Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 1030–1036

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Production of a reactive metabolite of troglitazone by electrochemical oxidation performed in nonaqueous medium

Kayoko Tahara[a,*], Takashi Nishikawa[b], Yutaka Hattori[a], Shiro Iijima[a], Yukiko Kouno[a], Yoshihiro Abe[a]

[a] Division of Clinical Physiology and Therapeutics, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan
[b] Department of Emergency and Critical Care Medicine, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

ARTICLE INFO

Article history:
Received 14 April 2009
Received in revised form 31 May 2009
Accepted 3 June 2009
Available online 11 June 2009

Keywords:
Troglitazone
Reactive drug metabolite
Electrochemical oxidation
Nonaqueous medium
LC-MS

ABSTRACT

In order to confirm the existence of reactive metabolites by LC-MS/MS analysis, they should be modified into stable compounds, because some reactive metabolites generated by biotransformation induce drug toxicity; however, they are unstable, with very short lives, and cannot be detected in their intact forms. To overcome these problems, electrochemical oxidation of troglitazone was performed in nonaqueous medium, since such reactive compounds are stable in the absence of water. Troglitazone, an antidiabetic agent, was withdrawn from the market because of serious hepatotoxicity in some patients. It has been considered that one or more reactive metabolites are involved in hepatotoxicity, although the mechanism of the adverse reaction is unclear. Using our method of electrochemical oxidation in nonaqueous medium, we obtained a product of troglitazone derivative that may be a clue to clarify the mechanism of toxicity. The product in the reaction mixture was separated by HPLC without chemical modification and detected using UV and ESI-MS. The mass spectrum of its molecular ion showed that it was an *o*-quinone methide derivative of troglitazone and identified as a reactive metabolite generated by liver microsome oxidation of the drug. The product was stable over 24 h at room temperature in anhydrous acetonitrile, but it reacted with *N*-(*tert*-butoxycarbonyl)-L-cystein methylester to produce an adduct that could be identified by its *m*/*z* value. Thus, the method of electrochemical oxidation in nonaqueous medium is considered to be useful to prepare and predict reactive metabolites of drugs that are unstable in aqueous medium or *in vivo*.

