Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 6, pp. 839–844, 1994

0731–7085/94 $7.00 + 0.00

0731–7085(93)E0031-H

Development and preliminary application of a high-performance liquid chromatographic assay for omeprazole metabolism in human liver microsomes

KAORU KOBAYASHI,†‡ KAN CHIBA,*† MASAYOSHI TANI,§ YUKIO KUROIWA‡ and TAKASHI ISHIZAKI†

† *Division of Clinical Pharmacology, Clinical Research Institute, National Medical Center, Toyama 1-21-2, Shinjuku-ku, Tokyo 162, Japan*
‡ *Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Showa University, Hatanodai 1-5-8, Shinagawa-ku, Tokyo 142, Japan*
§ *Division of General Surgery, National Medical Center, Toyama 1-21-1, Shinjuku-ku, Tokyo 162, Japan*

Abstract: A high-performance liquid chromatography assay was developed to measure the enzymatic activities of the 5-hydroxylation and sulphoxidation of omeprazole, a proton pump inhibitor, in human liver microsomes. The preliminary study was also undertaken to assess the assay's applicability for the enzyme kinetic analysis of omeprazole metabolism by human liver microsomes. The recovery of 5-hydroxyomeprazole, omeprazole sulphone and phenacetin (internal standard) after the precipitation of microsomal protein by acetonitrile was nearly complete. The intra-assay relative standard deviations (n = 6) were 8.2 and 12.8% for quantitation of the 5-hydroxylation and sulphoxidation activities of omeprazole, respectively. Eadie–Hofstee plots for the formation of 5-hydroxyomeprazole and omeprazole sulphone gave a biphasic relationship for all the microsomal samples studied (n = 6). The respective mean (±SD) high- and low-affinity component kinetic parameters for the 5-hydroxylation and sulphoxidation of omeprazole estimated by a two-enzyme kinetic analysis were: K_{m1} = 6.3 ± 1.7 and 10.4 ± 6.3 μM, K_{m2} = 183.2 ± 180.4 and 671.2 ± 639.4 μM, V_{max1} = 109.8 ± 75.4 and 77.5 ± 46.1 pmol mg^{-1} min^{-1}, and V_{max2} = 163.3 ± 94.1 and 318.3 ± 163.3 pmol mg^{-1} min^{-1}. The results suggest that the assay is reproducible, accurate and applicable for studying the metabolism of omeprazole in human liver microsomes.

Keywords: *HPLC assay; omeprazole; proton pump inhibitor; major metabolites; human liver microsomes.*

Introduction

Omeprazole is a selective inhibitor of H^+/K^+-ATPase proton pump in gastric parietal cells, which has recently been used for the treatment of duodenal ulcers, refractory gastroesophageal reflex disease, Zollinger–Ellison syndrome and other hypersecretory conditions [1, 2]. The drug is extensively metabolized by the liver primarily via oxidation. The major metabolites detected in plasma are 5-hydroxyomeprazole and omeprazole sulphone and those in urine are 5-hydroxyomeprazole and omeprazole acid in humans (Fig. 1) [1, 2].

Recently, Sohn *et al.* [3] and Andersson *et al.* [4] have reported that the areas under the plasma concentration–time curves (AUCs) of omeprazole and omeprazole sulphone were significantly greater, whereas the AUC of 5-hydroxyomeprazole was significantly less in poor metabolizers (PMs) than in extensive metabolizers (EMs) of *S*-mephenytoin. Moreover, the former investigators have shown that the urinary excretion of 5-hydroxyomeprazole was significantly less in PMs than in EMs of *S*-mephenytoin [3]. These findings suggest that the 5-hydroxylation of omeprazole is mediated by the cytochrome P450 responsible for the 4′-hydroxylation of *S*-mephenytoin but that the sulphoxidation of omeprazole appears to be catalysed by a different enzyme(s).

To further characterize the enzyme(s) responsible for the metabolism of omeprazole in humans, the establishment of an assay for its major metabolites formed by human liver microsomes appears to be essential. However,

* Author to whom correspondence should be addressed.

