Successful Preparation of Metabolite of Troglitazone by In-Flow Electrochemical Reaction on Coulometric Electrode

Kayoko TAHARA,* Yuka YANO, Kiyotada KANAGAWA, Yoshihiro ABE, Junko YAMADA, Shiro IIJIMA, Masataka MOCHIZUKI, and Takashi NISHIKAWA

Department of Pharmaceutical Sciences, Kyoritsu University of Pharmacy; 1–5–30 Shibakoen, Minato-ku, Tokyo 105–8512, Japan. Received April 7, 2007; accepted May 24, 2007; published online May 29, 2007

A simple, rapid and efficient system utilizing a coulometric electrode was developed for the preparation of drug metabolites. Trace amounts of reactants are usually generated in electrochemical reactions, which are not suitable for the sufficient preparation of products to obtain NMR and other spectral data for chemical structure confirmation or to obtain data from pharmacological activity screening tests of products. In the developed system, called the "in-flow electrochemical reaction system," a drug, troglitazone, was dissolved in a volatile flow solvent, and pumped into a coulometric electrode under optimized conditions, and the effluent was evaporated. Without any further purification, milligram amounts of a pure oxidation product of troglitazone could be obtained within several hours. The amount obtained was enough for ^{1}H- and ^{13}C-NMR analysis by which the structure could be confirmed and was found to be identical to one of the metabolites of troglitazone detected in human plasma. This system will be useful to prepare standard compounds of the required amount for pharmacokinetic study and for toxicokinetic study.

Key words drug metabolite; preparation; electrochemical reaction; troglitazone

Several types of chemical reaction are observed in drug metabolism such as oxidation, reduction, hydrolysis and conjugation. Among them, oxidation is the major reaction of drug metabolism and is catalyzed by the cytochrome P450 system. It is fundamentally mono-oxygenation, initiated by one-electron oxidation or direct abstraction of a hydrogen atom.[1)] Drug oxidation that requires the transfer of one or more electrons occurs also at the surface of an electrode. Electrochemical oxidation of a drug may produce a compound that is identical to the drug metabolite of that drug. Shono *et al.* reported that *N*-dealkylated compounds were generated from some drugs either by electrochemical oxidation and by treatment with rat liver microsomes.[2,3)] They determined the products by UV-spectrometry or HPLC-UV and compared the ratio of (*N*-demethylated/total of *N*-dealkylated) for the two methods and obtained good accordance of the selectivity. Hambitzer *et al.* developed on-line electrochemical cell/thermospray mass spectrometry, by which electrochemical oxidation products were detected directly.[4)] Getek *et al.* utilized on-line electrochemical cell/thermospray mass spectrometry for the oxidation of acetaminophen and succeeded in detecting an oxidation product as *N*-acetyl-*p*-benzoquinoneimin, and in synthesizing acetaminophen-glutathione conjugate and acetaminophen-cysteine conjugate only by mixing the product with the corresponding thiol compound.[5)] Other authors reported the electrochemical reaction of compounds by on-line electrochemistry/mass spectrometry[6—9)]; however, the electrochemical redox reaction has been rarely applied to actual studies on pharmacological metabolism using electrochemistry/thermospray tandem mass spectrometry. In recent years, the combination technique of liquid chromatography/mass spectrometry (LC-MS) has been remarkably improved and is widely available. Electrochemical cell (EC) could be combined with LC-MS in one flow, and the combined apparatus was found to be helpful for studies of pharmacological metabolism[10—14)] because the trace product of the electrochemical reaction could be directly detected and analyzed by MS. van Leeuwen *et al.* suggested that on-line EC-MS or on-line EC-LC-MS could give useful information for anticipating or elucidating the metabolic pathways of drug molecules[14)]; however, MS does not always give enough information for identification of its chemical structure. For that purpose, spectral data such as NMR are necessary, and they require pure analytes in milligram amounts.