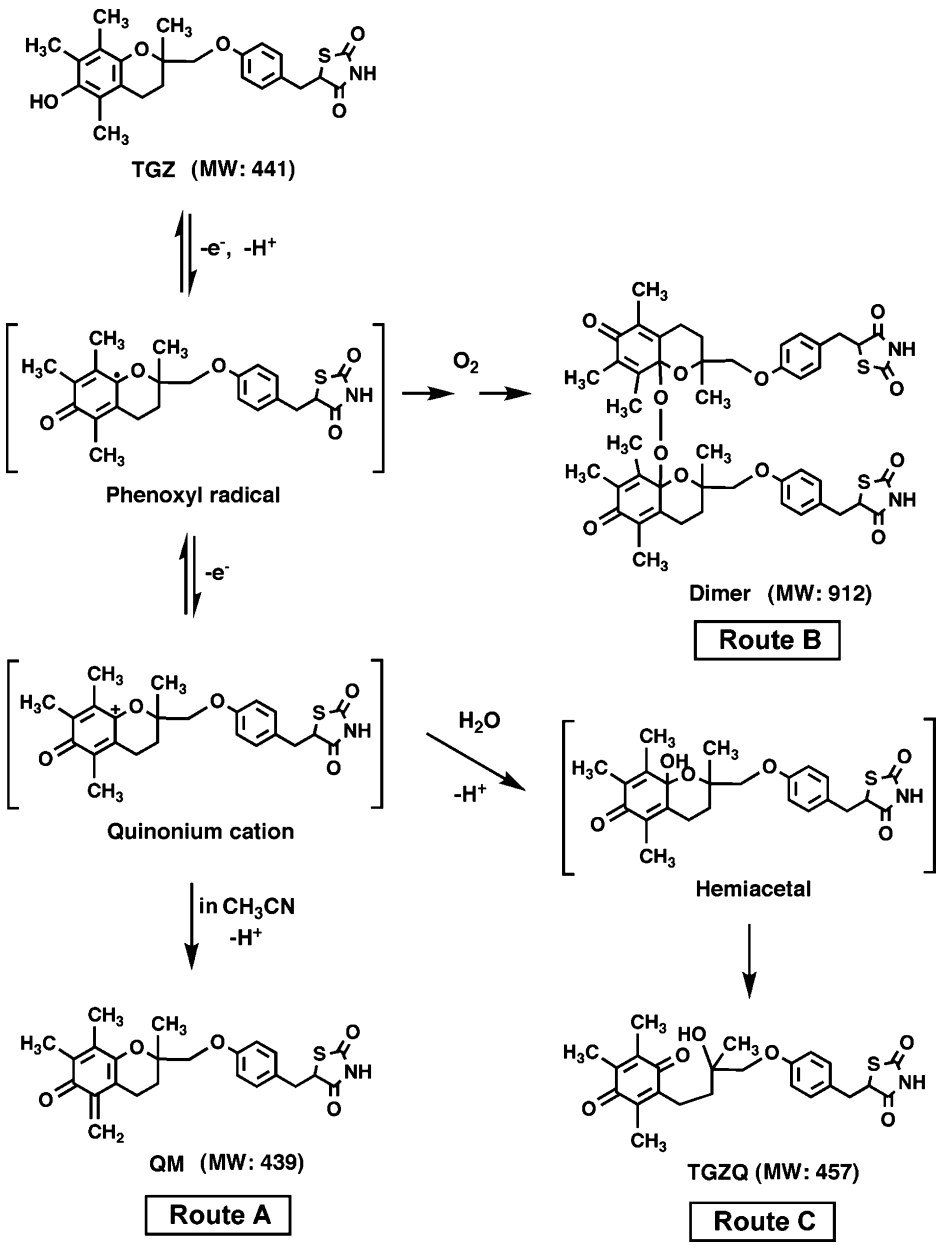
1. Introduction

It has been reported that reactive products generated by electrochemical oxidation are identical to reactive metabolites generated by liver microsome oxidation [1]. Reactive metabolites should bind covalently to biological macromolecules, such as proteins, nucleic acids and lipids, to modify these biological macromolecules irreversibly, which may trigger an allergic reaction, cell disorder, organ injury or carcinogenicity in the case of DNA damage [2,3]. We previously reported that electrochemical oxidation of troglitazone (TGZ, Scheme 1) in an aqueous medium generated *p*-quinone derivative (TGZQ), which is identical to a metabolite of TGZ found in human plasma after administration [4]; however, TGZQ was not a reactive metabolite. We had detected another product by electrochemical oxidation of TGZ using a nonaqueous liquid as a reaction medium, and it could be considered a reactive metabolite, *o*-quinone methide (QM) [5]. TGZ is a drug for type II diabetes that increases insulin sensitivity, but serious hepatotoxicity has been observed in a few patients after long-term administration of more than several months; therefore, toxicity has been considered to be induced by an idiosyncratic drug reaction, related to reactive metabolites generated by the biotransformation of TGZ [6,7]. QM was suggested to be one of the reactive metabolites involved in hepatotoxicity [8]; however, the mechanism of its toxicity, or the toxic metabolite, has been unclear.

Research into reactive metabolites has been performed to reduce the risk of drug-induced toxicity by liver microsome oxidation or electrochemical oxidation of drug candidates using various trapping agents, such as thiol compounds (glutathione, cystein and its derivatives), cyanide or semicarbazide [9–12]; however, it is difficult to detect all reactive metabolites as their trapped conjugate, much less in their intact form. Current researches of reactive drug metabolites using an on-line electrochemical cell-mass spectrometer (EC-MS) have been performed in aqueous medium, and reactive metabolites were detected as their conjugates using glutathione [1,13,14]; however, electrochemical oxidation of a drug in nonaqueous medium has rarely been reported in research into drug metabolism. The electrochemical oxidation method is supe-

* Corresponding author. Tel.: +81 3 3434 6241; fax: +81 3 5400 2672.
E-mail address: tahara-ky@pha.keio.ac.jp (K. Tahara).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.06.002



Scheme 1. Proposed routes of TGZ oxidation by electrochemical oxidation. TGZ is oxidized to form phenoxyl radical by oxidation of one electron and abstraction of one proton. The second electron oxidation should occur to provide a quinonium cation, which should provide QM (Route A). When the phenoxyl radical reacts with dissolved dioxygen to form a peroxy radical, the peroxy radical should react with another phenoxyl radical to produce a peroxy dimer (Route B). In aqueous medium, the quinonium cation should be transformed into TGZQ (Route C).

rior to *in vitro* oxidation by cytochrome P450 as it does not use any biomolecules, including enzymes or cofactors; thus, the products can be separated quite easily with little contamination, or detected without interference from biological matrices. When electrochemical oxidation is performed in nonaqueous medium, which does not represent *in vivo* conditions, we could obtain water labile reactive metabolites of a drug. Sufficient QM to detect its molecular ion without chemical modification could be separated and obtained by electrochemical oxidation in nonaqueous medium.

2. Experimental procedures

2.1. Reagents and chemicals

TGZ was supplied by Sankyo (Tokyo, Japan). Ammonium acetate (99.999%) as an electrolyte and *N*-(*tert*-butoxycarbonyl)-L-cystein methylester (Boc-Cys) as a nucleophilic reagent were purchased from Sigma–Aldrich (MO, USA). Acetonitrile and methanol were of LC-MS grade (Merck, Darmstadt, Germany). Distilled water of HPLC grade (Kanto Chemical, Tokyo, Japan) was purified with Milli-Q® (Millipore, Billerica, USA) before use.

