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Bunitrolol metabolism and its inhibition by cimetidine

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Abstract—A simple fluorometric assay method as well as a sensitive HPLC method for determination of bunitrolol, a β-adrenoreceptor blocking drug, and its 4-hydroxylated metabolite is described. More than 90% of bunitrolol metabolized was accounted for by the formation of 4-hydroxybunitrolol in rat hepatic microsomes. Bunitrolol 4-hydroxylation required NADPH, and was inhibited by CO, proadifen and metyrapone, indicating that this reaction is mediated by cytochrome P450. This reaction was also inhibited by cimetidine competitively, and the inhibition constant, K_i, was 40 ± 11·5 μM (mean ± s.e. n = 3).

Bunitrolol is a potent β-adrenoceptor blocking drug used in hypertension (Traunecker et al 1980). Its metabolic fate has been documented (Suzuki & Rikihisa 1979), but how it is metabolized and the effect of other drugs on the metabolism have not been investigated.

More than 90% of the bunitrolol metabolites in rat urine is reported to consist of 4-hydroxybunitrolol and its glucuronide (Suzuki & Rikihisa 1979). Our in-vitro metabolism study using rat liver microsomes also indicated that more than 90% of bunitrolol which disappeared from the incubation mixture could be accounted for by the formation of 4-hydroxybunitrolol. The formation of other metabolites, if any, must be minor. In the present study, we investigated the nature of bunitrolol 4-hydroxylase using various drug metabolism inhibitors including cimetidine (Gerber et al 1985; Sorkin & Darvey 1983).

Materials and methods

Chemicals. Bunitrolol hydrochloride and 4-hydroxybunitrolol were supplied by Boehringer Sohn, FRG. Cimetidine and proadifen hydrochloride (SKF 525-A) were gifts from Smith Kline and French Co., USA. All other chemicals were of analytical grade.

Preparation of microsomes. Wistar strain immature male rats, 100 g, were decapitated and liver microsomes were prepared according to the method of Omura & Sato (1964). Protein concentrations of microsomal preparations were determined by the method of Lowry et al (1951).

Fluorometric assay for bunitrolol metabolism. Bunitrolol 4-hydroxylase activity was assayed as follows. The microsomal reaction mixture contained in final concentrations, about 0·5 mg mL^{-1} of microsomal protein, 2 units mL^{-1} of glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 8 mM $MgCl_2$, and 50 μM bunitrolol in 0·1 M phosphate buffer, pH 7·4 in a final volume of 1·0 mL. The mixture was preincubated for 5 min at 37 °C. The reaction was then started by the addition of 10 μL of 50 mM NADP and incubated for 15 min. The reaction was stopped by the addition of 1 mL of 1·0 M carbonate buffer, pH 9·95. Ethyl acetate (6 mL) was then added and mixed well using a vortex mixer for 1 min. The mixture was centrifuged (1200*g*) for 10 min. Four mL of the ethyl acetate layer was removed and evaporated to dryness under vacuum. The residue was dissolved in 3 mL of methanol. Fluorescence intensity of 4-hydroxybunitrolol in the extract was measured at the excitation wavelength of 325 nm and emission wavelength of 365 nm.

The disappearance of bunitrolol was assayed by stopping the reaction by the addition of 1 mL of 1 M NaOH and extracting the bunitrolol with 15 mL of 1·5% isoamyl alcohol in heptane. After 10 min centrifugation (1200*g*), the heptane layer (10 mL) was transferred to a centrifuge tube containing 10 mL of 0·1 M HCl. The contents were mixed well for 5 min. The fluorescence intensity of the HCl layer was measured at the excitation wavelength of 295 nm and the emission wavelength of 330 nm. The reaction was linear for more than 15 min under these assay conditions.

Kinetic study. The microsomal reaction mixture similar to that described above containing 5–100 μM bunitrolol and 0–500 μM cimetidine was incubated for 1 min at 37 °C after 5 min preincubation followed by the addition of NADP to start the reaction. The 4-hydroxybunitrolol generated was extracted as above and assayed by an HPLC method (see below). The initial rate of 4-hydroxybunitrolol formation was determined as the amounts of 4-hydroxybunitrolol generated in 1 min by 1 mg of microsomal protein. 4-Hydroxybunitrolol formation was linear at least up to 3 min over the bunitrolol concentration range used in this kinetic study.

HPLC procedure for quantitation of 4-hydroxybunitrolol. After the reaction was terminated by the addition of 1 M carbonate buffer as described above, 0·5 mL of 1 μM 2-naphthol was added as an internal standard. Bunitrolol and its metabolite were extracted by ethyl acetate as described above. After evaporation of ethyl acetate under vacuum, the residue was dissolved in 0·1 mL methanol. About 10 μL of the methanol solution was applied to the HPLC column (Unisil Pack F3 50A, Gasukuro Kogyo, Japan). The mobile phase consisted of acetonitrile–methanol–water-acetic acid (37:10:52:1 v/v). 4-Hydroxybunitrolol and the internal standard were detected fluorometrically at an excitation wavelength of 325 nm and emission wavelength of 365 nm using a Hitachi 650-10S spectrofluorometer equipped with an HPLC flow cell.