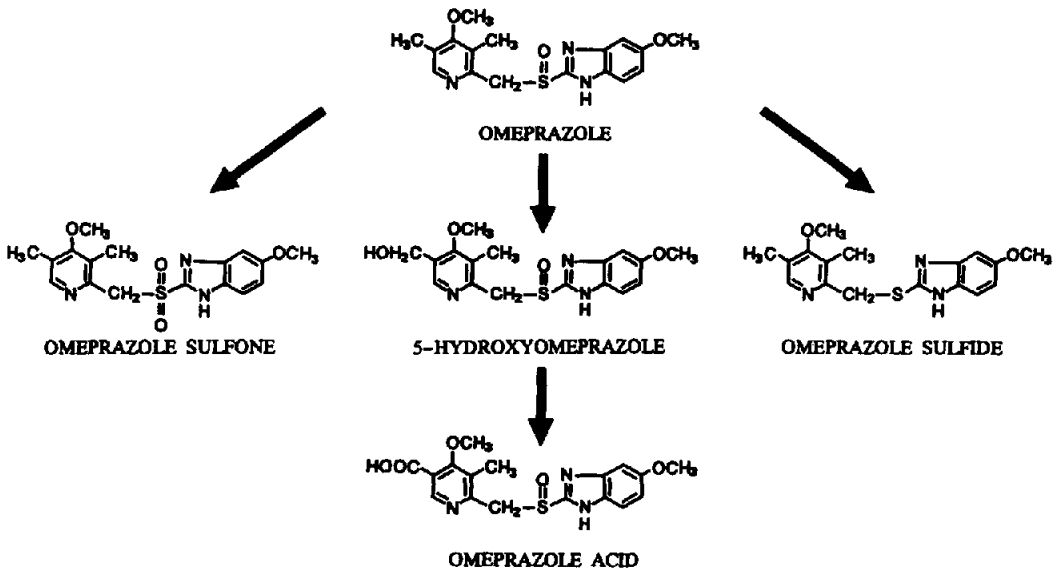

Figure 1
Metabolic pathways of omeprazole in humans.

several assay methods of omeprazole and its metabolites reported previously are those for plasma and urine samples obtained from humans in an *in vivo* experiment [5–8] that may not be applied directly for an *in vitro* human microsomal assay. In this report, we describe a high-performance liquid chromatographic (HPLC) assay of the major metabolites of omeprazole, which is considered to be reproducible and accurate enough to apply for investigating the metabolism of omeprazole in human liver microsomes.

Experimental

Drugs and chemicals

Omeprazole, omeprazole sulphone and 5-hydroxyomeprazole were gifts from Fujisawa-Astra (Osaka, Japan), Fujisawa (Osaka, Japan) and Astra Hässle AB (Mölndal, Sweden), respectively. Since omeprazole contained unknown contaminant(s), it was purified before use as follows: omeprazole was dissolved in dichlormethane and washed three times with sodium phosphate buffer (pH 11.0, 100 mM). By this procedure, more than 95% of the contaminant(s) was removed. Phenacetin (internal standard) was purchased from Aldrich (Milwaukee, USA). NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Acetonitrile and other reagents of analytical grade were purchased from Wako (Osaka, Japan).

Human liver microsomes

Fresh human liver samples were obtained from Japanese patients who underwent a partial hepatectomy at the Division of General Surgery, National Medical Center, Tokyo, as an excess material was removed during surgery on the liver as reported previously [9, 10]. The use of the human liver for the study had been approved by the Institutional Ethics Committee. It took less than 5 min from the removal of liver until collecting and freezing a sample in liquid nitrogen. The liver parenchyma of the non tumour-bearing part used for the study was shown later to be histopathologically normal in all the cases. The liver samples obtained from the following liver donors were not included in the study: patients with acute or chronic hepatitis, those with cirrhosis and those who were taking medications known to induce or inhibit the hepatic monooxygenase activity.

Microsomes were prepared by differential centrifugation, and the 105,000*g* pellet was washed and resuspended in 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. After the determination of protein concentration [11], the suspended microsomes were allocated, frozen and kept at −80°C until used.

Incubation and sample preparation

The basic incubation medium contained 0.1 or 0.2 mg ml^{-1} of microsomes, 0.5 mM NADP, 2.0 mM glucose-6-phosphate, 1 I.U. ml^{-1} of glucose-6-phosphate dehydrogenase, 4 mM $MgCl_2$, 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4) and 2.5–400 μM of omeprazole in a final volume of 250 μl. The mixture was incubated at 37°C, and the reaction was stopped by adding 100 μl of cold acetonitrile. After the termination of incubation, 50 μl of phenacetin solution (279 μM in methanol) was added to the sample as an internal standard. The mixture was centrifuged at 10,000*g* for 5 min, and the supernatant was injected into an HPLC apparatus as described below.

