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Evaluation of Tamoxifen and metabolites by LC-MS/MS and HPLC Methods

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Abstract

Epidemiological and laboratory evidence suggests that quantification of serum or plasma levels of tamoxifen and the metabolites of tamoxifen, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen), Z-4-hydroxy-tamoxifen (4HT), N-desmethyl-tamoxifen (ND-tam) is a clinically useful tool in the assessment and monitoring of breast cancer status in patients taking adjuvant tamoxifen. A liquid chromatographic mass spectrometric method (LC-MS/MS) was used to measure the blood levels of tamoxifen and the metabolites of tamoxifen. This fully automated analytical method is specific, accurate and sensitive. The LC-MS/MS automated technique has now become a widely accepted reference method. We analyzed a randomly selected batch of blood samples from participants enrolled in a breast cancer study to compare results from this reference method in 40 samples with those obtained from a recently developed high performance liquid chromatography (HPLC) method with fluorescence detection. The mean (SD) concentration for the LC-MS/MS (endoxifen 12.6 [7.5] ng/mL, tamoxifen 105 [44] ng/mL, 4-HT 1.9 [1.0] ng/mL, ND-tam 181 [69] ng/mL) and the HPLC (endoxifen 13.1 [7.8] ng/mL, tamoxifen 108[55]ng/mL, 4-HT 1.8 [0.8] ng/mL, ND-tam 184 [81] ng/mL), the methods did not show any significant differences. Our results confirm that the HPLC method offers an accurate and comparable alternative for the quantification of tamoxifen and tamoxifen metabolites.

Keywords

Tamoxifen; Endoxifen; 4-HT; ND-tam; High Performance Liquid Chromatography; LC-MS/MS; Breast cancer

Introduction

The biochemical mechanism of action of tamoxifen in treatment of breast cancer is widely understood to involve two active metabolites, 4-hydroxy-N-desmethyl-tamoxifen

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(endoxifen) and Z-4-hydroxytamoxifen (4HT). These metabolites are approximately 100 times more potent, relative to the parent drug.[1] Tamoxifen has been the most important drug worldwide for the prevention and treatment of hormone receptor positive breast cancer.[2] The overall response of the tumor is the result of the aggregate effect of the drug tamoxifen, and its metabolite which is more potent.[3] The concentration of tamoxifen and tamoxifen metabolites, including the ND-Tamoxifen (ND-T) metabolite, in the blood circulation is an accepted measure to assess treatment status.[4, 5] Several analytical methods have been used to determine the blood concentration levels of the parent drug and its metabolites. Advantages and disadvantages exist for each method, based on methodological characteristics.

One of the earliest described analytical methods was reported by Adam et al. in 1978.[6] The method is based on solvent extraction of the drug, followed by Thin Layer Chromatography (TLC) separation with UV light conversion and quantitation by densitometry. This densitometry quantitation was an improvement on the TLC separation method with radioactivity counting, first described by Fromson et al. in 1973.[7] The disadvantages of clinical treatment with radio labeled drugs are quite serious. A very elegant method for the quantitation of tamoxifen and one tamoxifen metabolite (4HT) involving a direct extraction from plasma or ion-paired extraction for whole blood, was described by Mendenhall et al. in 1978.[8] The major problem with the Mendenhall method is that large sample volume, 5mL, and large volumes of organic solvents were required for the extractions. These methods were slow, tedious, time consuming and not suitable for large automated runs, and only tamoxifen and one metabolite was measured.

The ion-paired HPLC chromatographic method with fluorescence detection described in 1980 by Golander and Sternson,[9] was similar in principle to the method of Mendenhall et al.,[8] with the major improvement that tamoxifen and 3 metabolites were measured. However, the disadvantages of this method are similar to those found with the Mendenhall method, and also include an additionally long delay time of the photochemical conversion, (20 minutes or more), and the use of a dry-ice acetone bath.

Between the years 1978 and 1987 several gas chromatography-mass spectrophotometric methods were described by Gaskell et al., Daniel et al., and Murphy et al.[10–12] In 1983, Brown et al.[13] described a HPLC method with post-column fluorescence activation. The disadvantages in this method involves the requirement of an air-cooled housing unit for the fluorescent activation of tamoxifen, aluminum foil reflectors, the generation of ozone, a three –way splitter valve and radio-labeled internal standard. Most importantly, not all the currently identifiable metabolites were detectable.

