Enzymatic Activities in the Microsomes Prepared from Rat Small Intestinal Epithelial Cells by Differential Procedures

Kiminori Mohri[1,2] and Yoshihiro Uesawa[1]

Received February 15, 2001; accepted April 27, 2001

KEY WORDS: small intestinal microsomes; rat small intestine; cytochrome P450; UDP-glucuronosyltransferase; enterocyte.

INTRODUCTION

In 1982, it was reported that the small intestinal extraction ratio of phenacetin in rats coincided with the result of a metabolic investigation using epithelial cells (enterocytes) of the rat small intestine (1). That is, when Klippert *et al.* administered phenacetin into the jugular vein, the portal vein, and the duodenum of rats pretreated with 3-methylcholanthrene, the extraction ratio by the gut was calculated to be 0.53. However, the extraction ratio obtained from the *in vitro* data on phenacetin metabolism using isolated mucosal cells of 3-methylcholanthrene-pretreated rats was between 0.31 and 0.53. Gupta *et al.* (2) first reported in humans that the contribution of the small intestine in the first pass metabolism of cyclosporine was significantly large.

In recent years, the metabolism of a drug when it passes through enterocytes has been the focus of attention (3–8). Although drug metabolism studies using rat intestinal microsomes have been increasing, their results vary quite widely among the researchers. It is conceivable that different procedures in the preparation of intestinal microsomes contribute severely to enzymatic stability; however, a comparative study of the preparation procedures of the microsomes from rat enterocytes has never been reported.

In this report, we describe the activities of cytochrome P450 and UDP-glucuronosyltransferase in the rat small intestinal microsomes prepared by four different procedures and the stability of the enzyme activities in the microsomes during storage at 4°C, –20°C, and –80°C for 30 days.

MATERIALS AND METHODS

Chemicals

6β- and 16α-hydroxytestosterone (6β- and 16α-TOH) and *p*-nitrophenol (p-NP) were purchased from Daiichi Pure Chemicals Co., Ltd., (Tokyo). Testosterone was obtained from Nacalai Tesque, Inc., (Kyoto). *p*-Nitroanisole (p-NA) was purchased from Tokyo Kasei Kogyo Co., Ltd., (Tokyo). Phenylmethylsulfonyl fluoride (PMSF) and *p*-nitrophenol β-D-glucuronide (p-NPG) were purchased from Sigma Chemical Co. (St. Louis, MO). Corticosterone (internal standard for TOHs), histidine, dithiothreitol, digitonin, glycerol, and UDP-glucuronic acid sodium salt (UDP-Ga) were obtained from Wako Pure Chemical Industries, Ltd., (Osaka). The methanol (MeOH), ethanol (EtOH), and acetonitrile (MeCN) used were of high-performance liquid chromatography grade (Wako). All other chemicals were reagent grade (Wako). Water was used after double distillation in a glass still.

Preparation of Small Intestinal and Hepatic Microsomes

Male Wistar-ST rats (Sankyo Labo Service Co., Ltd., Tokyo) weighing 280 to 300 g were used throughout the study. The rats were housed in stainless steel cages, five animals per cage, in a temperature-controlled (24–26°C) room with a 12-h light/dark cycle. The rats were allowed free access to standard rat chow (Sankyo) and water for 1 week before the experiments. The rats fasted overnight before the experiments. Each animal was anesthetized with 20% (w/v) urethane (1 g/kg body weight intraperitoneally). All the preparation procedures of the microsomes were undertaken in a cold room (4°C). A 30-cm portion (from pars pylorica ventriculi) of rat small intestine was rinsed with solution A, pH 7.3, which contained 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4 and 0.23 mM PMSF (9), and prepared as four kinds of small intestinal microsomes (G-mic) by the procedures described below in (A), (B), (C), and (D).

