Analysis of a *cis–trans* 4OHtam (30/70) sample produced only one 4OHtam peak. Comparing the present LC–MS–MS method with our standard LC-fluorescence method the correlation coefficients for tam, 4OHtam and NDtam were 0.93 ($p < 0.001$), 0.54 ($p < 0.05$) and 0.96 ($p < 0.001$), respectively. This shows a linear relationship over the range of values of the measurement between these two methods. For 4OHtam, the lowest concentrations examined were near the detection limit of our LC fluorescence assay. This may explain the lower correlation coefficient observed for 4OHtam compared to tam and NDtam that are present in higher concentrations.

We did not find any unexpected metabolite profiles in samples from 180 breast cancer patients using 20 mg tam per day. This indicates no interference from other drugs used by these patients.

4. Discussion

We have developed a sensitive and specific LC–MS–MS method for analysis of tam and five metabolites in serum. The sample processing is simple and requires no derivatization. The method allows simultaneous analysis of tam, 4OHtam, NDtam, NDDtam, tamNox and 4OHNDtam. It can be used for drug monitoring during low dose tam regimens, which requires selective and sensitive techniques, especially for the potent hydroxylated metabolites 4OHtam and 4OHNDtam that are present in low concentrations. We obtained a limit of quantification for tam and 4OHtam at 0.25 ng/mL whereas our LC fluorescence method has a detection limit of 1 ng/mL for these compounds.

Analysis of tam and its clinically relevant metabolites is analytically challenging because there are structurally similar tam metabolites that may be present in biological samples. With our LC fluorescence method, quantification of 4OHtam has often been difficult due to peaks from unidentified tam metabolites with nearly identical retention times. In this paper, we identified and separated one of these peaks as tamNox. We observed two major peaks on the tamNox product ion spectra which gave the transitions *m*/*z* 388 → 58 and *m*/*z* 388 → 72 in MRM mode. Both tamNox and 4OHtam have the protonated molecular $[M + 1]^+$ ions of *m*/*z* 388. They were separated on column and by selective ion monitoring. The first peak in transition *m*/*z* 388 → 72 was 4OHtam and the second peak was tamNox, as verified by spiking blank serum samples with either 4OHtam or tamNox authentic standards. Since MRM with transition *m*/*z* 388 → 72 was used to detect 4OHtam, a second fragment (*m*/*z* 58) was used to monitor tamNox. It has been suggested that tamNox may serve as a stored form of tam in vivo [12] and it may be an intermediate for the formation of DNA adducts [33,34].

Another active metabolite of tam, 4OHNDtam, has recently been subject to attention due to its high affinity to the estrogen receptor [9]. In this study, we used the protonated molecular $[M + 1]^+$ for 4OHNDtam (*m*/*z* 374) since it has a molecular weight of 373. Moreover, it has been observed as the dominant parent ion in a previous mass spectrometry

method [35]. The product ion spectra of the analytes show that they all fragmented between the oxygen atom and carbon atom of the side chain. By using fragmentation pattern seen for the other metabolites and especially for NDtam, we anticipate that 4OHNDtam will give a fragment of *m*/*z* 58 similar to that of NDtam (Fig. 3). Accordingly, 4OHNDtam can be detected in MRM mode with the transition: *m*/*z* 374 → 58. Further identification of 4OHNDtam will be possible after obtaining its authentic standard.

The high coefficient of variation observed for the highest concentration of tamNox may be due to the low recovery of this metabolite. More tamNox may have been lost through adsorption to glass and plastic surfaces following spiking serum as compared to spiking acetonitrile–distilled water solutions. Adsorption of tam and 4OHtam to glass and plastic surfaces has been described previously [36]. Higher adsorption was demonstrated for tam (less polar) than for 4OHtam (more polar). The polarity of tam and metabolites in the present system decreases in the order 4OHNDtam, 4OHtam, NDDtam, NDtam, tam and tamNox, as indicated by the retention time in Fig. 4. This suggests that higher adsorption may be observed on tamNox since it is the least polar substance.

Using samples from breast cancer patients, comparison of this LC–MS–MS method with our LC-fluorescence method showed good correlation for tam and NDtam. The LC–MS–MS method requires 6 min per sample and the maximal capacity of this assay is 200 samples per day, whereas the use of LC-fluorescence separation requires 15 min per sample. There is no need for derivatization, and sample preparation may be carried out by a robotic workstation.

In conclusion, an easy sample preparation and LC–MS–MS assay allowed a fast and reliable identification of tam, 4OHtam, NDtam, NDDtam, tamNox, and 4OHNDtam with precise, selective and sensitive quantification. The method is suitable for monitoring of tam and metabolite levels during dose finding studies and for therapeutic drug monitoring.

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