The determination of tamoxifen and four metabolites in serum by Low-dispersion Liquid Chromatography was reported by Lien et al. in 1987.[14] This method is based on a one-step protein precipitation with acetonitrile followed by direct column injection, with the possibility of automation of sample batches. However, the described internal standard was not readily available from a commercial source, and the HPLC system requires an automated column-switching valve and a post –column converter that was built in-house. Thus, this method may not be easily transferable or practical for most analytical laboratories.

In 1988, Stevenson et al.[15] used an adapted variation of the HPLC fluorescence detection method to quantitate tamoxifen and five metabolites in plasma. This adapted method required 1.0mL volumes of plasma and the extracting solvent is 10mL of diethyl ether, with 8mL of solvent being evaporated to dryness. Additionally, the post column UV exposure and conversion of tamoxifen and metabolites to the phenanthrene products required a 15 minutes exposure time.

The 1994 HPLC method developed by Fried and Wainer,[16] was designed to "handle large numbers of samples easily and economically." This method has few of the disadvantages seen in previously reported methods; however, based on the chromatographic representation, the analytical run time is greater than 70 minutes and so it was highly unlikely that large numbers of samples could be processed within a short time period.[16] During the years 1996 through 2011, several LC-Mass spec based methods were developed.[17–20]

Chromatographic and LC-MS/MS methods are very specific, highly sensitive, and offer shorter batched turn-around time. These techniques have the ability to separate and potentially more accurately quantify both tamoxifen and tamoxifen metabolites when compared to other methods. LC-MS/MS methods that are demonstrated to be accurate and sensitive have the major disadvantage of being too expensive for most research and clinical laboratories.

The primary aim of this study was to compare total serum or plasma tamoxifen and tamoxifen metabolites concentration from human samples quantified by a LC-MS/MS method and a recently developed HPLC fluorescent method. We also examined the correlations of circulating concentrations of total tamoxifen and tamoxifen metabolites using these two methods.

Materials and Methods

Formic Acid, Ammonium Hydroxide (30%), Propranolol Hydrochloride, Tamoxifen, 4 Hydroxytamoxifen and the UV Photochemical Reactor Enhanced Detection tube unit (PHRED) were obtained from Sigma Aldrich, St. Louis MO, USA. The HPLC grade reagents, methanol, acetonitrile, deionized water, Bovine serum albumin (BSA), Potassium Phosphate, 12×75mm polystyrene tubes from Fisher Scientific, Pittsburg, PA, USA. Endoxifen and ND-tamoxifen, were obtained from Toronto Research Chemicals, Ontario, Canada. The Solid Phase Extraction columns (SPE), STRATA-X-C 3, 3u, were obtained from Phenomenex, Torrance, CA, USA. The SPE Extraction Manifold was obtained from Varian, Walnut Creek, CA, USA. The HPLC Column, and guard cartridge, Spherisorb C18 CNRP, 4.6×250mm, 5u, from Waters, Milford, MA, USA. The Micro centrifuge tubes were obtained from USA Scientific, Ocala, FL, USA.

REAGENT Preparations

The formic acid solution was prepared by adding 2mL of formic acid to a final volume of 100mL with HPLC grade water. The 5% ammonium hydroxide reagent was prepared from ammonium hydroxide Stock (30%) and HPLC grade methanol, in the ratio of 1 volume

ammonium hydroxide to 5 volumes of methanol. Propranolol internal standard, 0.2ug/mL, was prepared in 20mm K3PO4 buffer (pH 7.0).

The HPLC system consisted of a ProStar 410 Auto-sampler with refrigeration and heating oven, a 323 Fluorescent detector, a Prostar 230 solvent delivery system, with Star Works 5.3 chromatography software (Varian, Walnut Creek, CA, USA).

The mobile phase reagent consisted of a 65% solution of 20mm potassium trihydrate, (4.25g/L), plus 35% acetonitrile. The final solution was adjusted to pH 3.0. The mixed reagent was filtered under vacuum through a 0.45um filter (Millipore, Bedford MA). The reagent was degassed before use with a solvent de-gas system. This reagent is stable at room temperature for at least 4 weeks.