2.2. Instruments and conditions

The electrochemical cell (EC) was a coulometric single-electrode Model 5021A Conditioning Cell (ESA, MA, USA) with a porous graphite electrode, and the potential was controlled by a potentiostat, Coulochem II (ESA).

The LC-MS system consisted of an HPLC system (Shimadzu, Kyoto, Japan) and a quadrupole mass spectrometer with a turbo spray ionization (ESI) source, API3200 (Applied Biosystems Japan). For LC-MS, negative or positive ion detection was employed in the

Q1 full scan mode. The ion source temperature was set at 600 °C. Ion spray voltage was set at 3500 V. The curtain gas was set at 20 psi and declustering potential at 65 V; GS1 and GS2 gases were set at 30 and 60 psi, respectively. The HPLC-UV system was the LC-2000 Plus Series (Jasco, Tokyo, Japan), with detection at 225 nm.

HPLC analysis was performed on a C18 column (Inertsil ODS-3; 100 mm × 3.0 mm I.D., 3 μm; GL Sciences, Tokyo, Japan) with a mobile phase consisting of acetonitrile/methanol/20 mM ammonium acetate (40/20/40, v/v) at a flow rate of 0.30 mL/min at room temperature. The ammonium acetate solution for the mobile phase was filtered through a 0.22 μm membrane (Millipore) before mixing with these organic solvents.

2.3. Electrochemical oxidation of troglitazone in nonaqueous medium

The solvent for electrochemical oxidation in nonaqueous medium was acetonitrile containing 10 mM ammonium acetate because of its limited solubility.

There are three experimental modes (Fig. 1): (1) "on-line" EC-MS system, (2) "off-line" oxidation method, and (3) "in-flow" oxidation method. In the latter two methods, separation and/or detection were carried out separately from electrochemical oxidation.

(1) On-line EC-MS system

In the on-line EC-MS system, the EC was placed between the injector and the MS of the LC-MS system, where the electrolyte solution was pumped at 0.30 mL/min to the EC, and TGZ was injected by flow injection analysis (FIA). Oxidation products were directly detected by ESI-MS.

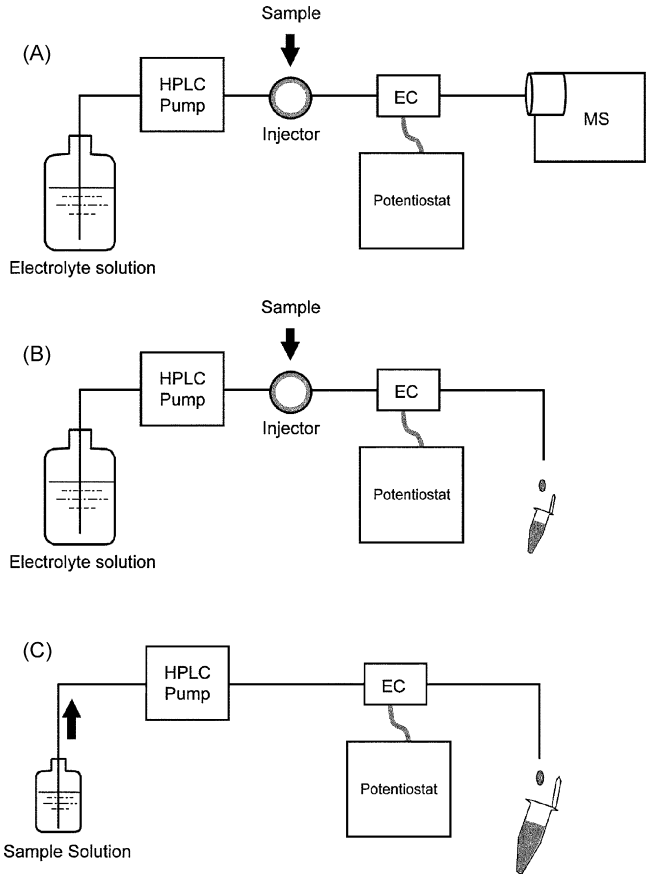


Fig. 1. Three modes of electrochemical oxidation. (A) On-line EC-MS system, (B) off-line oxidation method, (C) in-flow oxidation method.

(2) Off-line oxidation method

In the "off-line" oxidation method, the oxidation products were collected from the outlet of the EC. After evaporation of the solvent under reduced pressure at −50 °C, the oxidation products were reconstituted with the mobile phase, and analyzed by LC-UV or LC-MS.

(3) In-flow oxidation method

In the "in-flow" oxidation method, TGZ solution was continuously pumped into the EC to obtain relatively large-scale oxidation products [4].

