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Optimizing high-performance liquid chromatography method with fluorescence detection for quantification of tamoxifen and two metabolites in human plasma: Application to a clinical study

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Abstract

We set an improved high-performance liquid chromatography method with fluorescence detection HPLC-FLU assay with more sensitivity and precision for the quantification of tamoxifen and two metabolites: 4-hydroxytamoxifen and *N*-desmethyltamoxifen. The compounds and internal standard, mexiletine, were separated with an Agilent Extend C_{18} column set at 65 °C and a mobile phase of methanol–1% triethylamine aqueous solution (pH 11; 82:18, v/v). The detection system utilized offline ultraviolet irradiation to convert the analytes to their respective photocyclisation products, followed by fluorescence detection (λ_{ex} = 260 nm and λ_{em} = 375 nm). The limits of quantification for tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen in plasma were improved to 0.5, 0.5 and 0.1 ng/ml, respectively. And the retention times for tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen were minimized to 11, 10 and 3.9 min, respectively. A single stage liquid–liquid extraction method for determination of these triphenylethylene drugs in plasma was developed, with high extraction efficiency and rapid sample treatment for target compounds. The method has been validated for use in a clinical bioavailability research of tamoxifen.

Keywords: Tamoxifen; 4-Hydroxytamoxifen; *N*-Desmethyltamoxifen; Triphenylethylene fluorescence; Bioavailability

1. Introduction

Tamoxifen [*Z*-1-(4-β-dimethylaminoethoxy-phenyl)-1,2-diphenylbut-1-ene] (TAM), a non-steroidal selective estrogen receptor modulator (SERM), is a first-line drug in the treatment of breast cancer [1]. And it has also been approved in the Untied States as a chemopreventative agent in women at high risk in 1978 [2]. Recent random prevention trials showed that TAM can reduce the risk of ER-positive breast cancer clearly [3,4]. Likewise, the long-term safety of TAM is also a subject of controversy, because its estrogen agonistic properties may cause side effects like endometrial cancer and thromboembolic diseases [5,6]. And it has also been linked to numerous undesirable side effects. Symptoms such as mild nausea, vaginal dryness and discharge, irritability, headache, fever, inability to concentrate, and fatigue have also been described in samples of breast cancer patients taking TAM [7].

It is unclear why some women experience TAM-related side effects while others do not experience side effects. One hypothesis is that the appearance of symptoms is related to the metabolism of TAM [8]. Its side effects may be dose- and concentration-dependent [9] and an increased risk of endometrial cancer has been associated with duration of treatment and accumulated dose [10]. Furthermore, the activity and side effects of TAM may be attributed not only to concentrations of the parent drug but also to its biologically active metabolites and their accumulation in plasma.

TAM is extensively metabolized by the cytochrome P450 enzyme system in vivo into several metabolites that have variable potencies towards the estrogen receptor, including *N*-desmethyltamoxifen (DMT), 4-hydroxytamoxifen (OHT) and tamoxifen-*N*-oxide, α-hydroxytamoxifen, and *N*-didesmethyltamoxifen [11,12]. Among these, the metabolite OHT has been shown to be a potent anti-estrogen 30–100-fold

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more potent than TAM itself [13]. So TAM is considered as a prodrug increased activity after activation in vivo [14]. However, serum concentrations of OHT are only 2% of the parent drug levels, whereas DMT concentrations are about 1.5–2 times greater [15]. Recently some papers [14,16] have demonstrated 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), a metabolite of TAM, is equipotent to OHT in estrogen receptor-alpha (ERα) and receptor-beta (ERβ) binding, as well as in the inhibition of 17β-estradiol (E2)-induced proliferation in human breast cancer cells. Although DMT and OHT not the only metabolites of TAM, we chose to focus on them rather than others because they are predominant. Many of the other TAM metabolites are either difficult to detect or rapidly cleared from the body except endoxifen [8]. Because of lack of standards, we were not able to measure endoxifen in our paper.