We therefore devised a system for the preparation of an enough quantity of an oxidized drug utilizing an electrochemical reaction. In previous flow systems, the sample was injected into the flow with a sample injector or syringe pump, and the injected amount of the sample was less than a few microgram. In the devised flow system, this sample is dissolved in a flow solvent and a large quantity of sample can be introduced into the flow. Troglitazone (Fig. 1) was used as the model drug because an unknown metabolite of this drug was suggested to be involved in the serious hepatotoxicity of troglitazone.[15)]

Experimental

Chemicals Troglitazone was provided by Sankyo (Tokyo, Japan). Acetonitrile was of LC-MS grade (Merck, Darmstadt, Germany). Distilled water of HPLC grade (Kanto Chemical, Tokyo, Japan) was purified with Milli-Q® (Millipore, Billerica, U.S.A.) before use. Chloroform-d_4 (containing 0.03% v/v TMS) was purchased from Sigma-Aldrich (MO, U.S.A.).

Instruments and Conditions The electrochemical cell was a coulometric single electrode Model 5021 Conditioning Cell, (ESA, Chelmsford, MA, U.S.A.) that has a porous graphite electrode with controlled potential by a Coulochem II (ESA).

The LC-UV-MS system consisted of an HPLC pump L-7100, UV detector

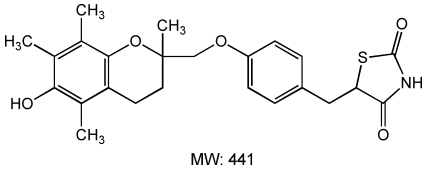

Fig. 1. Structure and Molecular Weight of Troglitazone

* To whom correspondence should be addressed. e-mail: tahara-ky@kyoritsu-ph.ac.jp

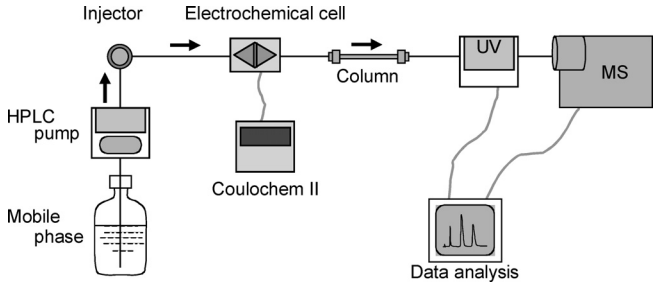

Fig. 2. Scheme of the On-Line EC-LC-UV-MS System

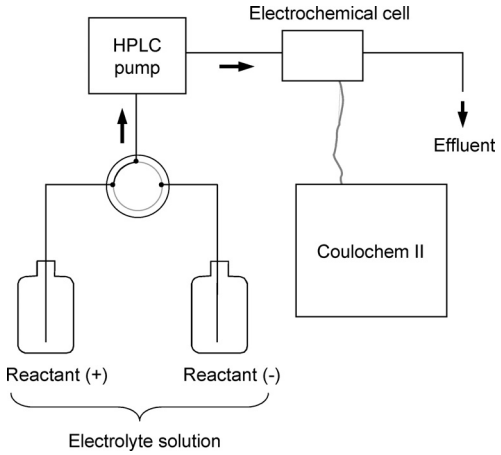

Fig. 3. Scheme of the In-Flow Electrochemical Reaction System

L-7400, column oven L-7300, mass spectrometer M-1200H with APCI (Hitachi, Tokyo, Japan), sample injection valve with a 20 μl loop (Rheodyne, U.S.A.), and an integrator with software HIT mass version 2.0 (Hitachi) for data acquisition and analysis. For LC-MS, negative ion detection was employed in the full scan mode. Temperatures of the nebulizer and desolvator were 200 and 400 °C, respectively. The needle electrode voltage and drift voltage were 3000 and 40 V, respectively. LC-MS was performed on a C18 column (Inertsil ODS-3; 250 mm×4.6 mm I.D., 5 μm; GL Sciences, Tokyo, Japan) with a mobile phase consisting of acetonitrile and 20 mM ammonium acetate (volatile salt) (60 : 40, v/v) at 1.0 ml/min flow rate with UV absorption detection at 225 nm. In the on-line EC-LC-UV-MS system (Fig. 2), the electrochemical cell was placed between the pump and the LC column.