2.4. Electrophilic reaction of electrochemical oxidation products with Boc-Cys

To elucidate the electrophilic reactivity of the products obtained by electrochemical oxidation in nonaqueous medium, Boc-Cys, which is soluble in anhydrous organic solvent, was used as a nucleophile. By the off-line oxidation method, collected oxidation products (0.05 mM) were incubated with Boc-Cys (1 mM) in a micro-tube at 37 °C for 5 h, and 10 μL of the mixture was injected into the LC-MS system by the FIA method. The concentration of oxidation products was described as the concentration of TGZ before oxidation.

3. Results and discussion

3.1. Electrochemical oxidation in nonaqueous medium

TGZ (0.1 μg) was injected into the on-line EC-MS system with varying potentials of 0, 300, 500, and 800 mV applied to the EC. Although we reported that TGZ did not react at 0 mV in aqueous medium [4], electrochemical oxidation of TGZ occurred in anhydrous acetonitrile solution, and TGZ reacted with electric potential over 0 mV, providing oxidation products with *m*/*z* 438, 455, 498 in negative ion mode. The molecule ion peak of TGZ disappeared with electric potential over 500 mV, and the mass spectrum obtained with electric potential 800 mV was identical to that with electric potential 500 mV. These products were separated into two peaks (5.8 and 15.5 min; in Fig. 2A) by HPLC analysis of the off-line oxidation method. Because the product of 5.8 min showed *m*/*z* 455 $[M-2H]^{2-}$ in the negative ion mode, and *m*/*z* 457 $[M+2H]^{2+}$, *m*/*z* 439 $[M-2H_2O+2H]^{2+}$, *m*/*z* 479 $[M+2Na]^{2+}$ in the positive ion mode (Fig. 2B), it was postulated as a dimer with a molecular weight of 912, although its *m*/*z* could not be detected. It has been reported that dimers, trimers or polymers can be formed by electrochemical oxidation of aromatic compounds [15,16]. The product of 15.5 min was postulated as QM based on its *m*/*z* 438 $[M-H]^{-}$, *m*/*z* 498 $[M+CH_3COO]^{-}$ in negative ion mode, and *m*/*z* 440 $[M+H]^{+}$, *m*/*z* 462 $[M+Na]^{+}$ in positive ion mode, respectively, indicating that the molecular weight should be 439, (Fig. 2C). Madsen et al. reported that QM should be generated by electrochemical oxidation of TGZ (at potential 200 mV) in aqueous medium based on the NMR spectrum of its conjugate with *N*-acetylcysteine [17]. Although they obtained the conjugate of QM by electrochemical oxidation in aqueous medium, and also detected thiol conjugates of QM by LC-MS/MS in liver microsome oxidation products with thiol compounds, they could not detect QM itself. We obtained intact QM for the first time by electrochemical oxidation in anhydrous acetonitrile solution without a trapping agent, which had a molecular weight of 439.

Because a large amount of un-reacted TGZ remained, the efficiency of TGZ oxidation by electrochemical oxidation in anhydrous acetonitrile solution was low compared to oxidation in an aque-