The analytical methods of TAM and its metabolites has been well documented, including GC–MS [17,18], HPLC [19–22], TLC [23,24], LC–MS [8,25], CE [26] and CE–MS [27]. Procedures based on gas chromatography or capillary electrophoresis–electrospray ionization with mass spectrometry are highly specific, but require derivatization of sample and involve equipment not generally available [28]. Several groups published thin-layer or high-performance liquid chromatographic methods [19–22,28–30] involve photochemical conversion of TAM and its metabolites to fluorescent phenanthrene derivates, which may be detected by fluorescence detectors with high sensitivity. In 1980, Golander and Sternson [29] described the high-performance liquid chromatography method with fluorescent detection (HPLC-FLU) by offline pre-column UV irradiation for the determination of TAM and metabolites. Later, many methods pointed out the technical problem of the assay: the broad, irregular peaks and irreproducible results of chromatograph and avoided the problem by using post-column irradiation [19]. But compared to other post-column methods, the offline pre-column HPLC-FLU method still had some advantages in the sensitivity and the convenience. So we optimized the chromatograph conditions of the assay and set an optimized HPLC-FLU assay with more sensitivity and precision. The detection system used offline direct ultraviolet irradiation to convert solutes into their respective photocyclisation products, followed by fluorescence detection ($\lambda_{ex} = 260$ nm and $\lambda_{em} = 375$ nm), with undetectable degradation of parent compound in our improved HPLC conditions.

Finally the method was validated in recovery, accuracy, precision and stability and applied for the bioequivalence study of the TAM in Chinese healthy subjects.

2. Experimental

2.1. Chemicals and reagents

Tamoxifen and 4-hydroxytamoxifen were purchased from Sigma (St. Louis, MO, USA). *N*-Desmethyltamoxifen was purchased from T.R.C. (North York, Canada). Internal standard mexiletine (purity > 99%) was provided by China Pharmaceutical University. All these were stored at −20 °C until use. The structures of the standards were described in Fig. 1.

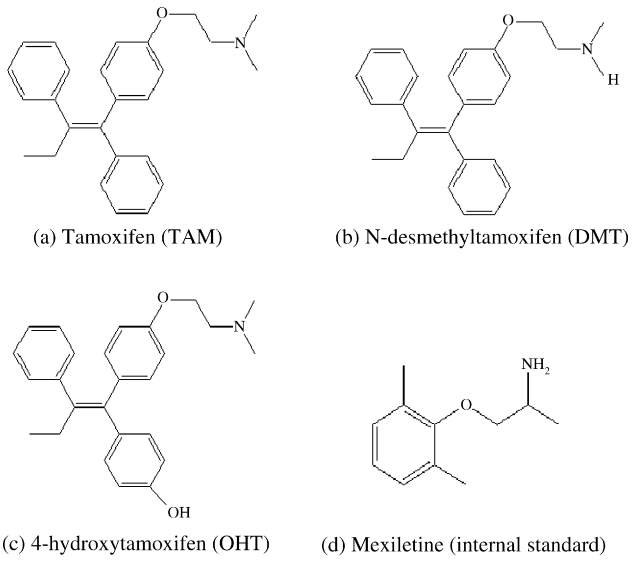

Fig. 1. Chemical structures of tamoxifen (TAM), *N*-desmethyltamoxifen (DMT), 4-hydroxytamoxifen (OHT) and mexiletine (internal standard).

The HPLC-grade methanol, hexane and butanol were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used were of good commercially quality available.

2.2. Standard solutions

Standard solutions of TAM, DMT and OHT were prepared by dissolving free-base of each compound 5 mg in 10 ml of methanol. Dilutions of the standard stock solutions for TAM and DMT, were made in methanol from 0.5 to 200 ng/ml to prepare for the standard curve and quality control (QC) samples, for OHT were made in methanol from 0.1 to 10 ng/ml. The internal standard stock solution of mexiletine was prepared by dissolving 1 mg free-base in 10 ml methanol, then diluting the solution to 100 ng/ml. All solutions were stored at −20 °C until analysis.