The effluent from an in-flow electrochemical reaction system (described in the following paragraph) was analyzed by a chromatographic system (HPLC-UV system) that consisted of an HPLC pump PU-980, UV detector UV-975, autosampler AS-950 and an integrator with software ChromNAV (Jasco, Tokyo, Japan). The HPLC in that system was performed on an Inertsil ODS-3 (150 mm×3.0 mm I.D., 3 μm; GL Sciences) at 0.30 ml/min flow rate with the same mobile phase and UV absorption at the same wavelength as under LC-MS conditions.

The mobile phase of the two systems was degassed by ultrasonication before use. The ammonium acetate solution for the mobile phase was filtered through a 0.22 μm membrane (Millipore) before mixing with acetonitrile.

Preparation of Milligram Amount of Reaction Product for NMR Figure 3 shows the "in-flow electrochemical reaction system" for continuous flow oxidation. In this system, reactant solution was not injected to the LC line flow but was introduced as a constituent in the mobile phase solvent, therefore, the reactant solution was directly pumped in to the flow into an electrochemical cell that has potential, and oxidation continuously occurred with a successive supply of reactant and successive release of product. The effluent containing the oxidation product was collected from the outlet of the electrochemical cell, and analyzed by the HPLC-UV system before evaporation. The reactant solution contained 50 μg/ml troglitazone in the mobile phase solvent, the same as in HPLC analysis.

Measurement of NMR After evaporation of the collected effluent in a vacuum, the residue was dissolved in $CDCl_3$, and the solution was analyzed on a 600 MHz FT-NMR spectrometer (JNM-ECP600, JEOL, Tokyo, Japan) for both ^{1}H- and ^{13}C-NMR spectra, referring to the TMS signal at 0 ppm.

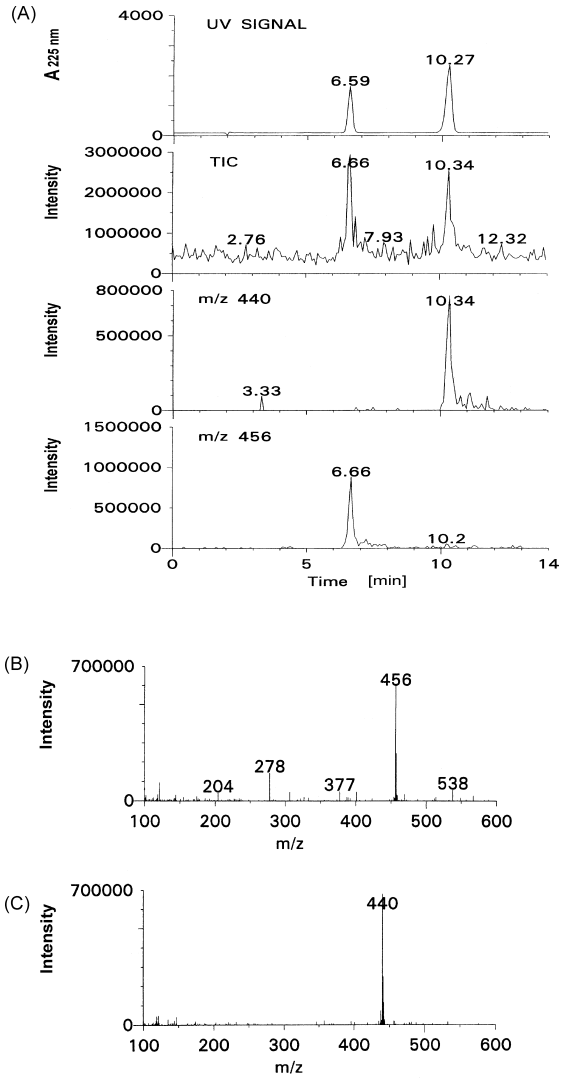

Fig. 4. Chromatograms and Mass Spectra Obtained on the On-Line EC-LC-UV-MS System, Electrochemical Cell Applying a Potential of 600 mV

(A) Chromatograms, (B) mass spectrum of the oxidation product (6.6 min), (C) mass spectrum of the troglitazone (10.3 min). LC-MS conditions are described in the text.