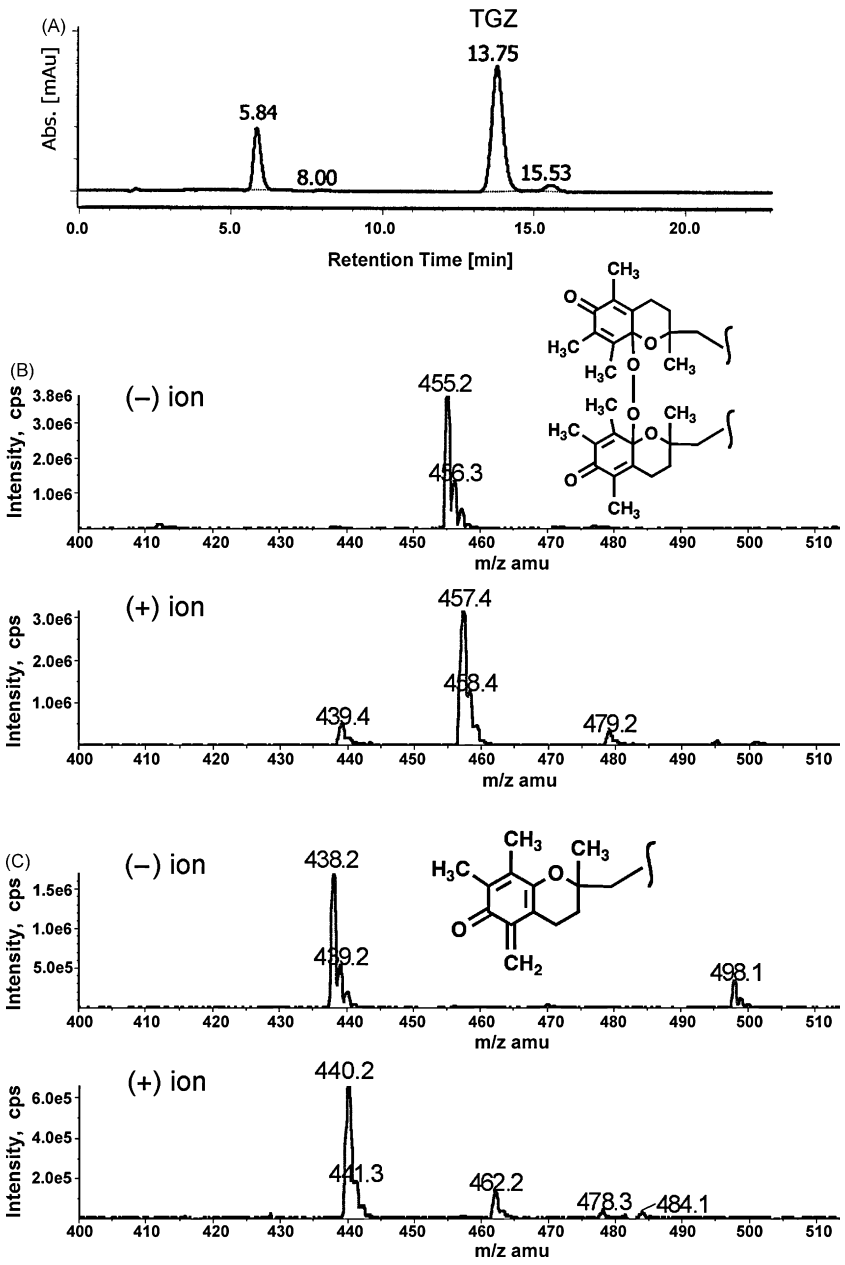


Fig. 2. LC-UV chromatogram and mass spectra of electrochemical oxidation products of TGZ by off-line oxidation performed in anhydrous acetonitrile solution. (A) HPLC separation of electrochemical oxidation products, (B) mass spectra of the oxidation product (5.8 min) postulated to be a dimer, (C) mass spectra of the oxidation product (15.5 min) postulated to be a QM.

ous solvent [4]. To obtain more QM, the flow rate of the solvent in the off-line oxidation method was reduced; however, the efficiency hardly improved; therefore, in-flow oxidation was carried out to identify the optimum condition. TGZ solutions in anhydrous acetonitrile were prepared at 10.0, 5.0, 3.3, 2.0, and 1.0 μg/mL, and were pumped continuously into the EC at 0.1, 0.2, 0.3, 0.5, and 1.0 mL/min, respectively. Here, the migration rate of TGZ through the EC per unit time (that is, equal to the product of the concentration of TGZ and the flow rate) remained constant as 1.0 μg/min. HPLC analysis of the in-flow oxidation effluent is shown in Table 1. At 0.5 or 1.0 mL/min,

Table 1
TGZ, dimer and QM in the oxidation effluent depending on the migration rate of solvents through EC.

Migration rate of solvent (mL/min)	Components of the oxidation effluent (%)[a]			Oxidation efficiency (%)[b]
	TGZ	Dimer	QM	
0.1	53.8	23.9	19.9	55
0.2	56.0	20.1	21.3	50
0.3	60.6	18.8	18.6	46
0.5	61.3	24.6	11.8	49
1.0	30.5	64.4	1.0	79

[a] Percentage (%) of components of oxidation effluent shown by ratio of HPLC peak area.
[b] Oxidation efficiency is the percentage (%) of (consumed TGZ)/(initial TGZ).

Table 2
Changes of the components of the EC effluent after addition of acetonitrile or water.

	Time after addition	Percentage of HPLC peak area of the four components in the mixture (%)			
		Dimer	QM	TGZQ	TGZ
EC effluent	0 h	19.4	16.7	0.8	63.1
Acetonitrile (+)	25 h	21.2	17.8	1.4	59.5
	75 h	21.8	15.5	2.0	60.8
Water (+)	25 h	10.9	13.8	18.4	56.8
	75 h	2.6	4.9	31.3	61.2

the amount of QM generated was small; however, at 1.0 mL/min, a large amount of dimer was generated. The optimum condition for obtaining QM was 0.2 mL/min.

