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Kinetic analysis of propranolol-induced impairment of its own metabolism in rats

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Abstract—The effect of repetitive oral administration of propranolol (100 mg kg^{-1} day^{-1}, 5 days) on the kinetics of liver microsomal propranolol metabolism was investigated in the rat. V_{max} values of the high-affinity phase for biphasic kinetics of propranolol 4- and 5-hydroxylase activities were decreased by propranolol pretreatment, while those of the low-affinity phase were unchanged. The V_{max} value of monophasic 7-hydroxylase activity was also decreased. On the other hand, the V_{max} value of *N*-desisopropylase activity in the propranolol-treated rats was increased more than 2-fold compared with non-treated (control) rats, resulting in a change from monophasic in control rats to biphasic kinetics in propranolol-treated rats. These findings indicate that repetitive administration of propranolol selectively impairs a CYP2D isozyme that is involved in the high-affinity phases for propranolol ring-hydroxylations.

It has been reported that the systemic availability of propranolol was increased after chronic administration in man (Evans & Shand 1973; Walle et al 1980; Straka et al 1987). Propranolol was shown to be a potent inhibitor of cytochrome P450 (P450) -dependent drug metabolism (Conrad et al 1980; Bax et al 1983; Suzuki et al 1993). Furthermore, repetitive oral administration of propranolol to rats caused a marked decrease in hepatic microsomal propranolol 4-, 5- and 7-hydroxylase activities and an increase in *N*-desisopropylase activity (Shneck & Pritchard 1981; Masubuchi et al 1991). It was proposed as an inhibitory mechanism that a reactive metabolic intermediate of propranolol covalently bound to P450 species catalysing propranolol ring-hydroxylations (Shneck & Pritchard 1981; Shaw et al 1987; Masubuchi et al 1992a, b).

Our recent study on enzyme kinetics showed that multiple forms of P450 were involved in propranolol metabolism in rat liver microsomes (Ishida et al 1992; Masubuchi et al 1993). We found that the high-affinity phases for propranolol 4- and 5-hydroxylase activities were deficient in the Dark Agouti rat, known as a poor-metabolizer animal model for debrisoquine 4-hydroxylation (Masubuchi et al 1993), and that sudan III, an inducer of a CYP1A isozymes, selectively increased the V_{max} of propranolol *N*-desisopropylase activity and of 4- and 5-hydroxylase activities in the low-affinity phases (Ishida et al 1992). In the present work, we have compared kinetic parameters of microsomal propranolol metabolism in non-treated (control) and treated rats to investigate the effects of propranolol administration on multiple species of P450 catalysing propranolol oxidation.

Materials and methods

Chemicals. Propranolol hydrochloride was purchased from the Sigma Chemical Co. (St Louis, MO, USA). 4-Hydroxy-propranolol hydrochloride and *N*-desisopropylpropranolol hydrochloride were obtained from Sumitomo Chemical Ind. (Osaka, Japan) and from ICI Pharmaceuticals Co. (Macclesfield, UK), respectively. 5- and 7-Hydroxypropranolol were synthesized as hydrochlorides according to the method of Oatis et al (1981). 4-Hydroxybunitrolol hydrochloride was obtained from Nippon C. H. Boehringer Sohn Co. Ltd (Osaka, Japan). Glucose-6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH) and NADPH were purchased from the Oriental Yeast Co. Ltd (Tokyo, Japan). All other chemicals and solvents were of analytical grade.

Pretreatment of animals and preparation of hepatic microsomes. Male Wistar rats (2 months old) were pretreated with propranolol (100 mg kg^{-1} day^{-1}, 5 days, p.o.). Treated rats and non-treated controls were killed by decapitation 40 h after the final administration. Hepatic microsomal fractions were prepared according to the method of Omura & Sato (1964). Protein concentrations were determined by the method of Lowry et al (1951).

Assay methods of enzymatic activities. Propranolol 4-, 5-, 7-hydroxylase and *N*-desisopropylase activities were assayed by high-performance liquid chromatography (HPLC) (Masubuchi et al 1991). The rates of oxidation of propranolol were measured in a 1-mL incubation mixture containing 0·2–1000 μM propranolol, 10 mM G-6-P, 2 units G-6-PDH, 0·5 mM NADPH, 8 mM $MgCl_2$ and 0·5 mg microsomal protein in 0·15 M potassium phosphate buffer (pH 7·4). After 5-min preincubation under air at 37°C, the reaction was started by adding NADPH and continued for 30 s. The reaction was stopped by adding 1 mL 1 M NaOH containing 25 mg sodium bisulphite. 4-Hydroxybunitrolol was then added as internal standard. After extraction of metabolites with 5 mL ethyl acetate, the organic phase was evaporated to dryness and the residue was dissolved in HPLC mobile phase (CH_3CN:CH_3OH:H_2O:CH_3COOH = 22:22:56:1). The samples were applied to a reversed-phase column (Inertsil ODS, GL Sciences, Tokyo, Japan). The fluorescent intensity of propranolol and its metabolites was continuously monitored at excitation and emission wavelengths of 310 and 380 nm, respectively.