Results and Discussion

Confirmation of Occurrence of Troglitazone Oxidation with Electrode by EC-LC-UV-MS System Troglitazone (5.0 μg in 20 μl) was injected into the on-line EC-LC-UV-MS. One peak of troglitazone was observed on the UV chromatogram and on the mass chromatogram with a potential of 0 mV applied to the electrochemical cell. With some potential applied, two peaks appeared, one for troglitazone and one for the oxidation product, on the two chromatograms. With an increase of the potential, the troglitazone peak decreased while the product peak increased in a complementary manner. The UV and mass chromatograms and mass spectra of the two peaks at a potential of 600 mV are shown in Fig. 4. The mass spectrum of the troglitazone peak (eluted at 10.3 min) showed a deprotonated molecule at *m/z* 440, and that of the product peak (eluted at 6.6 min) showed a deprotonated molecule at *m/z* 456, a molecular weight of 457. Three structures were postulated as the oxidation product of troglitazone having a molecular weight of 457 (Fig. 5). Two of the three postulated structures have a *para* quinone group,

because electrochemical oxidation of phenol compounds often results in the quinone group.[10,16)]

Effects of the Potential and the Flow Rate on the In-Flow Electrochemical Reaction System The troglitazone solution (50 μg/ml) in the mobile phase was introduced into the in-flow electrochemical reaction system under various potential and various flow rates for optimization of the conditions. A 10 μl portion of the effluent was injected into the HPLC-UV system for the determination of troglitazone and the product.

Figure 6A shows the relation between the potential applied to the electrochemical cell and reaction efficiency at a constant flow rate of 0.15 ml/min. The residual amount of troglitazone in the effluent decreased from 100 to 10% linearly with an increase of the potential 0 to 400 mV. It decreased gradually over potential 400 mV and to 0% at over 800 mV. Complementarily the oxidation product in the effluent increased from 0% and saturated to 100% at a potential of 800 mV, indicating molar-to-molar conversion from the reactant to the product. Figure 6B shows the relation between the flow rate and the reaction efficiency with a constant potential of 800 mV. At a flow rate slower than 0.20 ml/min, the efficiency was 100%, indicating complete conversion from troglitazone to the product. At a faster flow rate, the efficiency of the product decreased gradually, reaching 35% of the initial value at a flow rate of 1.0 ml/min. With a fixed concentration of troglitazone, the reaction efficiency was dependent on the potential of the electrochemical cell and flow rate. The surface of the electrode where oxidation occurs might not be able to catch up with the troglitazone molecules coming too fast after one after another. The optimized conditions for the preparation of a large quantity of the product were found to be over 800 mV potential and slower than 0.20 ml/min flow rate.

Milligram Preparation of Electrochemical Reaction Product Troglitazone solution was pumped into the cell with a potential of 800 mV at a flow rate of 0.10—0.20 ml/min for 5—10 h. The total volume of 60 ml containing 3.0 mg troglitazone was introduced into the system at various flow rates. The effluent from the system was fractionated in every 12 ml fraction. A 10 μl portion of the collected fraction was injected into the HPLC-UV system, and the result is shown in Table 1. Although oxidation efficiency declined gradually during continuous flow oxidation, it could be recovered to the initial efficiency by flushing the electro-

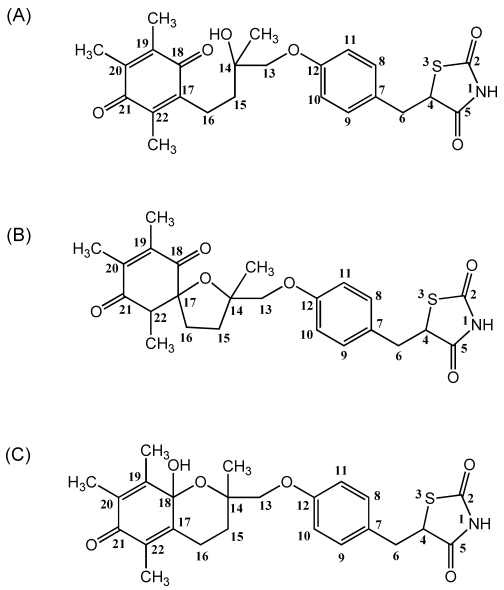

Fig. 5. Three Structures Postulated as an Oxidation Product Corresponding to *m*/*z* 456