Rat liver was minced and homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose at 20% (w/v) concentration. The homogenate was centrifuged at 700 g for 10 min at 4°C and then centrifuged at 10,000 g for 10 min. Two kinds of liver microsomes (L-mic) were prepared from the supernatant by the methods described in (E) and (F). Each of the microsomes was resuspended in 100 mM Tris-HCl (pH 7.4) containing 20% (w/v) glycerol and 10 mM EDTA and stored in liquid nitrogen until use. Protein concentration was measured by the method of Lowry *et al.* (10).

(A) Scraping of Enterocytes and Ca-Aggregation Procedures (G-mic/SCa)

Isolation of small intestinal epithelial cells was achieved by scraping the mucosa on the small intestine with a microscope slide on ice. The separated cells were homogenized in 5 mL of solution C, pH 7, which contained 5 mM histidine, 0.25 M sucrose, 0.5 mM EDTA, and 0.23 mM PMSF (9) per rat, and centrifuged at 15,000 g for 10 min at 4°C. After calcium chloride was added to a final concentration of 10 mM, the supernatant was kept standing on ice for 15 min. The microsome G-mic/SCa was obtained by centrifugation at 2,000 g for 10 min.

(B) Scraping of Enterocytes and Ultracentrifugation Procedures (G-mic/SU)

The microsome G-mic/SU was obtained from the homogenate of enterocytes described in (A) by centrifugation at

[1] Clinical Pharmaceutics Laboratory, Department of Pharmaceutics, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose-shi, Tokyo 204-8588, Japan.

[2] To whom correspondence should be addressed. (e-mail: k-mohri@my-pharm.ac.jp)

0724-8741/01/0800-1232$19.50/0

15,000 g for 10 min and then the supernatant at 100,000 g for 45 min.

(C) EDTA-Separation of Enterocytes and Ca-Aggregation Procedures (G-mic/ECa)

Rat small intestine was incubated for 15 min at 37°C in the solution A. The contents of the small intestine were replaced with ice-cold solution B, which contained 136.9 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1.5 mM EDTA, 0.5 mM dithiothreitol, and 0.23 mM PMSF, and the small intestine was placed on a glass plate on ice and tapped gently several times. The separation procedures of the enterocytes in solution B were repeated three times. The separated cells were washed with the solution C and centrifuged at 800 g for 10 min at 4°C. The cells were homogenized in 5 mL of solution C and centrifuged at 15,000 g for 10 min at 4°C. The microsome G-mic/ECa from the supernatant was obtained by the Ca-aggregation method described in (A) (9).

(D) EDTA-Separation of Enterocytes and Ultracentrifugation Procedures (G-mic/EU)

Homogenate of the enterocytes was prepared by the method described in (C). The microsome G-mic/EU was obtained from the homogenate by the ultracentrifugation method described in (B).

(E) Ca-Aggregation Procedures for Liver (L-mic/Ca)

The microsome L-mic/Ca was obtained from the supernatant of the liver homogenate instead of the enterocyte homogenate by the Ca-aggregation method described in (A).

(F) Ultracentrifugation Procedures for Liver (L-mic/U)

The microsome L-mic/U was prepared from the supernatant of liver homogenate by the ultracentrifugation method described in (B).

Measurement of Enzymatic Activities in the Microsomes

The assays of P450 isoform (CYP1A1, 2B/2C, and 3A) specific oxidation activities and UDP-glucuronosyltransferase (UGT) activity using testosterone, p-NA, and p-NP were performed using the HPLC according to the methods specified below.

(1) P450 Activities

The enzymatic reactions for testosterone 6β- and 16α–hydroxylation and p-NA demethylation were performed by the methods of Ding and Coon (11) and Kamataki *et al.* (12) with a modification. Briefly, a reaction mixture (final volume of 120μL in 0.1 M sodium phosphate buffer, pH 7.4) containing 1 mM NADPH, and 0.2 mM testosterone or 5 mM p-NA was incubated with each of the microsomes for 5 min at 37°C. The reaction was stopped by addition of ice-cold 2 μg/mL corticosterone/MeCN solution (120 μL) and 0.22 g of solid ammonium sulfate (for testosterone), or acetonitrile (120 μL) and saturated aqueous ammonium sulfate (120 μL) (for p-NA). The resulting mixture was vortexed vigorously, centrifuged at 12,000 g for 10 min, and the organic layer was injected onto the high-performance liquid chromatographer (HPLC).

