Development of a Rapid and Detailed Structural Identification System with an On-Line Immobilized Enzyme Reactor Integrated into LC-NMR

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Received November 2, 2009; accepted November 30, 2009; published online December 2, 2009

Immobilized enzyme reactors (IMERs) integrated into an LC-NMR system were developed for rapid and detailed structural identification of enzymatic reaction products. An on-column enzymatic reaction was achieved for immobilized cytochrome-*c* and dog microsomes. After the reaction, these products were analyzed by LC-NMR without any work-up processes. The immobilized cytochrome-*c* column integrated into LC-NMR was used to characterize the reaction product formed on *N*-demethylation by measurement of ^{1}H-NMR. In the case of reaction taking place on the microsome column, a glucuronidation product was identified by ^{1}H-NMR and ^{1}H–^{1}H correlated spectroscopy. The chemical structures of the enzymatic reaction products could be elucidated by IMER-LC-NMR without the need for authentic samples or isolation processes.

Key words immobilized enzyme reactor; LC-NMR; microsome; cytochrome-*c*

Immobilized enzyme reactors (IMERs) have been developed for use as high-performance liquid chromatographic (HPLC) columns and have been used as pre- or post-columns in HPLC systems for on-line analysis of biological substances.[1,2] IMERs have been used to study the enzymatic enantioselective recognition of racemic compounds[3] and the on-line synthesis and analysis of drug metabolites.[4,5] We have also previously prepared immobilized enzyme phospholipid columns with biomimetic characteristics for HPLC columns and examined their properties as enzyme reactors and their usage for drug development.[6] IMERs have been utilized in various applications combined with HPLC systems. However, such IMER-HPLC systems could not be used to identify reaction products without the use of authentic samples nor could they elucidate the detailed chemical structures of the products. Recently, IMERs have been combined with LC-MS for drug metabolism and inhibition studies. Nicoli *et al.*[7] incorporated two cytochrome P450-based immobilized enzyme reactors into an LC-MS system to perform automated on-line phase I drug metabolism studies. LC-MS has been often used for the identification of small amounts of drug metabolites and impurities in pharmaceuticals.[8] However, LC-MS by itself usually does not provide sufficient structural information for unambiguous identification. Therefore, time-consuming preparative chromatographic isolation is often necessary to obtain pure compounds for NMR spectroscopic analysis, if authentic compounds for comparison are not available commercially. Recently, LC-NMR has been increasingly applied for the structural determination of drug metabolites, impurities and degradation products in pharmaceuticals to obtain detailed structural information on compounds in mixtures without using isolation processes.[9,10] Also, NMR analysis allows identification of compounds with small molecular weights or poor ionization characteristics, which are likely to be difficult for MS detection.[11]

In this study, we developed an IMER-LC-NMR system for on-line enzymatic reaction and structural identification of the resulting products and evaluated the system. First, we developed a system using an immobilized cytochrome-*c* (Cyt-*c*) column as a model case to assess the performance of the system. Next, we used an immobilized dog microsome column for the system to identify glucuronide as a reaction product. Glucuronidation is one of the main metabolic pathways for drugs *in vivo*. Authentic samples of drug glucuronides can not be easily obtained by chemical synthesis or biosynthesis using free microsomes.[12] The results of this study demonstrate that an IMER-LC-NMR system can be used for on-line synthesis and characterization of the glucuronide. Drug development worked can be aided by being able to identify compounds which may be glucuronidated and to synthesize and characterize the resulting products.

Experimental

Materials Deuterium oxide was purchased from Sigma-Aldrich (Dorset, U.K.). Cyt-*c* and uridine 5′-diphosphate-glucuronic acid (UDPGA) were purchased from Nacalai Tesque (Kyoto, Japan). *N*-Methylaniline (MA), 4-nitrophenol (4NP) and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Wako (Osaka, Japan). Acetonitrile, methanol, water and trifluoroacetic acid were of HPLC grade and all other chemicals were of reagent grade. The HPLC column used for this study was Develosil ODS-UG-5 (35×4.6 mm i.d., 5 μm, Nomura Chemicals, Aichi, Japan).