2.3. Standard curves

Seven-point standard curves were prepared by adding known concentrations of TAM and its metabolites covering the range anticipated in this study to drug-free plasma. OHT was added at 0.1, 0.2, 0.5, 1, 2, 5 and 10 ng/ml, whereas the concentrations of the other compounds of interest were 0.5, 1, 3, 10, 30, 100 and 200 ng/ml. All solutions were stored at −20 °C until analysis.

2.4. Sample preparation

All plasma samples were thawed at room temperature. One millilitre plasma was placed into clean centrifuge tube, and then 50 μl internal standard solution mexiletine (100 ng/ml) were added to each tube and mixed. The mixtures were added to 200 μl NaOH solutions (NaOH in methanol) with a concentration of 1 mmol/l vortex-mixed for 20 s, and allowed to stand for at least 5 min before extraction with 5 ml mixture of hexane–butanol (98:2, v/v). Tubes were then mixed on a vortex mixer for 20 s and centrifuged for 10 min. Exactly 4 ml of the

organic phase was transferred to another tube and evaporated under dry nitrogen on a water bath at 40 °C The dry residue was re-dissolved by 200 μl mobile phase, and centrifuged at the speed of 16,000 rpm for 2 min. Ten microlitres volume of the supernatant was injected onto the column.

2.5. Instruments and chromatography conditions

High-performance liquid chromatography was accomplished by using an Agilent 1100 series liquid chromatography with a binary pump, on-line degasser, autosampler and column heater, a UV detector and a fluorescence detector.

An Agilent Extend C_{18} chromatography column (150 mm × 4.6 mm, 5μm, Agilent, USA), incorporating a unique patented bidentate silane, combined with a double-endcapping process that protects the silica from dissolution at high pH up to pH 11.5, was used with the following analytical conditions: a modified phase of methanol–1% triethylamine aqueous solution (pH 11; 82:18, v/v), a flow rate of 1.0 ml/min at 65 °C.

UV lamp set at 254 nm was used to convert the tamoxifen and its metabolites to highly fluorescent phenanthrene derivatives. The standard solutions and the plasma samples were converted to fluorescent derivatives by offline UV irradiation for 10 min, then injected 10 μl volume onto HPLC for analysis.

The fluorescent detector was set at an excitation wavelength of 260 nm and emission wavelength of 375 nm. Peak areas of each compound were generated from computerized software (Agilent, USA).

The chromatographic data were processed using the I.S. method of plotting peak area ratios of analytes/I.S. versus the relative concentration followed by least square regression of these data.

2.6. Pharmacokinetics study

The method was applied to evaluate the bioavailability of TAM in Chinese healthy volunteers ($n = 20$). In a randomized cross-over design, they were treated with a single dose of 20 mg of tamoxifen in tablets. After a wash-out period of 6 weeks the next single dose followed. Treatment A was a 20 mg TAM tablet from Xianhe Pharmaceuticals (China) and treatment B was a 20 mg TAM tablet from Leiras Pharmaceuticals (Finland).

They were asked to provide 10 ml of blood of every data point to measure concentrations of TAM and TAM metabolites. Blood samples were collected at the following data point: before and 1.0, 3.0, 5.0, 7.0, 9.0, 12, 24, 60, 108, 180, 276, 372 and 492 h post-dosing. Then the samples were stored in a dark freezer at −65 °C until detection. Prior to the trial, all procedures were approved by the Ethics Committee of Nanjing Medical University.

3. Results and discussion

3.1. Selectivity

In conditions described above, TAM, DMT and OHT exhibited good chromatography with baseline resolution of each compound. The method described in our paper was selective and specific. There were no foreign peaks interfered with analytes and internal standard at the retention times. The retention times for TAM, DMT, OHT and internal standard were 11, 10, 3.9 and 2.4 min, respectively (Fig. 2).