Determination of metabolites

The HPLC system consisted of a Model L-6000 pump (Hitachi, Tokyo, Japan), a Model L-4000 UV detector (Hitachi), a Model AS-2000 autosampler (Hitachi), a Model D-2500 integrator (Hitachi) and a 4.6 mm × 25 cm CAPCELL PAK C 18 SG120 column (Shiseido, Tokyo, Japan). The mobile phase consisted of acetonitrile–sodium phosphate buffer (0.05 M, pH 8.4) (26:74, v/v) and was delivered at a flow rate of 0.8 ml min^{-1}. The column temperature was maintained at 30°C. The eluate was monitored at 302 nm. Calibration curves were generated from 0.05 to 1 μM by processing the authentic standard substances throughout the entire procedure.

Kinetic analysis

Michaelis–Menten kinetic parameters for the formation of 5-hydroxyomeprazole and omeprazole sulphone were estimated by fitting the data to the following equation: $V = V_{max1} \cdot S/(K_{m1} + S) + V_{max2} \cdot S/(K_{m2} + S)$, where V is the velocity of the formation of 5-hydroxyomeprazole or omeprazole sulphone, S is the concentration of omeprazole in the incubation mixture, K_{m1} and K_{m2} are the affinity constants for the high- and low-affinity components, and V_{max1} and V_{max2} are the maximum enzyme velocities for the high- and low-affinity components, respectively. The kinetic parameters were initially estimated by the graphic analysis of Eadie–Hofstee plots, and the values obtained were used as the first estimate for the non-linear least-squares regression analysis, MULTI [12], where unweighted raw data were fitted to the model equation as described above.

Results

Representative chromatograms are shown in Fig. 2. The retention times for 5-hydroxyomeprazole, omeprazole sulphone and the internal standard (phenacetin) were 5.4, 7.7 and 10.4 min, respectively (Fig. 2(B)). No interference peak was found in the chromatogram of an incubation mixture incubated for 20 min without omeprazole (Fig. 2(A)). In addition, the peaks of 5-hydroxyomeprazole, omeprazole sulphone and phenacetin gave baseline separation from the peaks derived from microsomes or other omeprazole metabolites which appeared after the incubation of microsomes with omeprazole (Fig. 2(C)).

Recoveries after the deproteinization of an incubation mixture which contained 0.2 mg ml^{-1} of microsomal protein were 102.1 and 102.2% for 1 μM of 5-hydroxyomeprazole and omeprazole sulphone, and 102.1% for 40 μM of phenacetin, respectively. The calibration curves generated by processing the authentic standard substances through the entire procedure were linear over the concentration range (0.05–1 μM) examined, and the correlation coefficients were more than 0.999 for all

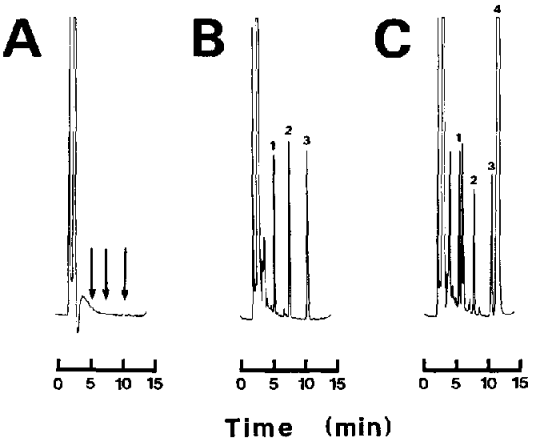

Figure 2
Representative chromatograms obtained from; the incubation mixture incubated without omeprazole for 20 min (A); the incubation mixture spiked with 0.3 nmol each of 5-hydroxyomeprazole, omeprazole sulphone and phenacetin (internal standard) (B); and the incubation mixture containing 0.2 mg ml^{-1} of microsomes and 100 μM of omeprazole which was incubated for 20 min (C). Arabic numerals in (B) and (C) indicate the peaks of: 1, 5-hydroxyomeprazole; 2, omeprazole sulphone; 3, phenacetin; and 4, omeprazole. The arrows in (A) indicate the anticipated elution times of the analytes and the internal standard.