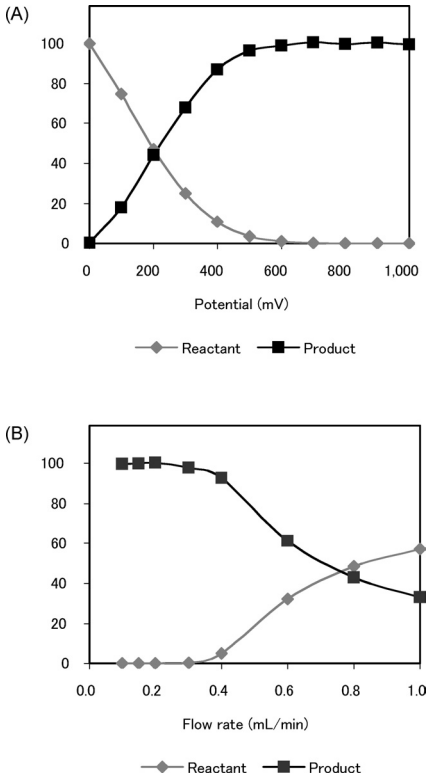

Fig. 6. Effects of the Potential and the Flow Rate on the In-Flow Electrochemical Reaction System

(A) Effect of the potential under constant flow rate, (B) effect of the flow rate under constant potential. Each plot is profiled for HPLC peak areas by injecting a portion (10 μl) of the effluent (1 ml) from the electrochemical cell. The reaction efficiency was defined as the percentage of the area of the formed oxidation product divided by the area of the oxidation product which should be formed after the complete consumption of troglitazone.

Table 1. Yield of the Oxidation Product in Each Fraction Obtained by the In-Flow Electrochemical Reaction System

Flow rate (ml/min)	Fraction No.				
	Fr. 1 (0—12 ml)	Fr. 2 (12—24 ml)	Fr. 3 (24—36 ml)	Fr. 4 (36—48 ml)	Fr. 5 (48—60 ml)
0.10	100.0%	100.0%	100.0%	99.7%	99.2%
0.15	100.0%	100.0%	99.6%	98.8%	97.5%
0.20	100.0%	99.7%	99.2%	97.7%	96.0%

It required 1 to 2 h to collect one fraction of 12 ml volume. The electrochemical cell was flushed with 50% acetonitrile, 50% acetone and 100% acetone for 1 h each at 1.0 ml/min after fractionation of each flow rate.

