Journal of Chromatography A, 1434 (2016) 64–69

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Reductive amination-assisted quantitation of tamoxifen and its metabolites by liquid phase chromatography tandem mass spectrometry

Shih-Shin Liang[a,b,*], Tsu-Nai Wang[c,d], Chien-Chih Chiu[a], Po-Lin Kuo[e], Mei-Fang Huang[a], Meng-Chieh Liu[a], Eing-Mei Tsai[f,g,**]

[a] Department of Biotechnology, College of Life Science, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[b] Institute of Biomedical Science, College of Science, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan
[c] Department of Public Health, College of Health Science, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[d] Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[e] Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[f] Headquarters of Research Centers, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[g] Department of Obstetrics and Gynecology, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan

ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 6 January 2016
Accepted 6 January 2016
Available online 11 January 2016

Keywords:
Tamoxifen
Reductive amination
Absolute quantification
Endoxifen
N-Desmethyltamoxifen

ABSTRACT

Tamoxifen, a hormonal therapy drug against estrogen receptor-positive breast cancer, can be metabolized by cytochrome P450 enzymes such as CYP3A4 and CYP3A5, and converted to *N*-desmethyltamoxifen, which is subsequently, metabolized by CYP2D6 and inverted to form 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). Conventional mass spectrometry (MS) analyses of tamoxifen and its metabolites require isotopic internal standards (ISs). In this study, endoxifen and *N*-desmethyltamoxifen amine groups were modified by reductive amination with formaldehyde-D_2 to produce new metabolite molecules. Both endoxifen and *N*-desmethyltamoxifen generated their corresponding D_2-methyl modified analogs. This method is expected to simplify MS detection and overcome the difficulty in selecting adequate ISs when tamoxifen metabolites are analyzed by absolute quantification. It identified tamoxifen, D_2-methyl modified endoxifen, and D_2-methyl modified *N*-desmethyltamoxifen with a linearity ranging from 2 to 5000 ng/mL with correlation coefficient (R^2) values of 0.9868, 0.9849, and 0.9880, respectively. Furthermore, this reductive amination-based method may enhance the signal intensities of D_2-methyl modified *N*-desmethyltamoxifen and endoxifen, thus facilitating the MS detection.

1. Introduction

The anti-breast cancer drug, tamoxifen [*Z*-(1-(4-(2-dimethylaminoethoxy)phenyl)-1,2-diphenyl-1-butene)] has proven useful for treating estrogen receptor (ER)-positive breast cancer patients [1]. Note that tamoxifen must affect ERs before forming active metabolites that exhibit over 100-fold higher ER activity than tamoxifen, such as (*Z*)-4-hydroxytamoxifen and (*Z*)-endoxifen, in the presence of cytochrome P450 (CYP) [2,3]. In particular, the cytochrome P450 enzyme CYP2D6 plays an essential role in transforming tamoxifen and *N*-desmethyltamoxifen into their corresponding 4-hydroxy analogs [3–5]. The CYP3A4/3A5 enzyme converts tamoxifen and 4-hydroxy tamoxifen into *N*-desmethyltamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), respectively [6–8]. In the primary metabolism, over 90% tamoxifen is transformed into *N*-desmethyltamoxifen by CYP3A4/3A5, whereas the rest undergoes CYP2D6-mediated hydroxylation to form 4-hydroxy tamoxifen. Eventually, the secondary metabolite endoxifen is generated by hydroxylation and demethylation [8].

According to previous studies, CYP2D6, which has been altered by genetic and environmental factors, may regulate tamoxifen and metabolites formation [8–10]. A reduction in CYP2D6 enzymatic activity limits the transformation of tamoxifen into metabolites such as 4-hydroxy tamoxifen and endoxifen, which are detected

* Corresponding author at: Department of Biotechnology, College of Life Science, Kaohsiung Medical University, 100, Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan. Fax:+886 7 3125339.
** Corresponding author at: Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, 100, Shih Chuan 1st Road, Kaohsiung 80708, Taiwan. Fax: +886 7 3112493.
E-mail addresses: liang0615@kmu.edu.tw (S.-S. Liang), tsaieing@kmu.edu.tw (E.-M. Tsai).

http://dx.doi.org/10.1016/j.chroma.2016.01.015
0021-9673/

the endoxifen plasma concentration of patients [11,12]. Moreover, this reduction significantly increases the recurrence risk and lowers the survival rate in breast cancer patients [13,14].