the analytes. The intra-assay relative standard deviations for determining the 5-hydroxylation and sulphoxidation activities of omeprazole measured at the substrate concentration of 100 μM were ±8.2 and ±12.8%, respectively. The detection limit, defined as a signal-to-noise ratio of 5, was 25 nM in the incubation mixture for both 5-hydroxyomeprazole and omeprazole sulphone. The formation rates of 5-hydroxyomeprazole and omeprazole sulphone were linear at 37°C for the incubation time of up to 30 min when 100 μM of omeprazole and 0.1 mg ml^{-1} of microsomal protein were used (Fig. 3(A) and (C)). Neither 5-hydroxyomeprazole nor omeprazole sulphone was formed in the absence of the NADPH-generating system, microsomes or omeprazole. A linear relationship was also observed between the production rates of the two metabolites of omeprazole over 20 min with protein concentrations of up to 0.2 mg ml^{-1} (Fig. 3(B) and (D)).

Eadie–Hofstee plots for the formation of 5-hydroxyomeprazole and omeprazole sulphone are shown in Fig. 4(A) and (B), respectively. Both the 5-hydroxylation and sulphoxidation of omeprazole gave a biphasic relationship in the six different human liver microsomes examined.

Because the data suggest that at least two enzymes are involved in the 5-hydroxylation and sulphoxidation of omeprazole in human liver microsomes (Fig. 4), the kinetic parameters for the formation of 5-hydroxyomeprazole and omeprazole sulphone were estimated by assuming that both of the metabolic reactions are catalysed by the two enzymes. The mean (±SD) high- and low-affinity component enzyme kinetic parameters for 5-hydroxyomeprazole and omeprazole sulphone obtained from the six different human liver microsomes are listed in Table 1.

Discussion

The assay procedure described herein re-

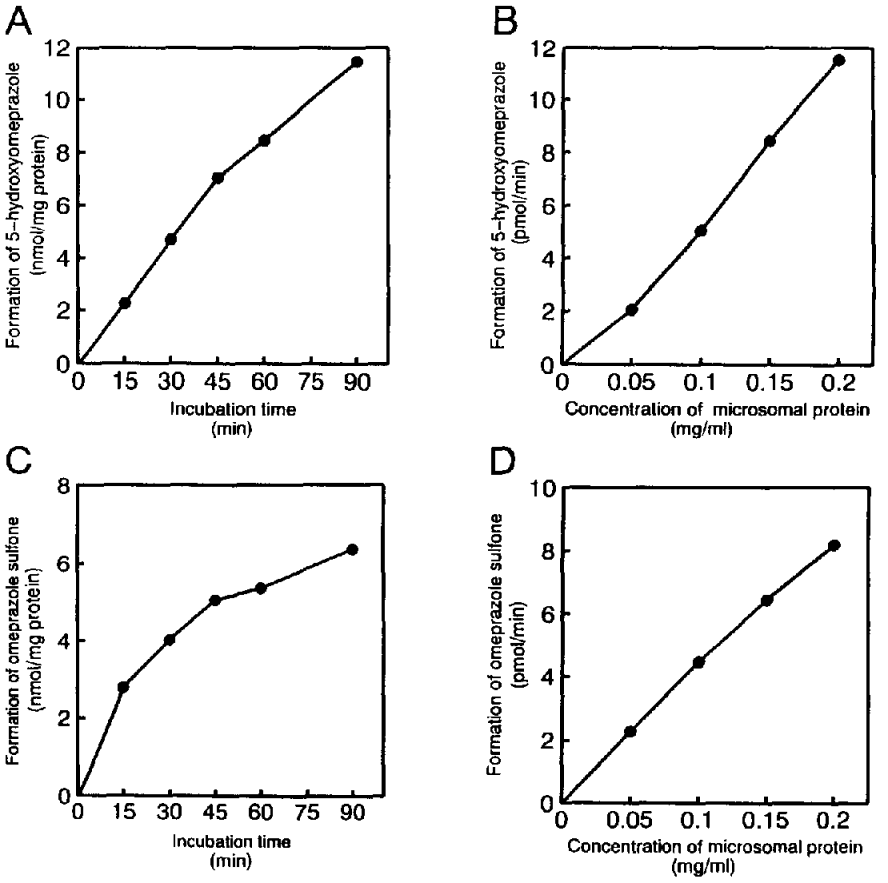

Figure 3
Influence of incubation time and microsomal protein concentration on the production of 5-hydroxyomeprazole, (A) and (B), and omeprazole sulphone, (C) and (D), from omeprazole (100 μM) by human liver microsomes.