Apparatus The HPLC system consisted of a Shimadzu LC-20AD (Kyoto, Japan) as a pump, a Shimadzu SIL-HTc automatic injector, a Shimadzu SPD-20AV spectrophotometer as a UV detector and a Shimadzu CTO-20AC as a column oven. The chromatographic data was analyzed using a Waters Empower software (Milford, MA, U.S.A.). The LC-NMR system consisted of a Varian INOVA 600-MHz spectrometer (Varian NMR Instruments, Palo Alto, CA, U.S.A.) equipped with a ^{1}H{^{13}C/^{15}N} pulse field gradient triple resonance 5 mm cryogenic probe with a flow cell (active volume: 60 μl) and a Varian HPLC system (a Prostar 230 pump and a Proster 335 photdiode array UV detector). This was operated in the loop-collection modes using Varian VNMRJ software. The field frequency was locked on the ^{2}H resonance of D_2O. Suppression resonances from HDO resonance were attained by using water elimination through a transverse gradients (WET) technique.[13] The ^{1}H-NMR spectra were obtained using an acquisition time of 3 s, a delay between the successive pulses of 1 s, and a spectral width of 9592 Hz. A total of 256 scans (measuring time *ca.* 17 min) were accumulated to obtain an appropriate signal-to-noise ratio. The ^{1}H–^{1}H correlated spectroscopy (COSY) spectrum was acquired with a spectral width of 5940 Hz into 2048 data points in t_2, with 256 increments in the t_1 dimension. Number of transients per increment was 24, and the measuring time was *ca.* 2 h. The cryogenic probe was used for increasing NMR sensitivity and reducing the measuring time.

Preparation of Dog Liver Microsomes Dog liver samples were obtained from female beagle dogs without drug treatment in our laboratory and the microsomes were prepared as described previously.[12] The microsomal pellets obtained were stored in a freezer at −80 °C until use. The microso-

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mal protein concentration was determined by using the Micro Bicinchoninic Acid Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as standard.[14] The microsomal protein concentration in this study was approximately 20 mg/ml.

Preparation of Immobilized Enzyme Columns The phospholipid column used to prepare the immobilized enzyme column was a Develosil ODS-UG-5 (35×4.6 mm i.d.) coated with DPPC. The phospholipid column was prepared by a dynamic coating technique developed in our laboratory.[15] The procedure was done under an ambient temperature of 25±1 °C. An immobilized Cyt-*c* column was prepared by recycling a solution of 20 mg of Cyt-*c* dissolved in 50 ml of a 10 mM sodium phosphate buffer in D_2O (pD 7.1) at a flow rate of 1 ml/min through the phospholipid column for up to 240 ml of total delivery. Similarly, an immobilized microsome column was prepared by recycling a solution of 31 mg of microsome added to 100 ml of a 10 mM sodium phosphate buffer in D_2O (pD 7.4) at a flow rate of 0.5 ml/min through the phospholipid column for total delivery of up to 360 ml. The immobilized enzyme columns were washed with a 10 mM sodium phosphate buffer in D_2O (pD 7.0) at 1 ml/min for 30 min. The amounts of DPPC coating on the octadecyl silica (ODS) columns and immobilized enzyme on the phospholipid column were calculated as described previously.[13] The activities of immobilized enzymes were estimated by *N*-demethylation of MA (Cyt-*c*) and glucuronidation of 4NP (microsomes).

IMER-LC-NMR Analyses On-line enzyme reaction, analytic separation and identification of products were performed using the IMER-LC-NMR system depicted in Fig. 1. The mobile phase was 10 mM sodium phosphate buffer in D_2O. NMR measurements were performed in the loop-collection mode. The loop-collection mode trapped chromatographic peaks of interest in nine loops according to UV detection, and the loop contents were transferred to an NMR flow cell for NMR measurements.

Results and Discussion

Evaluation of Preparation of Immobilized Enzyme Phospholipid Columns The amount of DPPC coating on the ODS columns and that of immobilized enzyme on the phospholipid columns were calculated. The amounts of DPPC coating on the ODS columns were 0.072 mmol (Cyt-*c* column, 35×4.6 mm i.d.) and 0.072 mmol (microsome column, 35×4.6 mm i.d.), respectively. The amounts of immobilized enzyme on the phospholipid columns were 16.3 mg (Cyt-*c* column) and 27.6 mg (microsome column), respectively. Thus, the immobilized enzyme phospholipid columns could readily be prepared by allowing a solution containing the desired ligands to flow through the HPLC column.

Development and Evaluation of IMER-LC-NMR Cyt-*c* is one of the electron transport hemoproteins located on the biomembrane surface and usually does not catalyze the oxidation reaction. However, Cyt-*c* is known to show *N*-demethylase activity on addition of an oxygenating agent.[16] We tried various methods of adding the oxygenating agent during the separation. First, a solution of *N*-methylaniline (MA) as the substrate and hydrogen peroxide as the oxygenating agent were simultaneously injected onto the immobilized Cyt-*c* column with a mobile phase of 10 mM sodium phosphate buffer in D_2O (pD 7.1). Since the yield of the oxidation product of MA was low, time-consuming measurement of ^{1}H-NMR was necessary to identify the product. Next, we tried to inject the hydrogen peroxide solution onto the column after injection of the substrate. The yield of the *N*-demethylation product was sufficient to easily obtain the ^{1}H-NMR spectrum. *N*-Demethylation was efficiently induced by successive injection of the oxygenating agent when hydrogen peroxide solution passed through the previously injected substrate band in the column. Figure 2 shows the chromatogram obtained when 16 μmol/50 μl hydrogen peroxide solution as the oxygenating agent was injected onto the column after injection of 3 μmol MA as the substrate. Both the substrate and the product were simultaneously separated on the on-line column and detected after *N*-demethylation. The ^{1}H-NMR spectra of peak 2 (MA) and the peak 1 (product) are presented in Fig. 3. The ^{1}H-NMR spectrum of the product showed no singlet at 2.73 ppm (compared with MA), indicating the product to be aniline. As a result, the IMER-LC-NMR system could be successfully developed and applied to identify the enzymatic reaction product.