We considered that dioxygen would be incorporated into the dimer, as shown in Scheme 1; thus, it is plausible that TGZ is oxidized on the electrode to lose one electron and proton to form the phenoxyl radical (TG·), which may react with dissolved dioxygen to form TG-peroxy radical (TG–O–O·) or its hydroperoxide (TG–O–O–H). Based on the *m*/*z* of the dimer, TG-peroxy radical should react with another phenoxyl radical to produce a peroxy

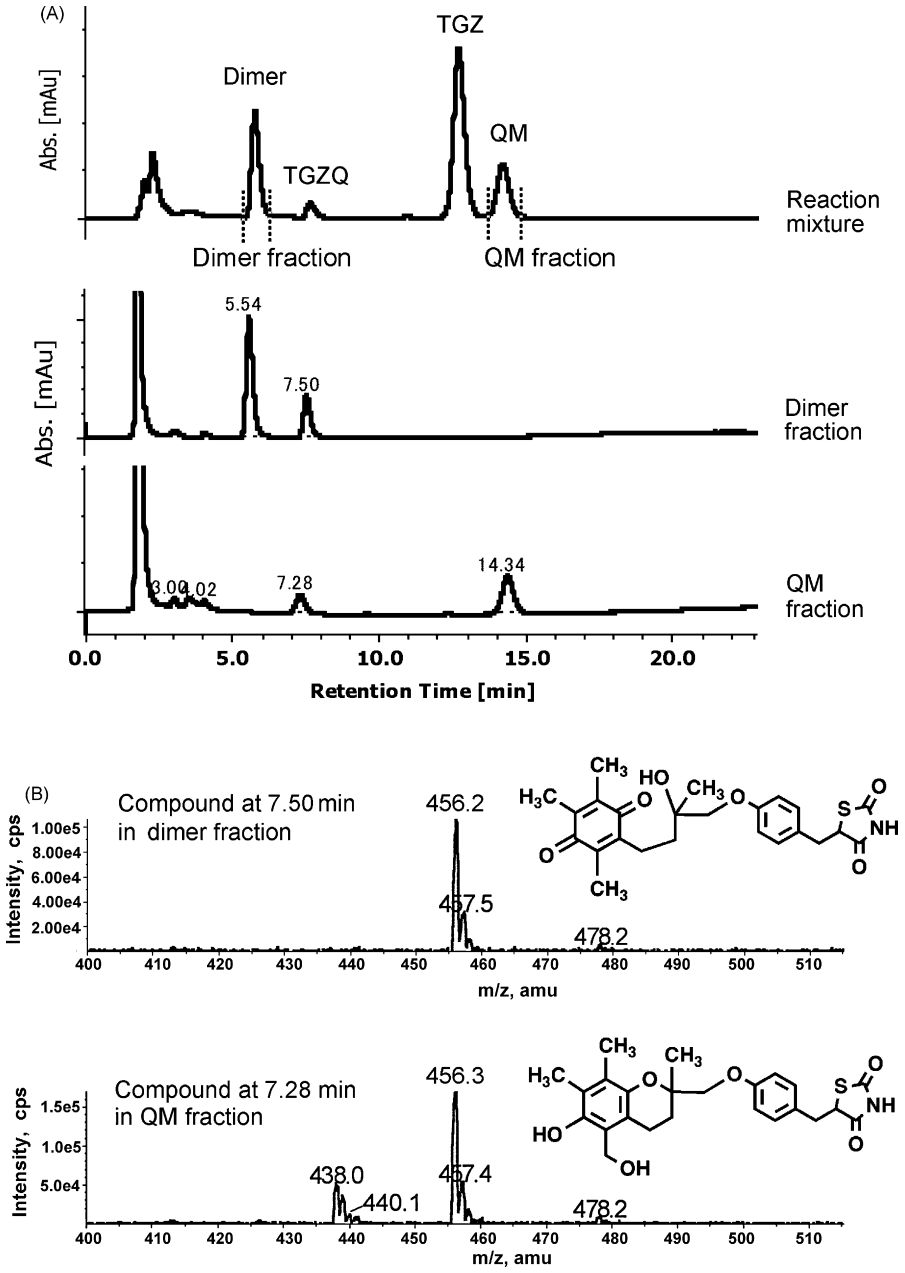


Fig. 3. LC-UV chromatograms showing TGZQ formation in the dimer fraction, and hydroxymethyl compound formation in the QM fraction, and their mass spectra. (A) Each dimer fraction and QM fraction was separated and collected from the effluent of in-flow oxidation, (B) TGZQ formed from a dimer in the fraction, and hydroxymethyl compound derived from QM in the fraction.

dimer (TG–O–O–TG) whose *m*/*z* 455 in the negative ion mode.

$$TGZ \xrightarrow{-e^-,-H^+} TG^{\bullet}$$

$$TG^{\bullet} \xrightarrow{O_2} TG\text{–}O\text{–}O^{\bullet} \quad \text{or} \quad TG\text{–}O\text{–}O\text{–}H$$

$$(TG\text{–}O\text{–}O\text{–}H \xrightarrow{-e^-,-H^+} TG\text{–}O\text{–}O^{\bullet})$$

$$TG\text{–}O\text{–}O^{\bullet} + TG^{\bullet} \rightarrow TG\text{–}O\text{–}O\text{–}TG$$

TG-hydroperoxide was previously reported in the photosensitized oxidation of TGZ, which changed to TGZQ and quinone epoxide [18]. We could not detect quinone epoxide, and there are reports that epoxide was not generated by electrochemical oxidation [17,19]. When the dioxygen molecule does not react with the phenoxyl radical, the second electron oxidation occurs to provide a quinonium cation (TG^+). Successive deprotonation occurs in nonaqueous medium on the ortho-methyl group of TG^+, and QM should be produced.

$$TG^{\bullet} \xrightarrow{-e^-} TG^+$$

$$TG^+ \xrightarrow{-H^+} QM$$

When methanol was used instead of acetonitrile, the methoxy form was generated (data not shown). In aqueous medium, TGZQ was generated by electrochemical oxidation [4].