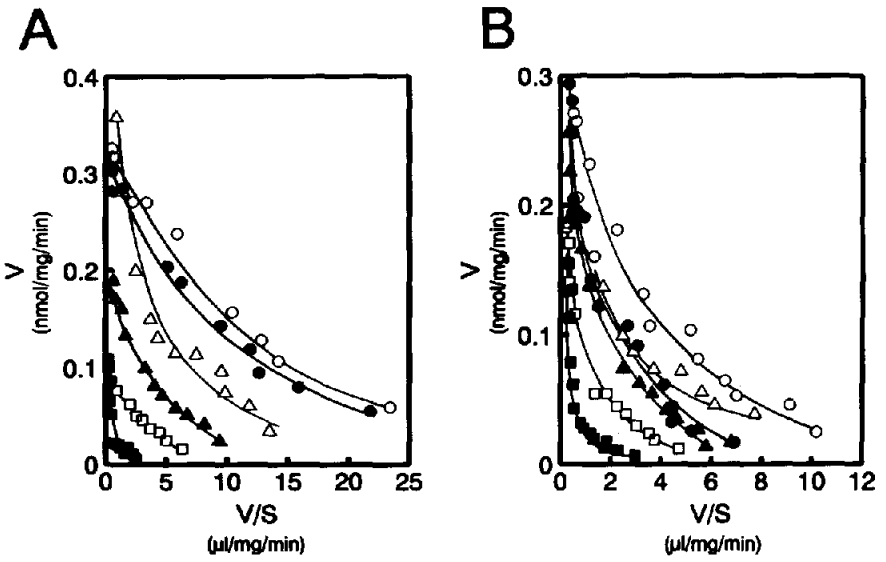

Figure 4
Eadie–Hofstee plots for 5-hydroxylation (A) and sulphoxidation (B) of omeprazole in six different human liver microsomal samples. The same individual data were plotted using the same symbols. Solid lines indicate plots computer-generated by fitting the individual data to a two-enzyme kinetic model as described in the Experimental section.

quires only one-step deproteinization with acetonitrile, which did not yield any interference peaks that may affect the quantitation of 5-hydroxyomeprazole and omeprazole sulphone (Fig. 2). In addition, the two metabolites of omeprazole and the internal standard were recovered nearly completely. Moreover, the intra-assay relative standard deviations were less than ±13% and the lower detection limits of the assay were as low as 25 nM for both 5-hydroxyomeprazole and omeprazole sulphone. Thus, the present assay was applicable for the kinetic study of the microsomal metabolism of omeprazole in the human liver. In fact, the preliminary kinetic study showed that the method was successfully applicable to estimate the Michaelis–Menten parameters of 5-hydroxylation and sulphoxidation of omeprazole in human liver microsomes. Therefore, the current assay is considered to be reproducible and sensitive enough to apply to the study of the metabolism of omeprazole mediated via 5-hydroxylation and sulphoxidation in human liver microsomes.

The present enzyme kinetic data on both the formations of 5-hydroxyomeprazole and omeprazole sulphone from omeprazole exhibited a biphasic profile (Fig. 4). The findings suggest that the formation of both the metabolites are catalysed by at least two enzymes. Using a two-enzyme kinetic approach, the activities were well described by the high- and low-affinity components. When compared between the high- and low-affinity components, the former component was considered to dominate both the 5-hydroxylation and sulphoxidation, because the mean V_{max}/K_m values of the high-affinity component (i.e. V_{max1}/K_{m1}) were about 10- to 15-times greater than those of the low-affinity component (i.e. V_{max2}/K_{m2}). Considering that a peak plasma concentration of omeprazole attained after the repeated oral doses of omeprazole (20 mg) is around 0.5 μM, which is far less than the K_{m1} of 5-hydroxylation and sulphoxidation, in EMs of omeprazole [13], the high-affinity component appears to play a predominant role in both the 5-hydroxylation and sulphoxidation even in an *in vivo* human status.