Analysis of kinetic parameters. Enzyme kinetic parameters (K_m and V_{max}) were analysed according to a nonlinear least-squares regression analysis based on a simplex method (Yamaoka et al 1981). Best fittings of the data were performed by weighting them with the reciprocal of their square. Statistical significance was calculated by the Student's *t*-test.

Results and discussion

Table 1 summarizes the results of kinetic analysis for propranolol 4-, 5- and 7-hydroxylase and *N*-desisopropylase activities of liver microsomes from control and treated rats. The formation of 4- and 5-hydroxypropranolol showed biphasic Michaelis–Menten kinetics over the substrate concentration range of 0·2–1000 μM in both control and treated rats. The formation of *N*-desisopropyl-propranolol in treated rats also showed biphasic kinetics. On the other hand, the formation of 7-hydroxypropranolol in both control and treated rats, and that of *N*-desisopropyl-propranolol in control rats exhibited monophasic kinetics.

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Table 1. Kinetic parameters of propranolol metabolism in liver microsomes from propranolol-treated rats.

Pathway	K_{m1} (μM)	V_{max1} (nmol min^{-1} mg^{-1})	K_{m2} (μM)	V_{max2} (nmol min^{-1} mg^{-1})
4-OH	0·254 ± 0·069	0·098 ± 0·027**	238 ± 122	0·384 ± 0·089
	(0·225 ± 0·029)	(0·230 ± 0·023)	(208 ± 25)	(0·470 ± 0·070)
5-OH	0·153 ± 0·063	0·023 ± 0·003*	188 ± 17	0·089 ± 0·019
	(0·113 ± 0·032)	(0·048 ± 0·005)	(123 ± 24)	(0·128 ± 0·028)
7-OH	0·188 ± 0·071	0·101 ± 0·011**	—	—
	(0·212 ± 0·016)	(0·255 ± 0·035)	—	—
N-DP	0·214 ± 0·002	0·023 ± 0·003	217 ± 82	2·21 ± 0·25**
			(172 ± 21)	(1·04 ± 0·22)

Abbreviations are: 4-OH, 4-hydroxylation; 5-OH, 5-hydroxylation; 7-OH, 7-hydroxylation; *N*-DP, *N*-desisopropylation. Values in parentheses are the activities in non-treated (control) rats, and are quoted from Masubuchi et al (1993). Data are means ± s.e. of 3–4 determinations. *$P < 0.05$, **$P < 0.01$ compared with control.

The V_{max} values of the high-affinity phases (V_{max1}) for 4- and 5-hydroxylase activities and the V_{max} value of monophasic 7-hydroxylase activity were decreased by 52–60% by pretreatment. The V_{max} values of the low-affinity phases (V_{max2}) for 4- or 5-hydroxylase activity was not significantly changed. Neither K_m value for these reactions was affected by the pretreatment. Therefore, the inhibitory effect is different between high-affinity and low-affinity enzymes responsible for propranolol hydroxylations. The K_m value of the low-affinity phase for *N*-desisopropylase activity in treated rats was close to that of monophasic *N*-desisopropylase activity in control rats, but the V_{max} value of this phase in treated rats was more than twice that in control rats.

These results indicated that propranolol pretreatment selectively impaired a P450 isozyme belonging to the CYP2D subfamily, because we reported that isozyme was involved in the high-affinity phases for propranolol ring-hydroxylase activities (Masubuchi et al 1993). The present observations are consistent with our previous results (Masubuchi et al 1991) showing selective impairment of CYP2D-dependent reactions such as debrisoquine 4-hydroxylation and imipramine 2-hydroxylation by propranolol pretreatment of rats. We proposed that a reactive metabolite formed by CYP2D isozymes binds covalently to microsomal protein, and causes a selective impairment of a CYP2D isozyme (Masubuchi et al 1992a, b).

The present kinetic study indicated that propranolol induced at least two kinetically different enzymes catalysing propranolol *N*-desisopropylation. Because the kinetics of *N*-desisopropylase activity in propranolol-treated rats were similar to those in sudan III (an inducer of CYP1A1/2)-pretreated rats (Ishida et al 1992), it is suggested that propranolol induces CYP1A1 or CYP1A2. However, no increase by propranolol pretreatment was observed in the content of the P450 isozymes that cross-react with the antibody against CYP1A1/2 in Western blot analysis (unpublished observation). Further studies are required to identify the P450 isozymes induced by propranolol pretreatment.

The metabolic activity at low substrate concentrations calculated from the sum of V_{max}/K_m for the primary metabolic reactions described above was markedly decreased by propranolol pretreatment, showing 2·66 and 1·18 mL min^{-1} (mg protein)$^{-1}$ for control and pretreated rats, respectively. Consequently, we suggest that the contribution of the induction of *N*-desisopropylase activity to the total propranolol metabolism was negligible.