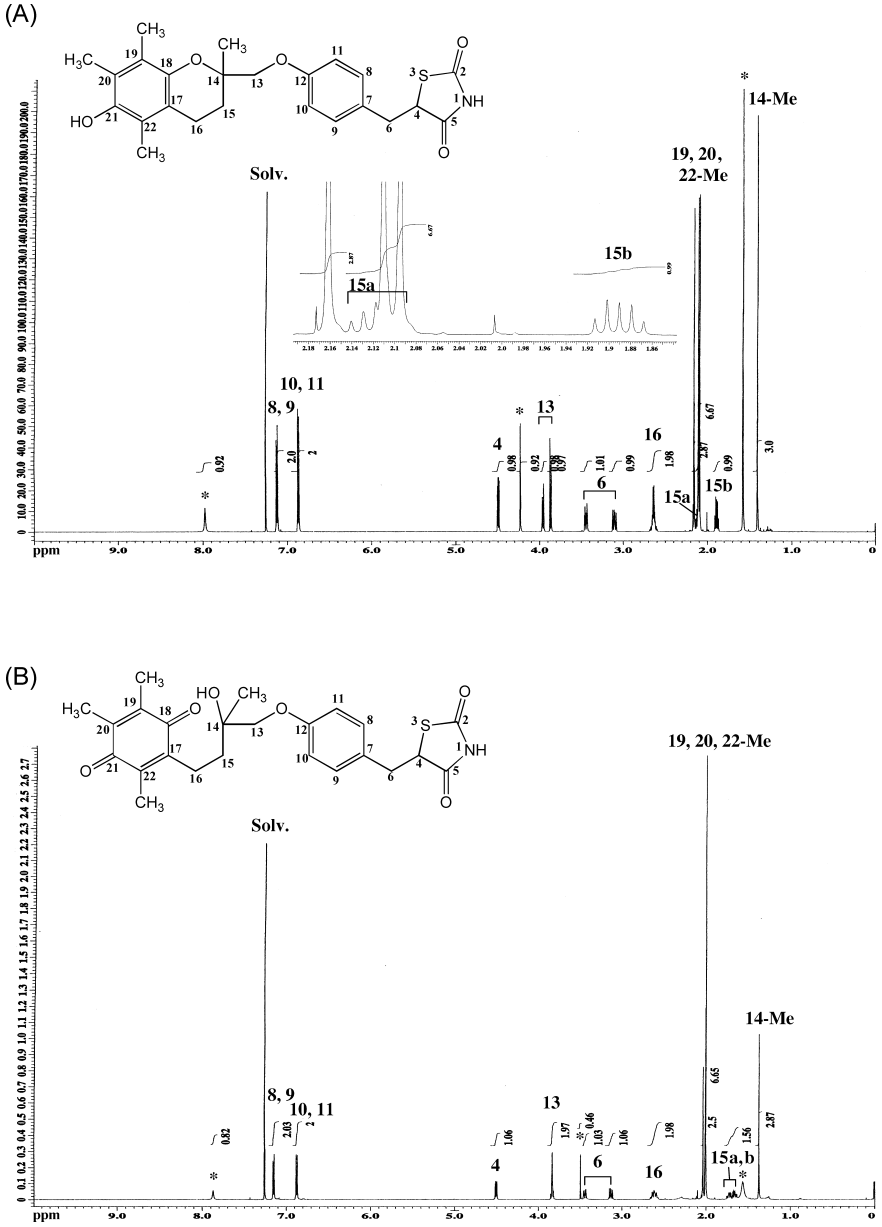

Fig. 7. [1]H-NMR Spectra of Troglitazone and the Oxidation Product

(A) Troglitazone (approximately 3 mg), scanning was carried out 32 times, (B) product (approximately 1.2 mg) scanning was carried out 256 times. Peaks marked with asterisks are attributed to protons of imide, phenol and H_2O because these peaks were quenched by the addition of D_2O.

chemical cell, which could be reused without losing efficiency. This decline may be due to the adhesion of some substances such as impurities and part of the oxidized troglitazone on the surface of the electrochemical cell.

Troglitazone generated one pure product with complete conversion by electrochemical oxidation, but other drugs may generate multiple products with incomplete conversion. In these cases, a purification technique such as HPLC is necessary after preparation; therefore, the decline of reaction efficiency during continuous flow oxidation will be a minor problem, because the unreactive reactant is separable by HPLC purification. However, in this experiment, the purity of the product was so high that further purification was unnecessary.

Structure Confirmation of Electrochemical Oxidized Product After evaporation of the initial two fractions of the 24 ml effluent performed at 0.15 ml/min, the residue (approximately 1.2 mg) was reconstituted in 0.5 ml $CDCl_3$ for measurement of NMR. From [1]H- and [13]C-NMR data, the structure of the purified product could be confirmed to be structure (A) in Fig. 5.

[1]H-NMR spectra of troglitazone and the oxidation product are shown in Fig. 7. A relatively large difference in the chemical shifts of the protons at 15-position was observed (2.1 ppm and 1.9 ppm in Fig. 7A). As these protons are fixed on the six-membered ring of troglitazone, they should have quasi-equatorial or quasi-axial conformation, thus, they are affected by the magnetically anisotropic effect of the ring. In the [1]H-NMR spectrum of the oxidation product, in contrast, this difference in chemical shifts at the 15-position became small and the peaks of the protons appeared more closely around 1.7 ppm (Fig. 7B). These differences in [1]H-NMR