A sample portion of each pure compound was weighed on a Mettler model AB204 balance (Mettler Instrument, Hightstown, NJ). The tamoxifen and each metabolite were dissolved and made up to volume with methanol to achieve a final concentration of 1mg/mL. This working stock standard was used to prepare a calibrator and quality control samples as required, by the established spiking technique.

The original blood samples were collected from a group of breast cancer survivors; with Institutional review Board approval and oversight. After blood processing, the serum samples were stored at minus 80 degrees Centigrade, until analysis. On the day of extraction of tamoxifen and its metabolites, the serum samples were thawed at room temperature under subdued lighting conditions (subdued lighting conditions is the natural working environment of choice for our laboratory). The samples were then extracted as outlined.[21] Briefly, a deuterated internal standard in buffer was mixed with an aliquot of serum. The diluted serum- internal standard mixes were subsequently extracted through solid phase extraction cartridges and the extracted eluents containing the tamoxifen and metabolites, were collected and dried under a stream of nitrogen gas. These dried extracts were then frozen overnight and subsequently shipped on dry ice for overnight delivery to the LC-MS /MS laboratory for analysis.[22] For the LC-MS/MS quantitation, a 3200 QTRAP Tandem/ion trap mass spectrometer was used. The reported linearity of this LC-MS /MS method is 250ng/mL for Tamoxifen and endoxifen, 500ng/mL for ND-tamoxifen and 6ng/mL for 4 hydroxy – tamoxifen.[21]

A 1mg/mL stock solution of propranolol hydrochloride was prepared in HPLC grade methanol. A working buffer solution of 0.2ug/mL was prepared by diluting into a 20mm solution of potassium phosphate trihydrate, pH 7.0.

To 400uL of standard, unknown and quality control samples 600uL of internal Standard buffer solution (0.2ug/mL) were added in 2mL sized micro centrifuged tubes (USA scientific, Ocala, FL, USA). After vortex mixing for 30 seconds, the micro centrifuge tubes were set aside. Next, Solid Phase Extraction columns (SPE) were selected and labeled, one for each sample to be analyzed. The SPE columns were installed into the manifold bracket, and the columns were prepared and conditioned by first drawing through 1mL of methanol with low vacuum pressure, followed by 1mL of DI water. Then the 1mL mixture of sample with internal standard buffer was loaded onto the SPE column. A low vacuum pressure was

applied and the sample mixture was eluted from the column. The eluents, the methanol and water conditioning reagents were discarded to waste. The next step in the procedure involved washing the eluted SPE columns with 1mL of a 2% formic acid solution, followed by a wash with 1mL of methanol. The retained Tamoxifen and metabolites were eluted from the SPE columns with 1.0 mL of a 5% solution of Ammonium hydroxide in methanol (1: 5 v/v). The eluent from each column was collected into individual polystyrene tubes. These polystyrene tubes containing the tamoxifen and metabolites of tamoxifen were dried under a stream of nitrogen gas using low heat setting.

The extracted dried product was re-suspended in 250uL of mobile phase reagent. The tubes were capped and mixed by vortex at medium speed for 30 seconds. The tubes were allowed to sit at room temperature in subdued light for at least 10 minutes, followed by repeat vortex mixing and transfer of the content of each tube to an injection sample vial for HPLC assay.

Tamoxifen and the metabolites of tamoxifen, (endoxifen, 4 hydroxytamoxifen, ND-tamoxifen), were separated and quantified by isocratic HPLC method, with post-column irradiation by exposure to UV light in a PHRED unit. Fluorescent detection after post UV irradiation occurred at an excitation wavelength of 256nm and emitting wavelength of 380nm. An aliquot of 50 uL was injected onto a reverse phase C18 column, heated at a constant 35 degrees Celsius, and eluted with a mobile phase containing 65: 35 v/v, K3PO4 20 mm: Acetonitrile, final pH to 3.0. The isocratic flow rate was 1.0 mL/min. Quantitation of tamoxifen and the metabolites of tamoxifen were by peak height ratio, compound to internal standard, and is based on a single point standard generated for tamoxifen and each metabolite, by using an external standard of the pure compound to spike a 3% solution of bovine serum albumin in a phosphate buffer matrix (Figure 1).