When the mean V_{max}/K_m values for the high-affinity component were compared between the sulphoxidation and 5-hydroxylation of omeprazole, the mean value for the sulphoxidation was less than the half of the 5-hydroxylation, suggesting that the sulphoxidation is a less efficient metabolic process of omeprazole compared with the 5-hydroxylation. Although to what extent omeprazole would be metabolized *in vivo* to omeprazole sulphone remains unknown, the present observation is consistent with an *in vivo* finding that 5-hydroxylation is a dominant metabolic pathway of omeprazole in humans, accounting for 51% of the metabolites of omeprazole excreted in the post-dose 0–2 h urine, equivalent to 24% of the dose [14].

Table 1
Michaelis–Menten kinetic parameters for the 5-hydroxylation and sulphoxidation of omeprazole obtained from six different human liver microsomes

Metabolite's formation	K_{m1} (μM)	V_{max1} (pmol mg^{-1} min^{-1})	V_{max1}/K_{m1} (μl mg^{-1} min^{-1})	K_{m2} (μM)	V_{max2} (pmol mg^{-1} min^{-1})	V_{max2}/K_{m2} (μl mg^{-1} min^{-1})
5-Hydroxylation	6.3 ± 1.7	109.8 ± 75.4	16.40 ± 10.31	183.2 ± 180.4	163.3 ± 94.1	1.18 ± 0.56
Sulphoxidation	10.4 ± 6.3	77.5 ± 46.1	7.86 ± 3.42	671.2 ± 639.4	318.3 ± 163.2	0.74 ± 0.48

Values are mean ±SD.

In conclusion, the assay method described is reproducible, sensitive and accurate enough to apply for the *in vitro* study of the metabolism of omeprazole in human liver microsomes. Such a study on the enzyme kinetic analysis of the metabolism of a drug like omeprazole, which is co-regulated with the genetically determined oxidation polymorphism of *S*-mephenytoin 4′-hydroxylase [3, 4, 9, 10], in an *in vitro* experiment by applying an appropriate assay method as described is considered to be a useful approach to understand further and scrutinize an *in vivo* drug disposition in humans.

Acknowledgements — We are grateful to Ms Yukiko Sawada for her excellent secretarial assistance. The study was supported by a grant-in-aid from the Japan Health Science Foundation (1-7-1-C) and from Drug Innovation Science Project (1-2-10), Tokyo, Japan.

References

[1] C.W. Howden, *Clin. Pharmacokinet.* **20**, 38–49 (1991).
[2] P.N. Maton, *N. Engl. J. Med.* **324**, 965–975 (1991).
[3] D.-R. Sohn, K. Kobayashi, K. Chiba, K.-H. Lee, S.-G. Shin and T. Ishizaki, *J. Pharmacol. Exp. Ther.* **262**, 1195–1202 (1992).
[4] T. Andersson, C.G. Regårdh, Y.-C. Lou, Y. Zhang, M.-L. Dahl and L. Bertilsson, *Pharmacogenetics* **2**, 25–31 (1991).
[5] P.-O. Langerström and B.-A. Persson, *J. Chromatogr.* **309**, 347–356 (1984).
[6] I. Grundevik, G. Jerndal, K. Balmér and B.-A. Persson, *J. Pharm. Biomed. Anal.* **4**, 389–398 (1986).
[7] M.A. Amantea and P.K. Narang, *J. Chromatogr.* *426*, 216–222 (1988).
[8] K. Kobayashi, K. Chiba, D.-R. Sohn, Y. Kato and T. Ishizaki, *J. Chromatogr.* **578**, 299–305 (1992).
[9] K. Chiba, K. Kobayashi, M. Tani, K. Manabe and T. Ishizaki, *Drug Metab. Dispos.* **27**, 747–749 (1993).
[10] K. Chiba, K. Manabe, K. Kobayashi, Y. Takayama, M. Tani and T. Ishizaki, *Eur. J. Clin. Pharmacol.* **44**, 559–562 (1993).
[11] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.* **193**, 265–275 (1951).
[12] K. Yamaoka, Y. Tanigawara, T. Nakazawa and T. Uno, *J. Pharmacobio.-Dyn.* **4**, 879–885 (1981).
[13] T. Anderson, C. Cederberg, G. Edvardsson, A. Heggelund and P. Landborg, *Clin. Pharmacol. Ther.* **47**, 79–85 (1990).
[14] L. Renberg, R. Simmonsson and K.-J. Hoffmann, *Drug Metab. Dispos.* **17**, 69–76 (1989).

[*Received for review 20 July 1993; revised manuscript received 19 November 1993*]