Each metabolite concentration produced by the enzymatic reactions was obtained from the calibration curves for each standard metabolite (6β- and 16α-TOHs: 2–10 μM, p-NP: 0.167–6.67 μM) added in the reaction mixture excluded each substrate. In the time-course experiments, the 6β- and 16α-TOHs and p-NP produced linear up to 7.5 min.

(2) UGT Activities

The UGT activities for p-NP in the microsomes were measured according to the previous reported methods with a modification (13). In our preliminary study for the glucuronidation of p-NP, the Michaelis–Menten plot was drawn. Because the V_{max} value was constant over 1 mM p-NP, we used 2 mM p-NP as the substrate amount. In the time-course experiments, p-NPG was produced linear up to 30 min. Briefly, a reaction mixture (final volume of 400 μ in 0.1 M Tris-HCl buffer, pH 7.4) containing 20 mM $MgCl_2$, 8 mM UDP-Ga, and 2 mM p-NP was incubated with each of the microsomes for 5 min at 37°C. The enzymatic reaction was stopped by addition of ice-cold EtOH (240 μL) and 0.32 g of solid ammonium sulfate. The resulting mixture was vortexed vigorously, centrifuged at 12,000 g for 10 min, and the organic layer was injected onto the HPLC. p-NPG concentrations produced by the enzymatic reaction were obtained from the calibration curves for standard p-NPG concentrations (1.2–360 μM) added in the reaction mixture excluded p-NP.

(3) Stability of Enzymatic Activities in G-mic/EU

G-mic/EU was stored at 4°C, –20°C, and –80°C for 30 days. Testosterone 6β- and 16α-hydroxylation, p-NA demethylation, and p-NP glucuronidation activities were determined with time.

HPLC Conditions

The HPLC system used consisted of a PU-980 pump (JASCO Co., Ltd., Tokyo) equipped with a UV-970 UV/VIS detector (JASCO), a SIL-9A auto-injector (Shimadzu Co., Ltd., Kyoto), and a C-R4A Chromatopac integrator (Shimadzu). TOHs, p-NP, and p-NPG were measured at wavelengths of 240 nm, 400 nm, and 300 nm, respectively. Each sample was analyzed using a reverse phase analytical Capcell Pak UG-ODS column [4.6 mm (inside diameter) × 25 cm; particle size 5 μm (Shiseido Co., Ltd., Tokyo)] equipped with a guard column packed with Capcell Pak SG-ODS [4.6 mm (inside diameter) × 1 cm; particle size 5 μm (Shiseido)]. Mobile phases consisting of 0.085 % phosphoric acid:MeOH:MeCN (50:30:15, v/v) for the analysis of 6β- and 16α-testosterone oxides and 0.05 M sodium phosphate (pH 7.1):MeOH (80:20, v/v) for p-NP and p-NPG were used. The mobile phases were pumped through the column at a speed of 1.0 mL/min. The detection limits of TOHs, p-NP and p-NPG in the reaction mixture by the HPLC methods used were equivalent to the enzymatic activities of 0.012, 0.0025, and 0.125 nmol/min/mg protein, respectively.