To date, various studies have addressed the detection of tamoxifen and its metabolites, such as endoxifen, 4-hydroxytamoxifen and *N*-desmethyltamoxifen, by liquid phase chromatography coupled to tandem mass spectrometry (LC–MS/MS) [1,7,8,15,16]. Tamoxifen and its metabolites in human plasma have been identified using analytical methods, such as LC [17], LC–MS/MS [18–22], micellar liquid chromatography [23], and gas chromatography coupled to mass spectrometry (GC–MS) [17,24]. However, a quantitative analysis requires internal standards (ISs) to guarantee a reliable MS detection. Regardless of their regular utilization in GC–MS and LC–MS, isotopic ISs are difficult to obtain [21,22]. Despite the existence of 4-hydroxytamoxifen-d_5, endoxifen-d_5, *N*-desmethyltamoxifen-d_5 and tamoxifen-$^{13}C_2$, ^{15}N solution, no proper ISs are available for tamoxifen-*N*-oxide, α-hydroxyltamoxifen, and β-hydroxyltamoxifen [8].

In this study, tamoxifen and *N*-desmethyltamoxifen were simultaneously characterized by absolute quantification using a reductive amination modification. Endoxifen was also derivatized via reductive amination to generate its D_2- and H_2-modified analogs, which acted as absolute quantification external and internal standards, respectively [25,26]. Reductive amination, or dimethyl labeling, has been extensively utilized in comparative proteomics for relative protein quantification [27–29]. However, we implemented reductive amination to metabolite detection with absolute quantification. Tamoxifen and its metabolites, including *N*-desmethyltamoxifen and endoxifen, were detected by LC–MS/MS with multiple reaction monitoring (MRM). Moreover, tamoxifen-$^{13}C_2$, ^{15}N solution was selected as an IS for the absolute quantification of tamoxifen and *N*-desmethyltamoxifen. However, formaldehyde-H_2-functionalized endoxifen was synthesized to generate its H_2-modified counterpart, i.e., 4-hydroxytamoxifen, which acted as an IS for the absolute quantification of endoxifen. The reductive amination enhanced the signal intensities of *N*-desmethyltamoxifen and endoxifen. Furthermore, this method simplified the detection of tamoxifen and *N*-desmethyltamoxifen and only one calibration curve was produced for external absolute quantification.

2. Experimental

2.1. Materials

The formaldehyde-H_2 solution (36.5–38% in H_2O), tamoxifen, *N*-desmethyltamoxifen, endoxifen hydrochloride hydrate, and formic acid (FA, 98–100%) were purchased from Sigma (St. Louis, MO, USA). Tamoxifen-$^{13}C_2$, ^{15}N solution, trifluoroacetic acid (TFA), sodium acetate, and sodium cyanoborohydride ($NaBH_3CN$) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Hydrochloric acid, sodium hydroxide, and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). The formaldehyde-D_2 solution (20% in D_2O) was purchased from Isotec Corp. (Miamisburg, OH, USA). Acetonitrile (MeCN, LC/MS grades) was acquired from Merck (Seelze, Germany). Deionized water, with a resistance of 18.3 MΩ, was produced using a Millipore system (Bedford, MA, USA). Moreover, polyvinylidene fluoride (PVDF) filters (pore size: 0.22 μm) were purchased from Millipore Co., to filter the re-dissolved precipitates.