Next, we applied this system to the identification of glu-

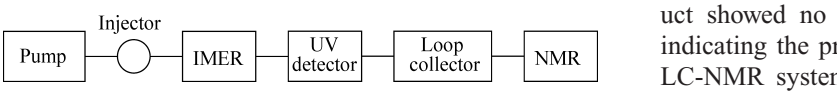

Fig. 1. Schematic Diagram of On-Line IMER Integrated into LC-NMR System

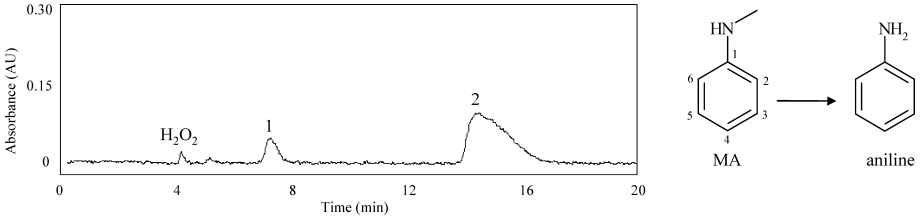

Fig. 2. UV Chromatogram of *N*-Demethylation of MA on Immobilized Cyt-*c* Column

Flow rate: 0.6 ml/min; detection: 280 nm; column temperature: 25 °C. Peaks: 1, aniline; 2, MA.

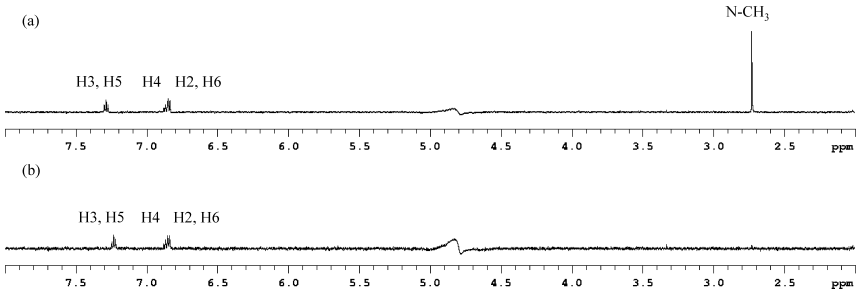

Fig. 3. ^{1}H-NMR Spectra of (a) MA and (b) Aniline

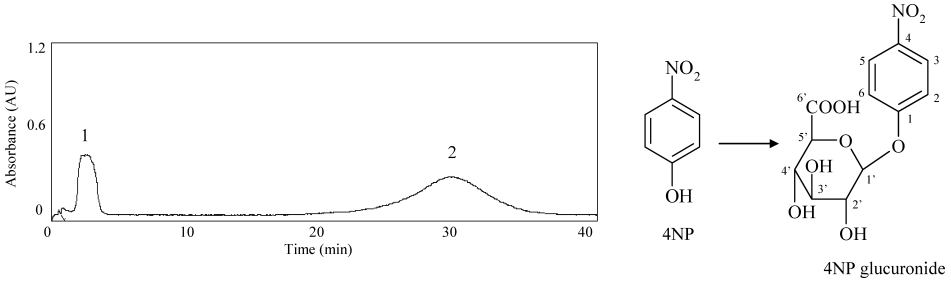

Fig. 4. UV Chromatogram of Glucuronidation of 4NP on Immobilized Microsome Column

Mobile phase: 10 mM sodium phosphate buffer in D_2O (pD 7.4) containing 10 mM magnesium chloride; flow rate: 0.5 ml/min; detection: 320 nm; column temperature: 37 °C. Peaks: 1, 4NP glucuronide; 2, 4NP.