A previous in-vivo study in man (Walle et al 1980) has demonstrated that the plasma 4-hydroxypropranolol/propranolol ratio after administration of therapeutic doses of propranolol is lowered in subjects receiving long-term treatment. The present result showing a decrease in high-affinity propranolol 4-hydroxylase activity is compatible with the study in man. Thus, the impairment of high-affinity enzyme activities for aromatic ring-hydroxylation may account for reduction in in-vivo clearance of propranolol following chronic administration (Evans & Shand 1973; Walle et al 1980; Straka et al 1987).

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Study of the mutual effects of sulphadiazine and ciprofloxacin on their uptakes by *Pseudomonas aeruginosa*

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Abstract—A high-performance liquid chromatography (HPLC) assay was developed for ciprofloxacin and sulphadiazine in Isosensitest broth. Combining the HPLC assay with cell dry-weight determinations indicated that both compounds were able to enhance the uptake of the other by log phase *Pseudomonas aeruginosa* cultured in the presence of the compounds. It is hypothesized that the increased bacterial uptakes are the reason for the enhanced antibacterial activity previously reported for combinations of ciprofloxacin and sulphadiazine.

It has been reported that both *Pseudomonas aeruginosa* and *Staphylococcus aureus* have developed resistance to ciprofloxacin in the clinical situation (Pedersen 1989; Ball 1990; Neu 1991). Combinations of quinolones together with other antimicrobial agents were used (Eliopoulos & Eliopoulos 1989) in order to improve activity against bacteria inadequately inhibited by the fluoroquinolones alone. The results obtained were somewhat variable with addition of activity being the most common effect, although synergism has also been documented. Synergism is the desired effect from antibacterial combinations and since this was demonstrated in-vitro against both *P. aeruginosa* and *S. aureus* with combinations of sulphadiazine and ciprofloxacin (Richards & Xing 1993), the mode of this enhanced activity is worthy of further investigation. Therefore, the object of the present study was to investigate this synergism by developing a single HPLC assay for both ciprofloxacin and sulphadiazine in Isosensitest broth. This would allow determinations to be made of bacterial uptakes from broth containing either antibacterial agent alone or in combination and thus provide evidence of whether the combination produced increased bacterial uptakes.

Materials and methods

Materials. The high-performance liquid chromatography (HPLC) system consisted of an M 6000A pump system (Waters Associates Inc.). Injection was by means of a Rheodyne 7125 valve fitted with a 20 μL fixed volume loop. The 80 mm long, 4·6 mm i.d. column was slurry packed with 5 μm ODS-Hypersil. Detection was at 280 nm using a Waters 440 UV-visible detector connected to a potentiometric recorder (BBC SE120).

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Pseudomonas aeruginosa NCTC 6750 was the test organism and was from the National Collection of Type Cultures, Colindale, London, UK. Isosensitest broth was obtained from Oxoid Ltd, Basingstoke, UK. Ciprofloxacin and sulphadiazine were obtained from Sigma, Poole, UK. Sodium sulphadiazine was prepared in the laboratory according to Richards et al (1991).

Buffer salt, disodium hydrogen orthophosphate was from Fisons, Loughborough, UK. Tetrabutylammonium bromide (TBA) was from Aldrich Chemical Co. Ltd, Dorset, UK. Acetonitrile was from Rathburn Chemicals Ltd, UK. Water for the HPLC was glass-distilled and further purified by a Millipore Milli-Q System.

Preparation of test sample. Samples of ciprofloxacin (10 μg mL^{-1}) and sulphadiazine (150 μg mL^{-1}) singly and in combination were prepared initially in distilled water and subsequently in Isosensitest broth. A 20 μL aliquot of this preparation was then injected.

Chromatography. The mobile phase system used consisted of 20 mM TBA, 10 mM disodium hydrogen orthophosphate in a 5% v/v acetonitrile/water mixture adjusted to pH 2·5. Detection was at 280 nm and the flow rate was 1·5 mL min^{-1}.

Quantification. The precision of the method was assessed by repeated analysis of Isosensitest samples containing the drugs.

Calibration was determined by adding a range of concentrations of each drug into Isosensitest broth. Peak heights were measured. The correlation coefficient (r^2) for the calibration lines showed good linearity. It was also demonstrated that the assay of ciprofloxacin was not affected by the presence of sulphadiazine and vice versa.

The detection limit of the assay for each of the drugs was determined by successive injections of smaller concentrations of the drug until a signal-to-noise ratio of three was obtained.

Chemical stability. The stability of ciprofloxacin and sulphadiazine in Isosensitest broth was determined by adding 5 μg mL^{-1} ciprofloxacin and 150 μg mL^{-1} sulphadiazine in combination to flasks containing Isosensitest broth. These flasks were incubated at 37°C in a water bath shaking at 100 oscillations min^{-1}. Five flasks were incubated under the conditions described.