RESULTS

Enzymatic Activities in the Microsomes

Testosterone 6β- and 16α-hydroxylation, p-NA demethylation, and p-NP glucuronidation activities are shown in Fig. 1, A–D. Both testosterone hydroxylation activities in G-mic/

SCa and G-mic/SU prepared by the scraping method could not be found in the HPLC system. On the other hand, testosterone 6β-hydroxylation activities in the G-mic/ECa and G-mic/EU prepared by the EDTA-separation method were 0.185 ± 0.020 and 0.289 ± 0.005 nmol/min/mg protein, respectively, and 16α-hydroxylation activities were 0.0875 ± 0.017 and 0.223 ± 0.041. The hydroxylation activities in the L-mic/U and G-mic/EU prepared by the ultracentrifugation method were higher than those in the L-mic/Ca and G-mic/ECa prepared by the Ca-aggregation method (Fig. 1, A and B). The activities in the G-mic/SCa and G-mic/SU for the oxidative demethylation from p-NA to p-NP were 0.00401 ± 0.00055 and 0.00440 ± 0.00111 nmol/min/mg protein, respectively, whereas the demethylation activities (0.00757 ± 0.00084 and 0.01140 ± 0.00020 nmol/min/mg protein, respectively) in the G-mic/ECa and G-mic/EU were higher than those in the G-mic/SCa and G-mic/SU prepared by the scraping method (Fig. 1C). The demethylation activities in the L-mic/Ca and L-mic/U were 0.0186 ± 0.0018 and 0.0317 ± 0.0004 nmol/min/mg protein, respectively.

p-NP glucuronidation activities in the G-mic/SCa, G-mic/SU, G-mic/ECa, G-mic/EU, L-mic/Ca, and L-mic/U were 0.055 ± 0.008, 2.21 ± 0.3, 2.49 ± 0.24, 6.92 ± 0.83, 10.5 ± 1.4 and 28.2 ± 1.7 nmol/min/mg protein, respectively.

Stabilities of the Enzymatic Activities in the Microsomes

The results of time course experiments for a period of 30 days for testosterone 6β- and 16α-hydroxylation, p-NA demethylation, and p-NP glucuronidation activities in the G-mic/EU are shown in Fig. 2, A–D. In all of the enzymatic reactions tested, the activities in the microsomes stored at –80°C did not decrease significantly. When the microsomes were stored at 4°C, both testosterone hydroxylation activities almost disappeared. The demethylation activity against p-NA remained at only 30% after 30 days at 4°C, whereas the glucuronidation activity against p-NP did not change significantly in the same microsomes at 4°C during 30 days.

DISCUSSION

In the recent reports, observations of drug metabolism in rat enterocytes using small intestinal microsomes varied widely among the researchers regarding enzymatic activities and the contents of the enzymes. Zhang *et al.* (14) reported that rat enterocytes included only a small amount of CYP3A, whereas other groups (3,15) reported that some drugs metabolized by the CYP3A subfamily were oxidized in the rat small intestinal microsomes (G-mic) with strong activities, comparable to that of hepatic microsomes (L-mic). Bonkovsky *et al.* (9) indicated that the P450 content in rat small intestinal microsomes obtained by the scraping method was lower than that prepared by EDTA treatment of small intestinal epithelial cells. We assumed that these different results might be attributable to the different procedures for the preparation of the small intestinal microsomes (G-mic).

Although many researchers select a procedure (the scraping method) that involves scraping the enterocytes with