Many compounds were tested for possible use as an internal standard, e.g. mexiletine, biphenyl and diazepam. Mexiletine exhibited the most suitable retention time and peak shape of all compounds tested, and its intensity of fluorescence was not changed by UV irradiation. Thus, mexiletine was selected as the internal standard for this assay.

3.2. Linearity

Calibration curves were determined by least squares linear regression analysis. Linear regression calibration curves based on seven data points, were constructed for each compound by plotting peak area ratio (f, f = As/Ai) of the compounds (As) to internal standard mexiletine (Ai) versus the concentrations (C) of plasma standard of each compound. The results were expressed as the regression equations with the weight values.

The calibration curves were linear from 0.5 to 200 ng/ml for TAM and DMT, 0.1 to 10 ng/ml for OHT. The mean values of regression equation of the analytes in plasma were as follows:

- TAM: $y = 0.0193x + 0.0064$, $r = 0.9994$ ($n = 5$);
- DMT: $y = 0.0017x + 0.0006$, $r = 0.9997$ ($n = 5$);
- OHT: $y = 0.0094x + 0.0008$, $r = 0.9979$ ($n = 5$).

The correlation coefficient values of all three compounds were greater than 0.99. The weight values (W) of every compound represent $1/(C \times C)$.

3.3. Sensitivity

Sensitivity was evaluated by the limit of detection (LOD) and the limit of quantification (LOQ) of every compound. LOD for our method was defined as the concentration of the analyte that produces a peak whose height is three times the height of the noise from blank sample (S/N = 3, parameter S/N represents the ratio of signal to noise). The LOD for TAM, DMT and OHT were 0.25, 0.25 and 0.05 ng/ml, respectively. LOQ was obtained for every compound by taking five replicates the lowest calibration standard (S/N = 10). The LOQ for TAM, DMT and OHT were 0.5, 0.5, 0.1 ng/ml, respectively.

Compared to the previous HPLC-FLU assay described in current literatures, we improved the LOQ of the target compounds to subnanogram, with regular and reproducible results.

3.4. Recovery

Recovery of all compounds of interest was tested in quality control (QC) samples. QC samples were made in plasma at three levels. The higher concentration sample contained OHT at 5.0 ng/ml and all other components at 100 ng/ml. The middle contained OHT at 1.0 ng/ml and all others at 10 ng/ml. For QC at a lower concentration the samples contained OHT at 0.2 ng/ml

and the remainder of components at 1 ng/ml. And the biological samples were extracted and detected as the procedure described above.

The recovery (*R*%) was calculated by peak area of the extracted samples (Ax) versus the non-extracted (As) at the similar concentration. Extraction efficiency is calculated by the following equation: $R\% = \mathrm{Ax/As} \times 100$ ($r = 5/4$, parameter r was the correction factor, it represents when extraction with the hexane–butanol, the volume ratio of organic phase pre- and post-extraction).

The present method produced a satisfactory recovery of 88.4–92.1%, thus implying that extraction of the plasma did