3.2. Stability of electrochemical oxidation products in anhydrous acetonitrile solution

One hundred microliters of acetonitrile or water was added to 400 μL EC effluent, and the mixtures were stored at room temperature for 25 or 75 h. When acetonitrile was added (Acetonitrile (+), Table 2), the compositions of the dimer and QM did not change, compared to at 0 h; however, they decreased gradually and TGZQ increased when water was added (Water (+)). Thus, the QM and dimeric compound were found to be stable in anhydrous acetonitrile solution over 24 h at room temperature after collection, although they were unstable in the presence of water.

From the effluent of in-flow oxidation, each dimer fraction and QM fraction was separated and collected, and analyzed by HPLC at 7 and 8 h after separation, respectively (Fig. 3). With a water-containing eluent in HPLC separation, the separated dimer changed to TGZQ (t_R = 7.50 min in Fig. 3A, *m*/*z* 456 $[M-H]^-$ in Fig. 3B). It was found that one of the water-reacted compounds of QM was eluted 0.2–0.3 min faster than TGZQ (t_R = 7.28 min in Fig. 3A), showing a different mass spectrum (*m*/*z* 456 $[M-H]^-$, and *m*/*z* 438 $[M-H_2O-H]^-$ in Fig. 3B) from TGZQ; thus, the amount of TGZQ in Table 2 included hydroxylated QM. These results show that QM reacts easily with water to generate a hydroxymethyl compound in HPLC eluent. Kassahun et al. also proposed the metabolic pathway of TGZ for this hydroxymethyl compound from QM *in vivo* [8].

3.3. Electrophilic reactivity of electrochemical oxidation products in anhydrous acetonitrile solution

To confirm the electrophilic reactivity of QM, the effluent collected by off-line oxidation of TGZ was mixed with a nucleophilic reagent, Boc-Cys (MW: 235). The reaction solution was incubated, and a portion of the mixture was analyzed by the FIA method in the negative ion mode of ESI-MS (Fig. 4). The molecule ion at *m*/*z* 673 $[M-H]^-$ was supposed to be the conjugate of QM with Boc-Cys; however, the conjugate of the dimer with Boc-Cys could not be detected. Both molecular ions of QM (*m*/*z* 438 $[M-H]^-$ and 498

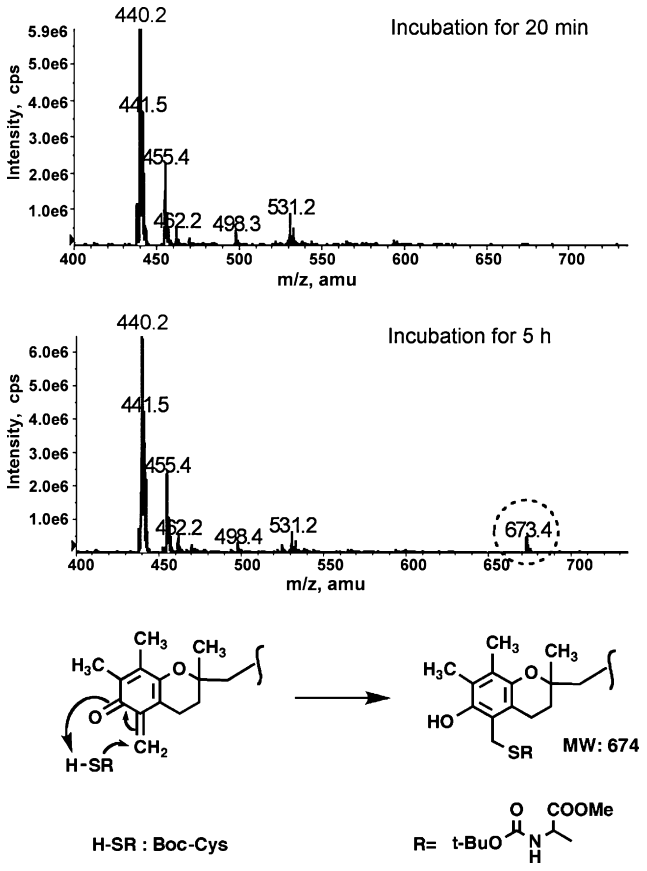


Fig. 4. Mass spectra of the off-line EC reaction mixture subjected to analysis 20 min and 5 h after the addition of Boc-Cys.