Results

The amount of added tamoxifen and tamoxifen metabolites were estimated in the high medium, medium and low concentration ranges (see Table 1 for concentration values). These sample pools were prepared by the standard addition technique. Five estimations were made on each sample pool. The results are summarized in Table 1 and indicate a recovery of tamoxifen and tamoxifen metabolites between 85–103% (Table 1).

The extraction efficiency of the method was determined by analyzing the neat solutions of tamoxifen and tamoxifen metabolites in the BSA matrix, containing the internal standard. Two different concentration pools were analyzed, see Table 2. Additionally the corresponding low and high levels of tamoxifen and tamoxifen metabolites in plasma /BSA matrix were extracted with internal standard. All samples were assayed in the same analytical run.

The results are summarized in Table 2. The extraction efficiency at the low concentration levels were greater than 300%. At the high concentration level, the extraction efficiency was greater than 240% of the non-extracted pool. The extraction efficiency of the internal standard was greater than 288%.

Accuracy and precision was assessed from results of replicate assays on one sample pool prepared in a BSA matrix, by the standard addition technique. Four estimations were made in three consecutive runs. The results are summarized in Table 3. The mean values (accuracy) of the assayed samples were between 92 and 102% of their actual concentrations. The within-run precision as measured by the coefficient of variation (CV) was less than 8% across all parameters, while the run-run precision was less than 6%.

Stability of tamoxifen and the metabolites of tamoxifen in plasma/BSA matrix were assessed from the results of replicate assays on 3 different sample pools. These sample pools were prepared by the standard addition technique. No preservative was added to any of the pool samples.

Five estimations were made on each pool during three freeze/thaw cycles. Each pool sample was assayed on day number one, and subsequently, the balance of each pool sample was frozen at minus 80 degrees C. On each succeeding day (cycle), the sample pool was removed from the freezer allowed to thaw at room temperature in the dark. After thawing the samples were mixed well by gentle vortexing. Next, an aliquot of 400uL was removed and assayed. The remainders of the pool samples were re-frozen. This procedure was repeated for three freeze thaw cycles.

The results are summarized in Table 4. With the exception of one possibly aberrant result, most likely due to technical error, the mean value of each sample pool after three freeze-thaw cycles were within 10% of their original prepared baseline values.

The limit of quantitation based on quantitative assay (N=5) for endoxifen was 1.74±0.1 ng/mL, ND-tam 4.0±0.1ng/mL, tamoxifen 1.56±0.2ng/mL. The LOQ for 4HT tam based on direct analytical comparison with the LC-MS/MS quantitation was 0.48ng/mL.

The assay upper linearity limits are: 250ng/mL for ND-Tamoxifen, 125ng/mL for tamoxifen, endoxifen, and 4-hydroxytamoxifen.

Method limitation

The presence of interfering substances was not apparent at the analyte recovery, sample preparation or chromatographic selectivity steps. By testing samples from cancer patients, no interferrent was seen except perhaps for the one 4-OH Tam outlier that may have been due to technical or analytical error.

Statistical methods

Serum samples from 40 breast cancer patients were analyzed by two laboratory methods to assay for tamoxifen and metabolites of tamoxifen. Pearson correlations between the two laboratory methods for tamoxifen and metabolites were: endoxifen r=0.95, tamoxifen r = 0.94, 4OH-tam r =0.49 overall and r= 0.81 excluding one outlier, and ND-tam r=0.98. Method differences were examined for each analyte using paired-tests, and no significant difference was identified for any analyte. Figure 2 shows scatter plots for each analyte using two methods. For the ND-tam assay nine samples were not assayed due to insufficient volumes. The samples were selected and subsequently thawed as before, extracted via solid

phase extraction columns from a different manufacturer, but using the identical reagents and procedures as previously described for the LC-MS /MS technique, and with propranolol[3, 5] being substituted for the deuterated internal standards. The dried extracted tamoxifen and metabolites were suspended in mobile phase and analyzed on a Varian HPLC system with Starworks Software after injection onto a reverse phase Spherisorb CNRP column heated at 35 degrees C temperature.