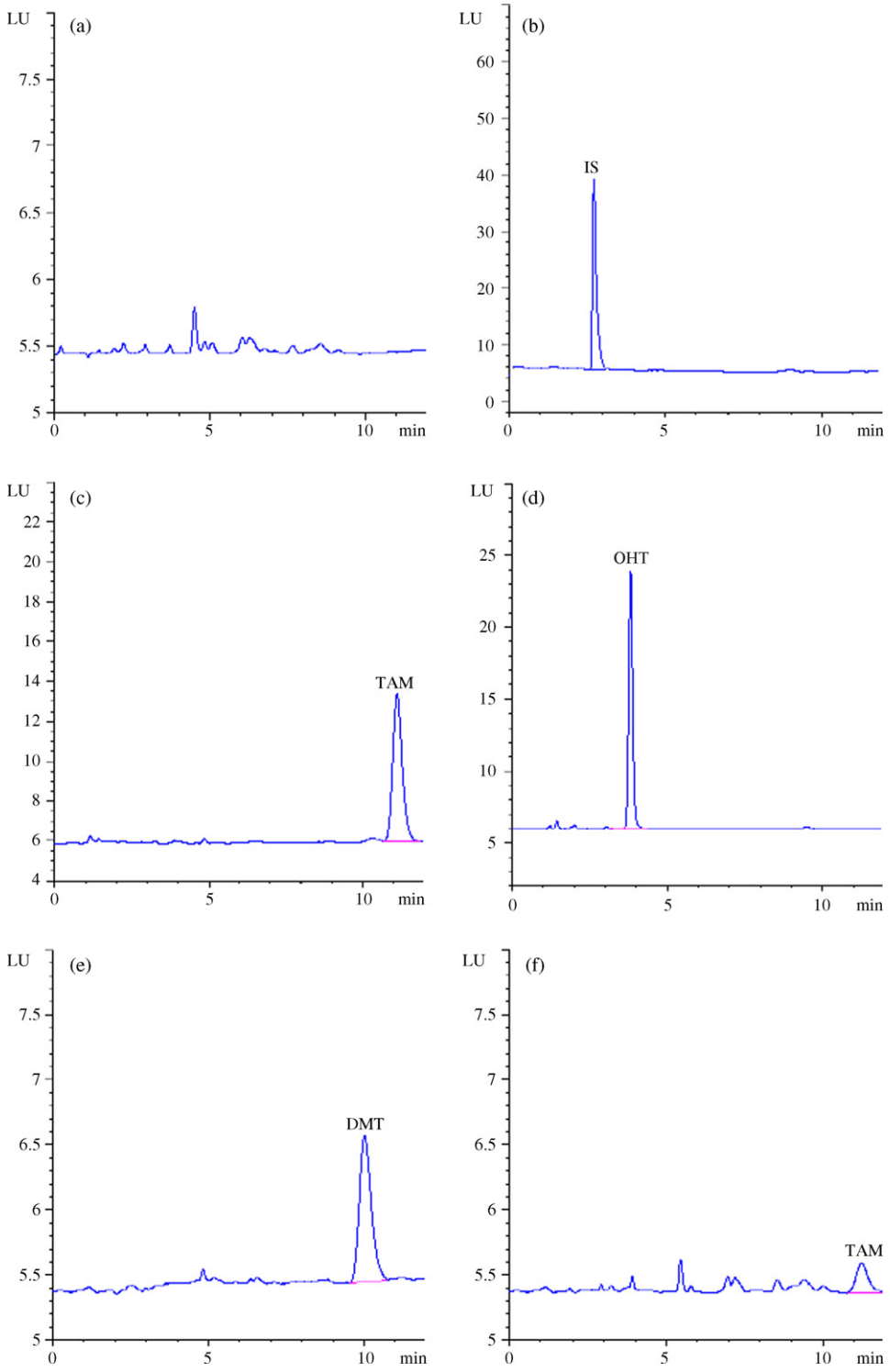

Fig. 2. Chromatograms of (a) blank plasma; (b) standard solution containing 100 ng/ml mexiletine (IS); (c) standard solution containing 50 ng/ml tamoxifen (TAM); (d) standard solution containing 5 ng/ml 4-hydroxytamoxifen (OHT); (e) standard solution containing 50 ng/ml *N*-desmethylamoxifen (DMT); (f) blank plasma containing 0.5 ng/ml TAM; (g) blank plasma containing 50 ng/ml TAM, 50 ng/ml DMT and 5 ng/ml OHT; (h) subjects taking oral tamoxifen (TAM).