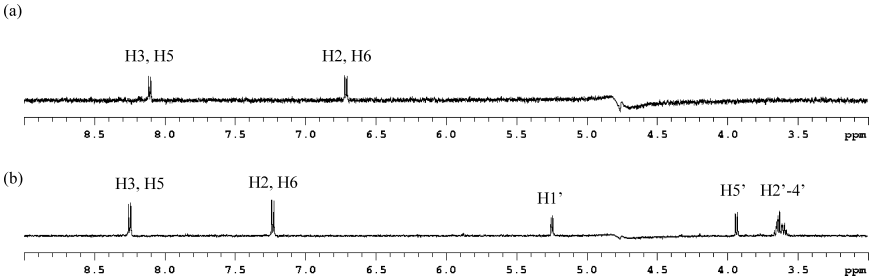

Fig. 5. ^{1}H-NMR Spectra of (a) 4NP and (b) 4NP Glucuronide

curonide formed from immobilized dog microsomes. Two micromoles of UDPGA as a coenzyme was injected onto the column after injection of 1 μmol 4-nitrophenol (4NP) as substrate at the mobile phase rate of 0.5 ml/min, and the flow was stopped after 55 s, enabling the substrate and UDPGA to be in contact with the microsome immobilized within the column. After 10 min, the mobile phase flow was restarted at 0.5 ml/min (Fig. 4). For identification, analyses of the ^{1}H-NMR of 4NP (peak 2) and 4NP glucuronide (peak 1) and ^{1}H–^{1}H COSY of 4NP glucuronide were performed (Fig. 5). The NMR spectra of the product supported glucuronidation with the presence of resonances at 5.25 ppm, 3.65 ppm, 3.63 ppm, 3.60 ppm and 3.94 ppm (compared with 4NP), attributable to protons H1′, H2′, H3′, H4′ and H5′ of glucuronic acid, respectively. The H1′ anomeric proton at 5.25 ppm showed a coupling constant of 7.3 Hz, which is the feature of diaxial coupling between H1′ and H2′ in the β-glucuronide.[12] Furthermore, the H2 and H6 of 4NP (6.71 ppm) was shifted downfield to 7.23 ppm by substitution of the molecule. These data clarified the product to be a glucuronide attached to the position 1 of 4NP *via* the ether linkage. As a result, the chemical structure of glucuronide as the enzymatic reaction product could be clearly elucidated by using IMER-LC-NMR without the need for authentic samples or isolation processes.

Conclusion

Coupling of immobilized enzyme reactors with an LC-NMR system enabled precise and rapid structural characterization of the enzymatic reaction products without the need for other commercially available authentic samples, chemically synthetic authentic samples or complicated isolation procedures. In this report, we presented two examples of the enzymatic reaction and the subsequent identification of the products using simple compounds. The IMER-LC-NMR system should be applicable for the structural identification of various drug metabolites by selecting the appropriate column and enzyme.

Acknowledgement We thank Takashi Kitade of M&S Instruments for many helpful discussions.

References

1) Kiba N., Goto Y., Furusawa M., *J. Chromatogr.*, **620**, 9—13 (1993).
2) Matsumoto K., Takahashi M., Takiyama N., Misaki H., Matsuo N., Murano S., Yuki H., *Clin. Chim. Acta*, **216**, 135—143 (1993).
3) Alebić-Kolbah T., Félix G., Wainer I. W., *Chromatographia*, **35**, 264—268 (1993).
4) Kim H. S., Wainer I. W., *J. Chromatogr. B*, **823**, 158—166 (2005).
5) Alebić-Kolbah T., Wainer I. W., *Chromatographia*, **37**, 608—612 (1993).
6) Kamimori H., Konishi M., *Anal. Sci.*, **17**, 1085—1089 (2001).
7) Nicoli R., Bartolini M., Rudaz S., Andrisana V., Veuthey J.-L., *J. Chromatogr. A*, **1206**, 2—10 (2008).
8) Lim C.-K., Lord G., *Biol. Pham. Bull.*, **25**, 547—557 (2002).
9) Dear G. J., Plumb R. S., Sweatman B. C., Ayrton J., Lindon J. C., Nicholson J. K., Ismail I. M., *J. Chromatogr. B*, **748**, 281—293 (2000).
10) Feng W., Liu H., Chen G., Malchow R., Bennett F., Lin E., Pramanik B., Chan T. M., *J. Pharm. Biomed. Anal.*, **25**, 545—557 (2001).
11) Elip M. V. S., Huskey S.-E. W., Zhu B., *J. Pharm. Biomed. Anal.*, **30**, 1431—1440 (2003).
12) Kamimori H., Ozaki Y., Okabayashi Y., Ueno K., Narita S., *Anal. Biochem.*, **317**, 99—106 (2003).
13) Smallcombe S. H., Patt S. L., Keifer P. A., *J. Magn. Reson. A*, **117**, 295—303 (1995).
14) Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J., Klenk D. C., *Anal. Biochem.*, **150**, 76—85 (1985).
15) Kamimori H., Konishi M., *Biomed. Chromatogr.*, **16**, 61—67 (2002).
16) Hamachi I., Fujita A., Kunitake T., *J. Am. Chem. Soc.*, **116**, 8811—8812 (1994).