Discussion

The primary goal of our study was to measure the concentration levels of tamoxifen and tamoxifen metabolites in serum samples taken from volunteer cancer survivors who had been treated with tamoxifen. The aim was to investigate the possible association of serum levels of tamoxifen and metabolites of tamoxifen, breast cancer outcomes and the CYP2D6 gene. The results of this primary study have been reported elsewhere.[21] The secondary goal of our study was to develop an alternative method for use in place of the LC-MS/MS method, for the quantification of the tamoxifen and its metabolites.

The HPLC separation and quantitation was by fluorescence detection, and calculation was based on peak height. One calibrator sample containing tamoxifen and its metabolites was analyzed with each batch of samples. While the use of a single calibrator is unusual, it is not uncommon in analytical quantitation assays. Others may choose to include additional calibrator points and quality control samples to monitor this HPLC assay. The upper limit of sensitivity for 4-OH tam by LC-MS /MS is reported to be 6ng/mL. The upper limit for our HPLC fluorescent detection method is 125ng/mL. We believe this difference is explained by the sample extraction column used in both methods. We compared the performance of the SPE Waters Oasis column used for the LC-MS /MS and the SPE column supplied by Phenomenex (data not shown). We found that the trough between the near eluting peaks were 12 second greater in retention times on the Oasis column extracted samples; however, the sensitivity of the peak heights in the SPE column from Phenomenex was greater by about 12%.

Results obtained for our HPLC fluorescent method for recovery of added drug (Table 1), extraction efficiency (Table 2), the estimation of accuracy and precision (Table 3) and the freeze-thaw exercise (Table 4) indicates that this HPLC method has performed in a manner consistent with the performance characteristics of the LC-MS /MS method. The ruggedness of the HPLC method is evident by the fact that similar results were obtained in different laboratories, and the robustness of the method seen with the similarity of results after the variation of analytical conditions. Additional proof of the reliability and accuracy of the new HPLC method was obtained when it was decided that the LC-MS /MS method used for comparative purposes, would itself be audited for accuracy and reliability. A second aliquot of the original serum samples, not previously thawed, were assayed by another LC-MS/MS method at the Mayo Medical Laboratories (Rochester, MN), and the obtained results showed concordance for tamoxifen, endoxifen, and ND-tamoxifen, but less concordance for 4-OH-tamoxifen.[21]

In conclusion, the results for tamoxifen and its metabolites, obtained by our HPLC fluorescent method, has shown concordance with results obtained in 2 independent laboratories using LC-MS/MS techniques. This study confirms that the HPLC method offers a useful, accurate and comparable alternative for the quantification of tamoxifen and its metabolites. The LC-MS/MS method is accurate and reliable; however, this and some of the liquid chromatographic methods reviewed here do require more expensive and specialized equipment and a higher level of technical expertise that could be considered too expensive for routine analytical and most research laboratories. This HPLC-fluorescent method also requires instrumentation; however, the equipment and software are not specialized and can be used for many laboratory analyses.