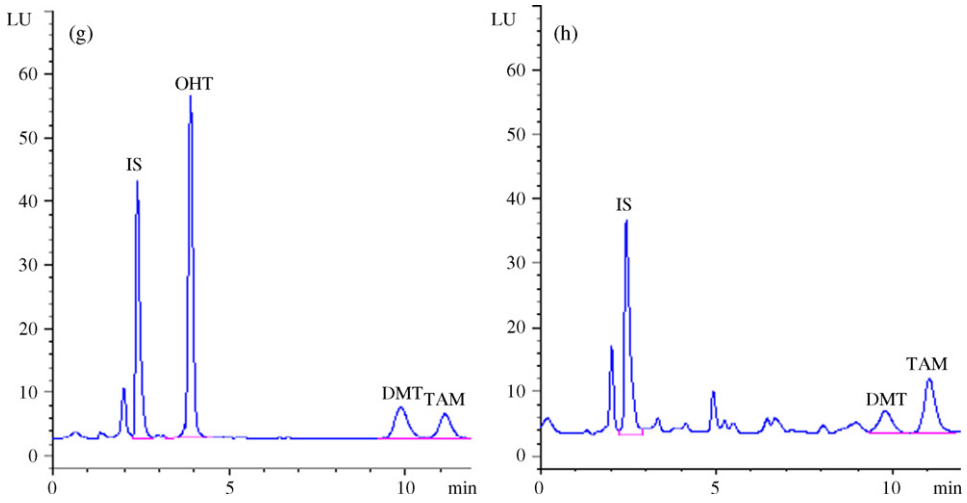

Fig. 2. *(Continued)*

not result in any substantial loss of the chemical constituents (Table 1). Data are expressed as mean ± S.D. ($n = 5$).

3.5. Assay accuracy and precision

The precision and accuracy of the method were assessed in plasma by performing replicate analyses of spiked samples against calibration standards. The procedure was repeated on the same day and between three different days on the same spiked standard series. The within-day and between-day precision and accuracy of the method are shown in Table 2. The precisions (R.S.D.%) were all less than 10%. The data indicated that the precision and accuracy of the method are acceptable.

3.6. Stability

In order to assess autosampler stability QC samples were included at the start, at half-way and at completion of the analytical runs. Autosampler temperature was set at 4 °C, while the anticipated batch run time was less than 12 h. The mean result at the completion of the runs should be ≥90% of the mean result at the start for at least three levels tested.

To evaluate freeze–thaw stability, plasma samples containing three concentration levels of target compounds were used. A freeze and thaw cycle was defined as the storage of QC samples at −65 °C, followed by thawing at room temperature. All samples were analyzed after the fourth cycle in 1 month, along with fresh reference samples of the same concentration. The results (back-calculated concentrations) of four freeze–thaw cycles verus fresh ones, which should not vary more than 10%.

To evaluate the long-term stability, the QC samples were initially frozen at −65 °C for 30 days, thawed and analyzed. Stock and working solutions stability (stored at 4 °C) was estimated by comparing fresh and old dilutions in mobile phase. All of the mean variations were below 10% (data not shown). The results proved that our method was stable and suitable for studies involving pharmacokinetics of TAM and its metabolites.

3.7. Improvements of our assay

The optimized UV irradiation time was determined to be 10 min by experimenting with various exposure time to high intensity UV light. Exposure time less than 12 min produced insufficient activation, while longer exposures led to the breakdown of the parent compounds as determined the appearance of additional peaks (data not shown).

Some factors may contribute to our HPLC results were discovered in the process of the optimization of chromatograph system; the interference of endogenous peaks decreased at high

Table 1
Recovery of TAM, DMT and OHT in human plasma ($n = 5$)

Analyte	Added (ng/ml)	Recovery (%) (mean ± S.D.)	R.S.D. (%)
TAM	1.0	92.1 ± 3.0	3.3
	10.0	95.5 ± 2.2	2.3
	100.0	93.8 ± 2.1	2.2
DMT	1.0	88.4 ± 6.5	7.4
	10.0	91.2 ± 3.9	4.3
	100.0	93.0 ± 3.3	3.5
OHT	0.2	89.6 ± 4.4	4.9
	1.0	90.6 ± 2.7	3.0
	5.0	95.1 ± 3.0	3.2