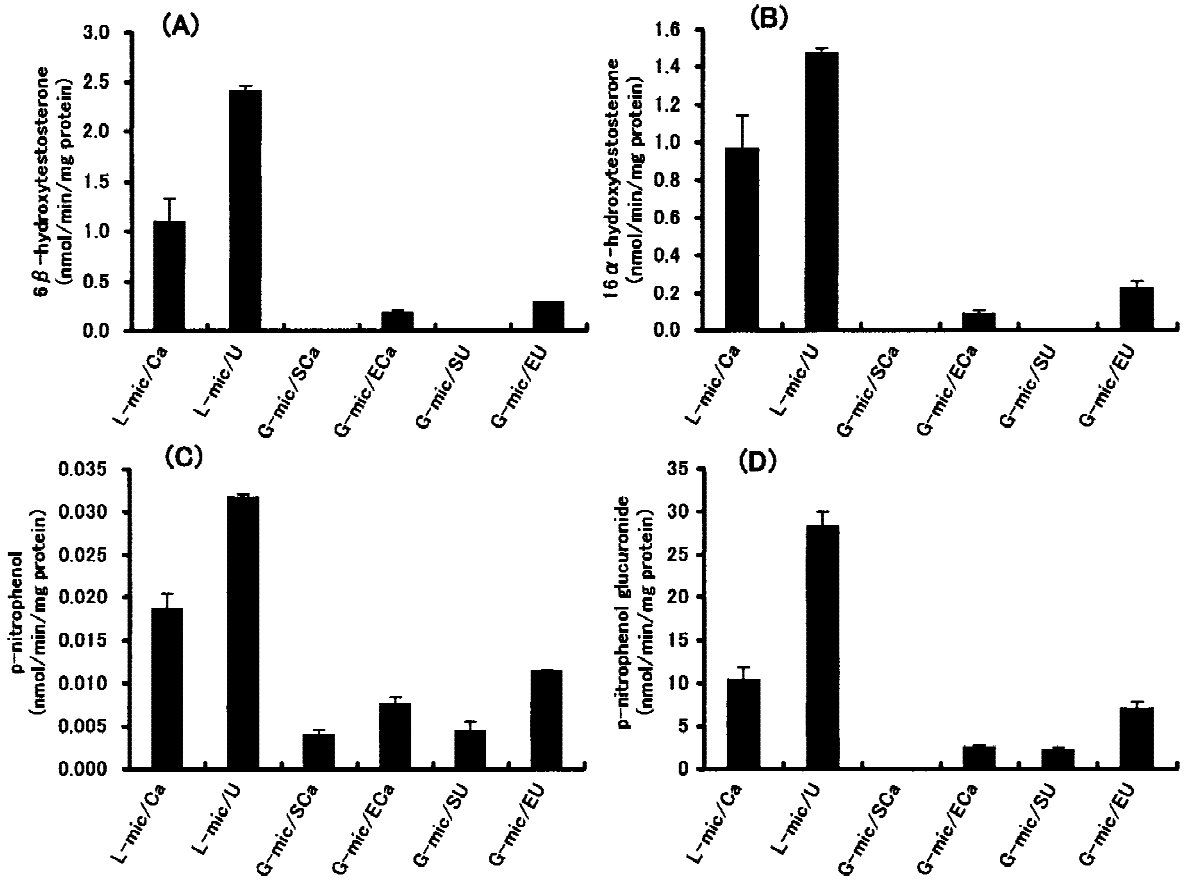

Fig. 1. The activities of (A) testosterone 6α-hydroxylation, (B) testosterone 16α-hydroxylation, (C) p-nitroanisole demethylation, and (D) p-nitrophenolglucuronidation in each of the microsomes. Each point and vertical bar represents the mean ± SD of five experiments.