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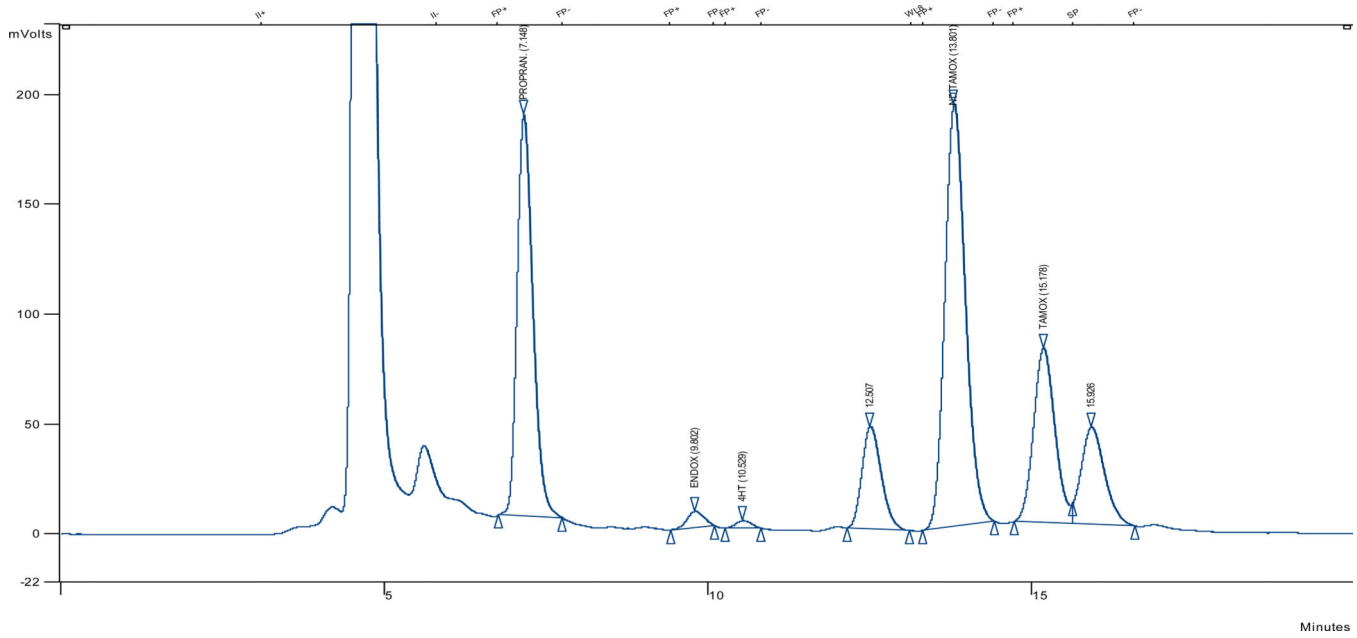

Figure 1.

Typical serum chromatogram of tamoxifen and metabolites obtained using the recommended high performance liquid chromatography column and conditions at 256 nm excitation and 380 nm emitting wavelength and flow rate of 1.0 mL/min