Table 2
Within-day and between-day precision of TAM, DMT and OHT

Analyte	Added (ng/ml)	Within-day (%), R.S.D. ($n = 15$)	Between-day (%), R.S.D. ($n = 15$)
TAM	1.0	7.7	9.8
	10.0	6.2	8.8
	100.0	5.3	7.6
DMT	1.0	4.5	6.8
	10.0	4.2	5.2
	100.0	3.5	4.9
OHT	0.2	5.2	7.1
	1.0	4.0	6.6
	5.0	4.2	5.4

Table 3
Calculated pharmacokinetics parameters for two groups of Chinese healthy subjects

Parameters	Group A	Group B
C_{max} (ng/ml)	71.8 ± 14.4	68.5 ± 15.8
t_{max} (h)	6.3 ± 2.2	6.7 ± 2.4
$AUC_{0-\tau}$ (ng h ml^{-1})	4608.4 ± 2054.5	4586.1 ± 2026.2
$AUC_{0-\infty}$ (ng h ml^{-1})	5016.7 ± 2253.1	5074.9 ± 2241.1
F (%)	101.3 ± 12.9	–
$t_{1/2}$ (h)	143.3 ± 24.5	153.01 ± 32.91

Data given are the mean ± S.D. values from 20 subjects. C_{max} is the maximal concentration; t_{max} is the time when the maximal concentration is reached; $AUC_{0-\tau}$ is the peak area under the curve from predose to the last sampling time; $AUC_{0-\infty}$ is the peak area under the curve from predose extrapolated to infinity; F is the relative bioavailability (Group A vs. Group B); $t_{1/2}$ is the mean elimination half-life.

pH up to 11, with the sensitivity of the assay increased. And the retention times were improved by the cooperation of the changes in organic phase and the growth of column temperature. In our HPLC system, the composition of organic phase methanol improved to more than 80% and the column temperature set at 65 °C in the alkaline mobile phase (pH 11) provides the best chromatogram, with better peak shapes and no observable parent compounds degradation.

Another major feature of our method was the improvements of LOQ. Compared with previous assays, we improved the LOQ of TAM, DMT and OHT to 0.5, 0.5 and 0.1 ng/ml. The sensitivity of our assay was higher than the following HPLC-FLU methods, which can afford the trace amount determination of target compounds.

3.8. Pharmacokinetics study

The results of pharmacokinetics study were described in Table 3. We compared the calculated parameters of tamoxifen in two groups. Group A was the pharmacokinetics results of the subjects taking oral of tamoxifen (Xianhe Pharmaceuticals, China), and Group B was the results of control group—the results of subjects taking oral of tamoxifen (Leiras Pharmaceuticals, Finland).

4. Conclusions

The method presented here describes a specific, sensitive and reproducible human plasma assay using HPLC with internal standard and fluorescence detection for the determination and quantification of TAM and its metabolites. The chromatogram performance criteria for the target compounds have been assessed and were within the SFDA (State Food and Drug Administration) recommended guidelines. Finally the method was validated by the pharmacokinetics of TAM in a clinical study.

Also there are limitations of our present study. First, because of lack of standards, we were not able to measure metabolite 4-hydroxy-*N*-desmethyltamoxifen (endoxifen) in the paper, which would be discussed in our further study. Second, the single dose study of TAM for bioavailability cannot demonstrate the tendency of accumulation for TAM and its metabolites. However, our assay may be useful for further study of the plasma concentrations of these compounds.

In another way, many works were focused on the low-dose study of TAM, which seems to be as active as the conventional dose on tumor cell proliferation. The positive correlation between the concentrations of TAM supports the argument that therapeutic drug monitoring may be introduced to optimize TAM treatment [21,22]. But the plasma concentrations of some metabolites in low-dose study were always so slow that cannot be detected conveniently. As the high sensitivity and simplicity of performing the analyses, our method is very suitable for the studies of pharmacokinetics, anti-cancer activity, therapeutic drug monitoring, and even the low-dose study of TAM.

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