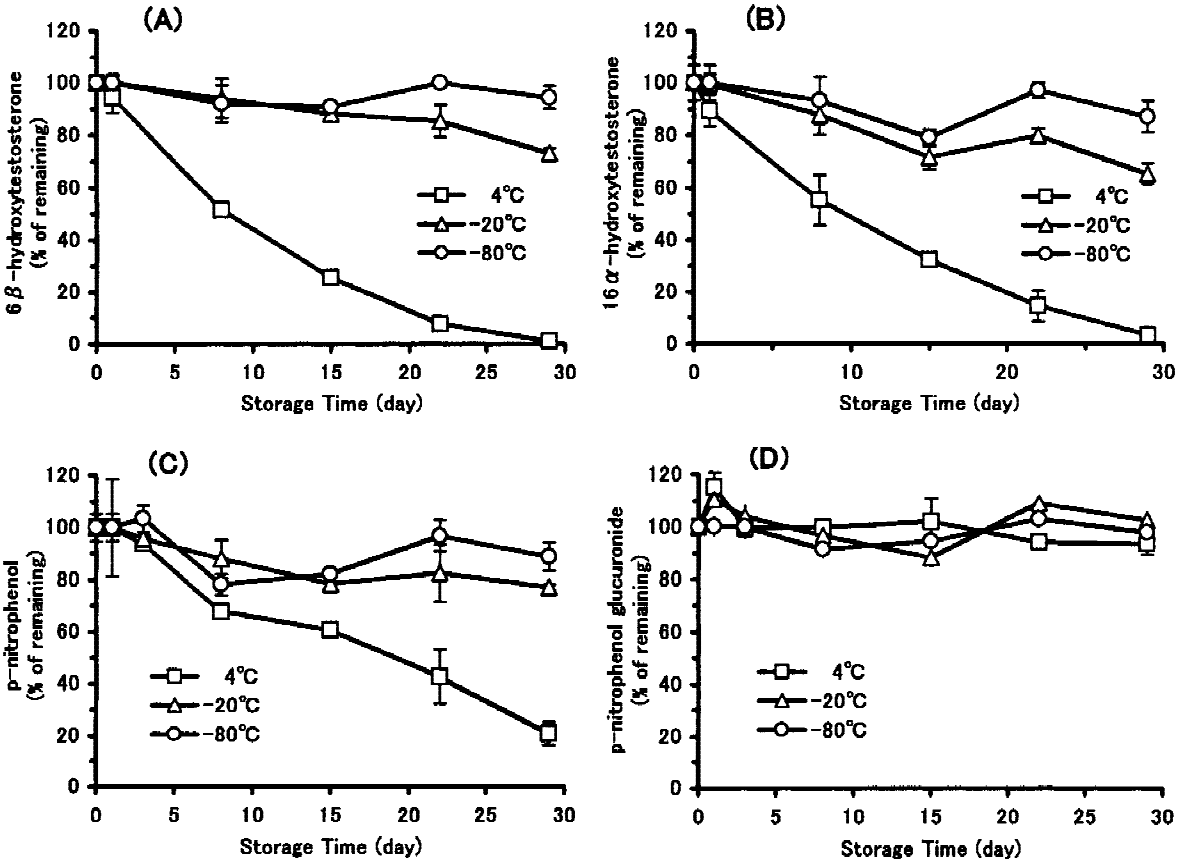

Fig. 2. The effects of storage temperature on the activities of (A) testosterone 6β-hydroxylaiton, (B) testosterone 16α-hydroxylation, (C) p-nitroanisole demethylation, and (D) p-nitrophenol glucuronidation in the rat intestinal microsomes (G-mic/EU). Each point and vertical bar represents the mean ± SD of five experiments.

a microscope slide and then preparing microsomes (G-mic/SU) by ultracentrifugation of the enterocyte homogenate, there is another procedure (EDTA-separation method) that separates the enterocytes by EDTA treatment of the small intestinal epithelial cells and then prepares the microsomes (G-mic/E). Furthermore, the preparation of microsomal fractions from the enterocytes is classified in two procedures; one is a procedure (Ca-aggregation method) that obtains microsomal fractions (G-mic/ECa) by aggregation adding Ca ion, and the other is a procedure (ultracentrifugation method) that obtains microsomal fractions (G-mic/EU) by ultracentrifugation of the enterocyte homogenate.

In this study, we prepared four kinds of small intestinal microsomes (G-mic/SCa, G-mic/SU, G-mic/ECa, and G-mic/EU) by combinations of these methods described previously in the "Materials and Methods" section and investigated testosterone 6β- and 16α-hydroxylation activities, oxidized with CYP3A and CYP2B/2C. Although it is known that CYP2B and CYP2C cause testosterone 16α-hydroxylation to progress in rat liver (16), Fasco *et al.* (17) reported that the CYP2C activity on warfarin oxidation could not be detected in rat G-mic prepared by a combination of EDTA-separation and ultracentrifugation procedures, being very similar to our G-mic/EU. Therefore, the testosterone 16α-hydroxylation in the rat G-mic might be caused by the CYP2B subfamily, which exists in rat enterocytes.