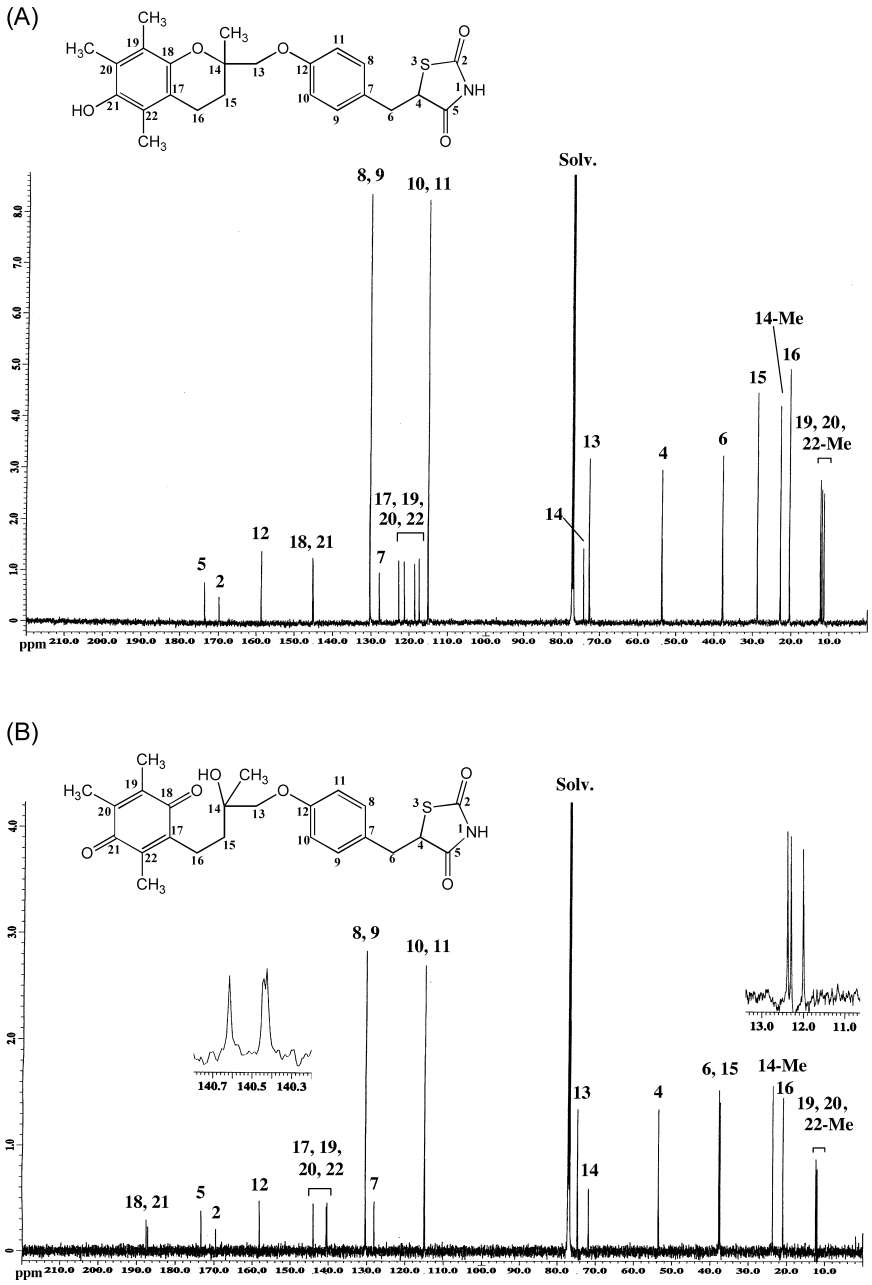

Fig. 8. ^{13}C-NMR Spectra of Troglitazone and the Oxidation Product

(A) Troglitazone (approximately 3 mg), scanning was carried out 36000 times, (B) product (approximately 1.2 mg) scanning was carried out 35478 times.

spectra suggest that the structure of the oxidation product should be a ring-opened structure. The chemical shifts (around 2 ppm) of the protons in the 22-methyl group of the product were not different from that of troglitazone, supporting that the double bond between the 17- and 22-position was retained. These results also support structure (A) in Fig. 5 rather than (B) or (C) for the product.

The ^{13}C-NMR spectra of the two are shown in Fig. 8. The spectrum of the product showed two peaks of carbons at the 18- and 21-positions at an extremely lower field of 187 ppm (Fig. 8B). These peaks of carbons at the 18- and 21-positions of troglitazone were not found in such a lower field (Fig. 8A). This supports that the two carbons in the *para* quinone of structure (A) or (B) in Fig. 5 should give two peaks at such a low field. The peaks of the carbons at the 17-, 19-, 20- and 22-positions of the product appeared at 140—144 ppm, indicating that the double bond of these carbons was retained, and this supports structure (A) and (C).