Results and discussion

The rate of formation of 4-hydroxybunitrolol from bunitrolol and the rate of disappearance of bunitrolol from the reaction mixture (Fig. 1) showed similar values indicating that almost all the bunitrolol molecules metabolized are converted to 4-hydroxybunitrolol in the microsomal reaction mixture (Fig. 1). After 10 min incubation, the concentration of 4-hydroxybunitrolol formed was 93·7% of that of bunitrolol metabolized. Since the bunitrolol 4-hydroxylase reaction is likely to be mediated by cytochrome P450, the microsomal assay mixture was treated with various inhibitors of P450. As indicated in

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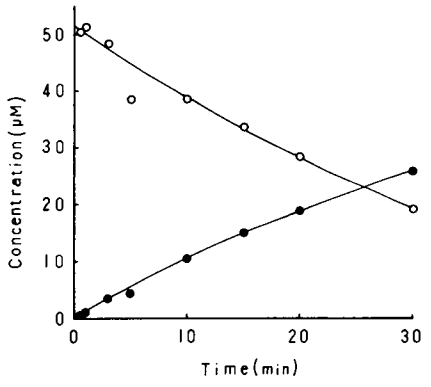

FIG. 1. The relationship between the formation of 4-hydroxybunitrolol and the disappearance of bunitrolol in rat liver microsomes. Bunitrolol (initial concentration, 50 μM) was incubated with microsomal reaction mixture for 0·5, 1, 3, 5, 10, 15, 20 and 30 min. The disappearance of bunitrolol (—○—) and the formation of 4-hydroxybunitrolol (—●—) were assayed by the fluorometric method.

Table 1. Effects of various inhibitors of drug metabolism on bunitrolol 4-hydroxylase activity.

Inhibitors	Bunitrolol 4-hydroxylase activity[a]	% Inhibition
Control[b]	2·15 ± 0·06	0
−NADPH	0·01 ± 0·00	100
+CO 80%, O_2 20%	0·94 ± 0·04	56
+Proadifen (250 μM)	0·15 ± 0·22	93
+Metyrapone (500 μM)	0·30 ± 0·06	86
+Cimetidine (500 μM)	0·91 ± 0·08	58
+Theophylline (3·0 mM)	2·00 ± 0·03	7

Bunitrolol 4-hydroxylase activities were assayed using an initial substrate concentration of 50 μM in the microsomal reaction mixture. The concentrations of the inhibitors are indicated in parentheses.

[a] Bunitrolol 4-hydroxylase activities are expressed as nmol 4-hydroxybunitrolol formed (mg microsomal protein)$^{-1}$ min. Values are mean ± s.d. of four determinations.

[b] Control assay mixture contained as a complete system, microsomes, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $MgCl_2$, bunitrolol, and NADP as described in the text.

Table 1, bunitrolol 4-hydroxylase required NADPH, and was inhibited by CO, proadifen, metyrapone and cimetidine. It is clear from these results that, as expected, bunitrolol 4-hydroxylase is mediated by cytochrome P450.

Fig. 2 is a Lineweaver–Burk plot for bunitrolol 4-hydroxylase activity in rat liver microsomes in the presence of various concentrations of cimetidine. It is apparent from this Figure that the inhibition is competitive. The inhibition constant K_i (mean ± s.e. n = 3) was 40 ± 11·5 μM. This is in contrast to the reported inhibition kinetics for aminopyrine *N*- demethylase (Pelkonen & Puurunen 1980) in the presence of cimetidine, which was shown to be non-competitive. Apparently, the inhibition mechanisms of cimetidine differ depending on the substrate, Drew & Grygiel (1981) demonstrated that cimetidine inhibited predominantly cytochrome P448-mediated monooxygenase reactions. This might suggest that bunitrolol 4-hydroxylation is mediated by cytochrome P448 in untreated animals. However, a high concentration of theophylline, a known substrate for cytochrome P448 (Nebert 1982) did not inhibit this reaction (Table 1). Furthermore, our preliminary study using liver microsomes from rats treated with 3-methylcholanthrene showed no increase in bunitrolol 4-hydroxylase activity under the assay conditions used in this study*, but rather, showed a slight decrease, indicating that the P450 species engaging in bunitrolol 4-hydroxylation in untreated rats is not cytochrome P448. Apparently, cimetidine can inhibit reactions mediated by species of P450 other than P448. This is in agreement with Speeg et al (1982), and Pelkonen & Puurunen (1980), who showed that cimetidine inhibited aminopyrine *N*- demethylase activity, which is mediated by constitutive P450 in non-induced microsomes and a phenobarbitone inducible type of P450.

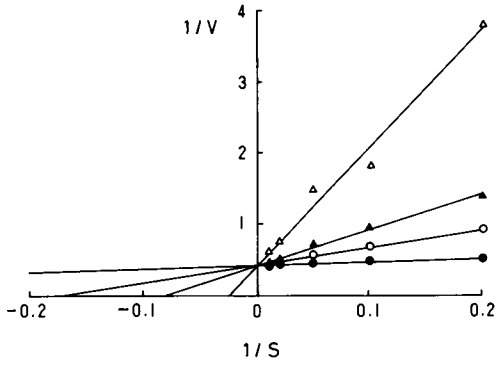

FIG. 2. Lineweaver–Burk plot for hepatic microsomal bunitrolol 4-hydroxylase activity in the presence of cimetidine. Various concentrations of cimetidine were added to the microsomal bunitrolol 4-hydroxylase assay mixture. 4-Hydroxybunitrolol formed was quantified by the HPLC method described in the text. Key: ● 0, ○ 100, ▲ 200, △ 500 μM cimetidine. V = nmol min^{-1} mg protein^{-1}, S = μM.

The fact that cimetidine inhibited bunitrolol metabolism is of clinical importance, because both drugs are currently in use and their simultaneous administration may cause increased bioavailability of bunitrolol, resulting in unexpected adverse reactions.

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* With a higher substrate concentration (500 μM), bunitrolol 4-hydroxylase activity was higher in liver microsomes from rats treated with 3-methyl-cholanthrene.