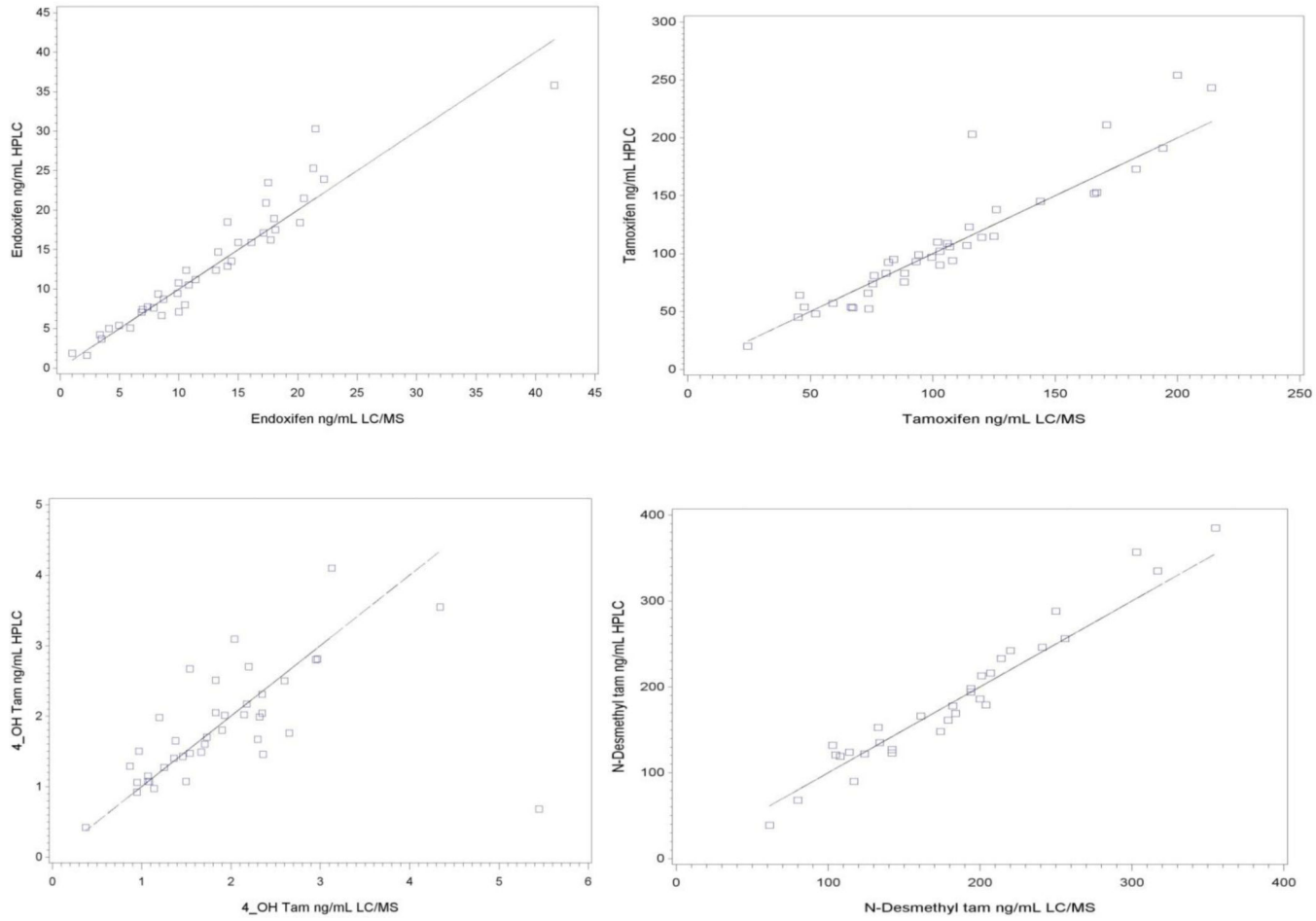

Figure 2.

Total serum tamoxifen and tamoxifen metabolites (ng/mL) measured by high performance liquid chromatography compared with concentrations measured by LC-MS/MS

Table 1

Summary of results for recovery of added tamoxifen and metabolites

	Pool HIGH	Pool MEDIUM	Pool LOW
ENDOX CALC. CONC. ng/mL	98	49	1.95
Number of assays	5	5	5
Recovered conc. (Mean[SD]) ng/mL	95 [2.3]	45 [1.1]	1.74 [0.09]
Coeff.of Variation %	2.4	2.6	5.1
Recovery %	97	92	89
4HT CALC. CONC. ng/mL	101	50	2.0
Number of assays	5	5	5
Recov. conc. (Mean[SD]) ng/mL	99 [2.9]	47 [1.1]	1.70 [0.1]
Coeff.of Variation %	2.9	2.4	6.6
Recovery %	98	94	85
ND-Tam Calc. Conc. ng/mL	200	100	4.0
Number of Assays	5	5	5
Recov. Conc.(Mean[SD]) ng/mL	206 [6.0]	93 [1.8]	4.0 [0.1]
Coeff. Of Variation %	2.9	0.2	3.6
Recovery %	103	93	100
Tamox. Calc. Conc. ng/mL	90	45	1.8
Number of assays	5	5	5
Recovery Conc.(Mean [SD]) ng/mL	92 [2.8]	43 [0.6]	1.6 [0.06]
Coeff. Of Variation %	3.0	1.3	3.5