The activities in the G-mic/SCa and G-mic/SU, however, were not detected under the detection limits (equivalent to 0.012 nmol/min/mg protein) in the HPLC system. On the other hand, G-mic/ECa and G-mic/EU indicated enzymatic activities in both hydroxylation reactions (Fig. 1, A and B). These findings elucidated that the scraping procedure injured the enzyme activities in the G-mic significantly. The demethylation activities of p-NA by CYP1A1 in the G-mic/SCa and G-mic/SU were about half of those in the G-mic/ECa and G-mic/EU, respectively.

The p-NP glucuronidation activity in the G-mic/SCa was almost zero. The activity in the G-mic/SU was almost equal to that in the G-mic/ECa and was approximately 0.3 times that in the G-mic/EU, respectively.

The results obviously suggested that the scraping process in the preparation of the intestinal microsomes caused some damage to the enzyme activities in the enterocytes and that the damage level was different with the species of enzymes and subfamilies. Although we added PMSF as a serine protease inhibitor in the microsomal preparation process, probably, this damage might be caused by the effect of digestive enzyme(s) in the alimentary canal. It may be speculated that since the scraping handling crushes the enterocytes directly, the enzymes in the endoplasmic reticulum received large damage by the digestive enzyme(s), before PMSF shows its effect. Furthermore, the effect of the digestive enzyme(s) may be different among CYP subfamilies (as CYP3A and CYP1A, Fig. 1).

The CYP1A1, 3A, 2B, and UGT activities in the G-mic/EU were higher than those in the other G-mic (G-mic/SCa,

G-mic/Eca, and G-mic/SU) (Fig. 1). The oxidation and glucuronidation activities in the L-mic/U prepared from rat liver were higher than those in L-mic/Ca.

We examined the stability of enzymes in the small intestinal microsomes using G-mic/EU. Not only CYP1A1, 2B, and 3A but also UGT activities were almost stable for 30 days when stored at –80°C (Fig. 2). However, testosterone 6β- and 16α-hydroxylation activities by CYP3A and 2B were gradually decreased at 4 and –20°C, respectively (Fig. 2, A and B). The decline in p-NA demethylation activity by CYP1A1 was slower than those of CYP3A and 2B (Fig. 2C). The UGT activity detected by p-NP glucuronidation was very stable for 30 days even at 4°C and did not change the activity significantly (Fig. 2D).

In conclusion, the enzymatic activities (CYP 1A1, 3A, 2B, and UGT) in small intestinal microsomes differed with the preparation procedures. The small intestinal microsomes prepared by the following procedure possessed high CYP3A activity: Separating the enterocytes with EDTA from the small intestinal mucosa and then obtaining the microsomal fraction (G-mic/EU) by ultracentrifugation, regardless of the kind of enzyme. In the case of hepatic microsomes, the microsomes (L-mic/U) obtained from the liver homogenate by the ultracentrifugation method possessed high enzyme activities. To understand the first-pass metabolism of an oral drug when absorbed through the alimentary canal mucosa, it is very important to employ the small intestinal microsomes with high enzymatic activities in in vitro studies.

ACKNOWLEDGMENTS

We thank Hitomi Nomura at Meiji Pharmaceutical University for skillful technical assistance.