Only structure (A) suffices for these results of ^{1}H- and ^{13}C-NMR; therefore, the structure of the product was confirmed to have an opened chroman ring with a *para* quinone group. The product of electrochemical oxidation was identical to one of the metabolites of troglitazone detected in patients plasma collected after troglitazone administration.[17,18]

Conclusion

The devised in-flow electrochemical reaction system could prepare milligram amounts of a pure oxidation product of a drug. The structure of the product could be confirmed by NMR analysis. There are several methods to obtain pure

drug metabolites, such as chemical synthesis, purification from biological samples, and *in vitro* biosynthesis using various preparations of cytochrome P450; however, these methods are complicated for purification to measure NMR spectra. Our method has an advantage in that only drug metabolites could be obtained without nonvolatile components such as enzyme, cofactors of enzyme and any other biological matrix; therefore, it is easy to prepare and efficient to purify the drug metabolite in milligram amounts. This in-flow electrochemical reaction system will be a good tool to prepare the oxidation product of drugs and will be useful for drug metabolism research.

Acknowledgements The authors acknowledge MC Medical Inc. (Tokyo, Japan) for the support instrument.

References

1) Guengerich F. P., MacDonald T. L., *FASEB J.*, **4**, 2453—2459 (1990).
2) Shono T., Toda T., Oshino N., *Drug Metab. Dispos.*, **9**, 481—482 (1981).
3) Shono T., Toda T., Oshino N., *J. Am. Chem. Soc.*, **104**, 2639—2641 (1982).
4) Hambitzer G., Heitbaum J., *Anal. Chem.*, **58**, 1067—1070 (1986).
5) Getek T. A., Korfmacher W. A., McRae T. A., Hinson J. A., *J. Chromatogr.*, **474**, 245—256 (1989).
6) Volk K. J., Yost R. A., Brajter-Toth A., *Anal. Chem.*, **61**, 1709—1717 (1989).
7) Volk K. J., Yost R. A., *J. Chromatogr.*, **474**, 231—243 (1989).
8) Hambitzer G., Heinz P. P., Stassen I., Heitbaum J., *Synthetic Metals*, **55**, 1317—1322 (1993).
9) Corazon M., Regino S., Brajter-Toth A., *Anal. Chem.*, **69**, 5067—5072 (1997).
10) Jurva U., Wikstrom H. V., Bruins A. P., *Rapid Commun. Mass Spectrom.*, **14**, 529—533 (2000).
11) Nesmerak K., Nemec I., Sticha M., Nemcova I., Horka V., *Anal. Lett.*, **35**, 1617—1629 (2002).
12) Jurva U., Wikstrom H. V., Weidolf L., Bruins A. P., *Rapid Commun. Mass Spectrom.*, **17**, 800—810 (2003).
13) Jurva U., Bissel P., Isin E. M., Igarashi K., Kuttab S., Castagnoli N., Jr., *J. Am. Chem. Soc.*, **127**, 12368—12377 (2005).
14) van Leeuwen S. M., Blankert B., Kauffmann J.-M., Karst U., *Anal. Bioanal. Chem.*, **382**, 742—750 (2005).
15) Watanabe I., Tomita A., Shimizu M., Sugawara M., Yasumo H., Koishi R., Takahashi T., Miyoshi K., Nakamura K., Izumi T., Matsushita Y., Furukawa H., Haruyama H., Koga T., *Clin. Pharmacol. Ther.*, **73**, 435—455 (2003).
16) Zhang T. Y., Zhu S. M., *Anal. Chim. Acta*, **309**, 111—115 (1995).
17) Loi C. M., Randintis E. J., Vassos A. B., Kazierad D. J., Koup J. R., Sedman A. J., *J. Clin. Pharmacol.*, **37**, 1114—1120 (1997).
18) Yamazaki H., Shibata A., Suzuki M., Nakajima M., Shimada N., Guengerich F. P., *Drug Metab. Dispos.*, **27**, 1260—1266 (1999).