Table 2

Summary of extraction efficiency results

	ENDOXIFEN 92ng/mL Peak Height, MicroVolts Mean [SD], CV%	**4-HT 95ng/mL Peak Height, MicroVolts** Mean [SD], CV%	**ND-TAM 197ng/mL Peak Height, MicroVolts** Mean [SD], CV%	**TAM 89ng/mL Peak Height, MicroVolts** Mean [SD], CV%	**INTERNAL STD. 200ug/mL Peak Height, MicroVolts** Mean [SD], CV%
NON-EXTRACTED GROUP					
Pool A, (N=3)	18,594 [2008] 10.8	22,950 [906] 3.9	103,450 [2774] 2.7	17,552 [560] 3.2	42,513 [1134] 2.7
EXTRACTED GROUP					
Pool A, (N=3)	44,796 [981] 2.2	59,774 [1154] 1.9	306,300 [14,398] 4.7	57,673 [1618] 2.8	122,626 [3249] 2.7
EXTRAC. EFF	240%	260%	296%	329%	288%
	ENDOXIFEN 46ng/mL Peak Height, MicroVolts Mean [SD], CV%	**4-HT 49ng/mL Peak Height, MicroVolts** Mean [SD], CV%	**ND-TAM 93ng/mL Peak Height, MicroVolts** Mean [SD], CV%	**TAM 43ng/mL Peak Height, MicroVolts** Mean [SD], CV%	**INTERNAL STD. 200ug/mL Peak Height, MicroVolts** Mean [SD], CV%
NON-EXTRACTED GROUP					
Pool B, (N=2)	5,238 [103] 2.0	6,969 [28] 0.4	34,047 [448] 1.3	4,267 [33] 0.8	37,761 [162] 0.4
EXTRACTED GROUP					
Pool B, (N=2)	15,724 [214] 1.4	21,019 [293] 1.4	103,103 [3891] 3.8	15,927 [309] 1.9	115,232 [1847] 1.6
EXTRAC. EFF	300%	302%	303%	373%	305%

Table 3

Within-run and run-to-run variation results

	ENDOX 125ug/mL	4HT 125ug/mL	ND-TAM 250ug/mL	TAMOX 125ug/mL
RUN 1	132	130	267	134
	132	132	275	135
	122	123	252	125
	118	118	235	119
MEAN	126	126	257	128
SD	7.1	6.4	17.6	7.6
CV	5.6	5.1	6.9	6
RUN 2	124	123	278	137
	115	112	244	122
	118	115	252	124
	126	121	249	113
MEAN	121	118	256	124
SD	5.1	5.1	15.2	9.9
CV	4.2	4.3	5.9	7.9
RUN 3	118	115	250	128
	117	114	245	119
	118	115	250	124
	119	115	250	122
MEAN	118	115	249	123
SD	8.2	0.5	2.5	3.8
CV	6.9	0.43	1	3.1
RUN-RUN				
MEAN	122	119	254	125
SD	5.8	6.5	12.8	7.2
CV	4.7	5.5	5	5.8

Table 4

Summary of freeze-thaw results

N=5 ng/mL	Pool HIGH Mean [SD] CV	Pool MEDIUM Mean [SD] CV	Pool LOW Mean [SD] CV
ENDOX	95 [2.3]	45 [1.1]	1.74 [0.1]
Baseline	2.4	2.6	5.1
ENDOX	91 [0.7]	43 [1.4]	1.86 [0.2]
Day 1	0.8	3.3	9.0
ENDOX	86.4 [3.4]	38 [0.83]	2.2 [0.1]
Day 2	3.9	2.2	3.2
ENDOX	96.4 [1.5]	47 [0.84]	2.2 [0.2]
Day 3	1.6	1.8	9.3
4-HT	99 [2.9]	47.4 [1.14]	1.68 [0.11]
Baseline	2.9	2.4	6.5
4-HT	97.4 [1.34]	46.2 [1.8]	2.2 [0.1]
Day 1	1.34	3.9	5.3
4-HT	96 [4.1]	43 [0.9]	2.3 [0.2]
Day 2	4.3	2.1	8.3
4-HT	100 [2.0]	47 [0.84]	2.2 [0.1]
Day 3	2.0	1.8	5.6
ND-Tam	206 [6.0]	93 [1.82]	4.0 [0.14]
Baseline	2.9	0.2	3.6
ND-Tam	207 [2.54]	92 [3.2]	4.3 [0.2]
Day 1	1.23	3.4	4.5
ND-Tam	212 [3.9]	93 [0.9]	4.9 [0.2]
Day 2	1.8	1.0	3.4
ND-Tam	232 [5.2]	105 [3.8]	4.8 [0.3]
Day 3	2.2	3.7	6.5
Tam	92 [2.8]	43 [0.6]	1.64 [0.1]
Baseline	3.0	1.3	3.5
Tam	86.2 [1.92]	38 [1.4]	1.64 [0.1]
Day 1	2.2	3.7	5.5
Tam	85 [0.83]	34 [0.4]	1.92 [0.2]
Day 2	1.0	1.3	7.6
Tam	83 [1.8]	33.4 [0.9]	1.56 [0.2]
Day 3	2.2	2.7	9.7