REFERENCES

1. P. Klippert, P. Borm, and J. Noordhoek. Prediction of intestinal first-pass effect of phenacetin in the rat from enzyme kinetic data —Correlation with in vivo data using mucosal blood flow. *Biochem. Pharmacol.* **31**:2545–2548 (1982).
2. S. K. Gupta, A. Bakran, R. W. Johnson, and M. Rowland. Erythromycin enhances the absorption of cyclosporine. *Br. J. Clin. Pharmacol.* **25**:401–402 (1988).
3. J. C. Kolars, P. L. Stetson, B. D. Rush, M. J. Ruwart, P. Schmiedlin-Ren, E. A. Duell, J. J. Voorhees, and P. B. Watkins. Cyclosporine metabolism by P450IIIA in rat enterocytes—Another determinant of oral bioavailability? *Transplantation* **53**:596–602 (1992).
4. M. F. Hebert, J. P. Roberts, T. Prueksaritanont, and L. Z. Benet. Bioavailability of cyclosporine with concomitant rifampin administration is markedly less than predicted by hepatic enzyme induction. *Clin. Pharmacol. Ther.* **52**:453–457 (1992).
5. D. Y. Gomez, V. J. Wacher, S. J. Tomlanovich, M. F. Hebert, and L. Z. Benet. The effects of ketoconazole on the intestinal metabolism and bioavailability of cyclosporine. *Clin. Pharmacol. Ther.* **58**:15–19 (1995).
6. L. C. Floren, I. Bekersky, L. Z. Benet, Q. Mekki, D. Dressler, J. W. Lee, J. P. Roberts, and M. F. Hebert. Tacrolimus oral bioavailability doubles with coadministration of ketoconazole. *Clin. Pharmacol. Ther.* **62**:41–49 (1997).
7. M. F. Fromm, D. Busse, H. K. Kroemer, and M. Eichelbaum. Differential induction of prehepatic and hepatic metabolism of verapamil by rifampin. *Hepatology* **24**:796–80 (1996).
8. K. E. Thummel, D. O'Shea, M. F. Paine, D. D. Shen, K. L. Kunze, J. D. Perkins, and G. R. Wilkinson. Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin. Pharmacol. Ther.* **59**:491502 (1996).
9. H. L. Bonkovsky, H. P. Hauri, U. Marti, R. Gasser, and U. A. Meyer. Cytochrome P450 of small intestinal epithelial cells. Immunochemical characterization of the increase in cytochrome P450 caused by phenobarbital. *Gastroenterology* **88**: 458–467 (1985).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265275 (1951).
11. X. Ding and M. J. Coon. Steroid metabolism by rabbit olfactory-specific P450 2G1. *Arch. Biochem. Biophys.* **315**:454–459 (1994).
12. T. Kamataki, M. Kitada, H. Shigematsu, and H. Kitagawa. The involvement of cytochrome P-448 and P-450 in NADH-dependent O-demethylation of p-nitroanisole in rat liver microsomes. *Jpn. J. Pharmacol.* **29**:191–201 (1979).
13. G. F. Rush, J. F. Newton, and J. B. Hook. Sex differences in the excretion of glucuronide conjugates: The role of intrarenal glucuronidation. *J. Pharmacol. Exp. Ther.* **227**:658–662 (1983).
14. Q. Y. Zhang, J. Wikoff, D. Dunbar, and L. Kaminsky. Characterization of rat small intestinal cytochrome P450 composition and inducibility. *Drug Metab. Dispos.* **24**:322–328 (1996).
15. T. Koudriakova, E. Iatsimirskaia, S. Tulebaev, D. Spetie, I. Utkin, D. Mullet, T. Thompson, P. Vouros, and N. Gerber. In vivo disposition and metabolism by liver and enterocyte microsomes of the antitubercular drug rifabutin in rats. *J. Pharmacol. Exp. Ther.* **279**:1300–1309 (1996).
16. M. Paolini, J. Barillari, M. Broccoli, L. Pozzetti, P. Perocco, and G. Cantelli-Forti. Effect of liquorice and glycyrrhizin on rat liver carcinogen metabolizing enzymes. *Cancer Lett.* **145**:35–42 (1999).
17. M. J. Fasco, J. B. Silkworth, D. A. Dunbar, and L. S. Kaminsky. Rat small intestinal cytochromes P450 probed by warfarin metabolism. *Mol. Pharmacol.* **43**:226–233 (1993).