2.2. Instrumentation and software

The separation was performed using a Thermo Finnigan Acella 1250 ultra-high-pressure liquid chromatography (UHPLC) system (Thermo Fisher Scientific Inc., Waltham, MA, USA), which was coupled with a tandem MS detector equipped with a triple quadrupole MS (Thermo Fisher Scientific Inc.). Diluted and extracted samples were sequentially injected using an Acella 1250 autosampler (Thermo Fisher Scientific Inc.) fitted with a 10 μL loop. They were separated on a Shiseido CAPCELL PAK C18 MG II column (i.d. 1.5 mm × 150 mm, 3 μm, Tokyo, Japan) using 0.1% FA in water (A) and 0.1% FA in 100% MeCN (B) as mobile phases at a flow rate of 200 μL/min. The UHPLC linear gradient was set as follows: 30% (B) for 1 min from injection, 30–60% (B) for 5 min, 60–98% (B) for 3 min, and maintained at 98% (B) for 2 min.

The MS detection was conducted using an applied voltage of 3000 V in positive ion mode and vaporization and capillary temperatures were set at 300 and 350 °C, respectively. Sheath gas and auxiliary gas pressures were fixed nitrogen gas at a flow rate of 35 arbitrary units and a flow rate of 10 arbitrary units, respectively, while the collision chamber pressure was maintained at a flow rate of 1.5 arbitrary units, and the collision energy was adjusted between 22 and 25 V. MS acquisition and control methods, such as MRM transitions, were set to the *m*/*z* values of precursor and product ions (Table 1). UHPLC system control and MS data acquisition were performed using the Xcalibur software (Version 2.2, Thermo Finnigan Inc., San Jose, CA).

2.3. Dimethyl labeling of tamoxifen metabolites

Tamoxifen, endoxifen, and *N*-desmethyltamoxifen were dissolved individually in ethanol to prepare 1000 μg/mL solutions. These solutions (10 μL) were transferred into two tubes and their pH was adjusted to 5.6 using sodium acetate buffer (150 μL). The first sample was allowed to react for 5 min with 10 μL 4% formaldehyde-D_2 solutions. The modified sample was reduced for 1 h in the presence of 0.6 M $NaBH_3CN$ and then its pH was adjusted to 2–3 using 10% TFA before mixing with its unmodified counterpart. Individual mixtures were injected into the mass spectrometer to establish *m*/*z* values, MRM transitions between modified and unmodified compounds, and signal enhancement.

2.4. Tandem MS with MRM detection mode

Tamoxifen and its metabolites were quantified using the triple quadrupole tandem MS in the MRM mode. MRM transitions were set according to the following *m*/*z* values: 372.2 > 72 and 372.2 > 129 for tamoxifen, 375.4 > 75 and 375.4 > 129 for tamoxifen-$^{13}C_2$, ^{15}N solution (IS), 358.2 > 58 and 358.2 > 129 for *N*-desmethyltamoxifen, 374.4 > 74 and 374.4 > 178 for D_2-methyl modified *N*-desmethyltamoxifen, 374.2 > 58 and 374.2 > 129 for endoxifen, 390.4 > 74 and 390.4 > 129 for D_2-methyl modified endoxifen, and 388.4 > 72 and 388.4 > 129 for H_2-methyl modified endoxifen as an IS. These MRM transitions are listed in Table 1.

3. Results and discussion

3.1. Reductive amination with formaldehyde-D_2

In this study, tamoxifen metabolites were modified by reductive amination with formaldehyde-D_2 solutions, providing a novel method to simplify their detection. In this method, external quantification tools, such as calibration curves, and the internal quantification standard tamoxifen-$^{13}C_2$, ^{15}N solution were used for the absolute quantification of tamoxifen and *N*-desmethyltamoxifen. Unlike isotopic formaldehyde, such as formaldehyde-D_2, formaldehyde-H_2 was unsuitable for this application because the modified *N*-desmethyltamoxifen would generate tamoxifen while endoxifen would be transformed into 4-hydroxy tamoxifen. Therefore, the MRM scanning mode would not distinguish the original 4-hydroxytamoxifen from