$[M+CH_3COO]^-$) decreased to about 0.6 times, whereas that of the conjugate of QM with Boc-Cys (*m*/*z* 673 $[M-H]^-$) increased about 16 times after 5 h, compared to at 20 min, respectively, although the intensity of molecular ions of TGZ (*m*/*z* 440 $[M-H]^-$) and of the fragment ion derived from the dimer (*m*/*z* 455) did not change at all in this period.

Because QM reacted with water and nucleophilic reagent, QM is an unstable reactive metabolite with a very short life when reacted with nucleophiles *in vivo*. Electrochemical oxidation of drugs in nonaqueous medium could provide highly reactive oxidation products without conjugation, and could also perform the conjugation reaction with appropriate nucleophiles separately from the oxidation process.

4. Conclusion

Using nonaqueous medium for electrochemical oxidation of TGZ, we obtained QM, not in the conjugated form, which is suspected to be a reactive metabolite related to the hepatotoxicity of TGZ. QM was stable in anhydrous acetonitrile for over 24 h at room temperature, and could be separated by HPLC and its molecular ion detected by ESI-MS. It was found that QM reacted with water to form a hydroxymethyl compound. Electrochemical oxidation of a drug in nonaqueous medium with an on-line EC-MS system will be a powerful tool for the preparation of a pure compound of unstable metabolites, and the prediction of their biotransformation *in vivo*.

Acknowledgement

The authors thank MC Medical Inc. (Tokyo, Japan) for the supporting instrument.

References

[1] K.G. Madsen, J. Olsen, C. Skonberg, S.H. Hansen, U. Jurva, Chem. Res. Toxicol. 20 (2007) 821–831.
[2] D.A. Hess, M.J. Rieder, Annal. Pharmacother. 31 (1997) 1378–1387.
[3] D.E. Amacher, Curr. Drug Metab. 7 (2006) 219–229.
[4] K. Tahara, Y. Yano, K. Kanagawa, Y. Abe, J. Yamada, S. Iijima, M. Mochizuki, T. Nishikawa, Chem. Pharm. Bull. 55 (2007) 1207–1212.
[5] K. Tahara, Ph. D Thesis of Kyoritsu University of Pharmacy, Tokyo, 2008.
[6] E.J. Murphy, T.J. Davern, A.O. Shakil, L. Shick, U. Masharani, H. Chow, C. Freise, W.M. Lee, N.M. Bass, Digest. Dis. Sci. 45 (2000) 549–553.
[7] D.C. Evans, A.P. Watt, D.A. Nicoll-Griffith, T.A. Baillie, Chem. Res. Toxicol. 17 (2004) 3–16.
[8] K. Kassahun, P.G. Pearson, W. Tang, I. McIntosh, K. Leung, C. Elmore, D. Dean, R. Wang, G. Doss, T.A. Baillie, Chem. Res. Toxicol. 14 (2001) 62–70.
[9] N. Chauret, D. Nicoll-Griffith, R. Friesen, C. Li, L. Trimble, D. Dube, R. Fortin, Y. Girard, J. Yergey, Drug Metab. Dispos. 23 (1995) 1325–1334.
[10] D. Argoti, L. Liang, A. Conteh, L. Chen, D. Bershas, C. Yu, P. Vouros, Chem. Res. Toxicol. 18 (2005) 1537–1544.
[11] J.C.L. Erve, S.C. Vashishtha, W. DeMaio, R.E. Talaat, Drug Metab. Dispos. 35 (2007) 908–916.
[12] N. Masubuchi, C. Makino, N. Murayama, Chem. Res. Toxicol. 20 (2007) 455–464.
[13] T.A. Getek, W.A. Korfmacher, T.A. McRae, J.A. Hinson, J. Chromatogr. 474 (1989) 245–256.
[14] S.M. van Leeuwen, B. Blankert, J.M. Kauffmann, U. Karst, Anal. Bioanal. Chem. 382 (2005) 742–750.
[15] G. Hambitzer, J. Heitbaum, Anal. Chem. 58 (1986) 1067–1070.
[16] S. Lin, J. Chen, M. Liu, O.Y. Su, M. Leung, J. Org. Chem. 63 (1998) 5059–5063.
[17] K.G. Madsen, G. Grönberg, C. Skonberg, U. Jurva, S.H. Hansen, J. Olsen, Chem. Res. Toxicol. 21 (2008) 2035–2041.
[18] Y. Fu, C. Sheu, T. Fujita, C.S. Foote, Photochem. Photobiol. 63 (1996) 615–620.
[19] U. Jurva, H.V. Wikstrom, L. Weidolf, A.P. Bruins, Rapid Commun. Mass Spectrom. 17 (2003) 800–810.