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Note

Stereoselective determination of plasma pindolol in endotoxin-pretreated rats by high-performance liquid chromatography

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β-Adrenergic blocking agents, which are widely used in the treatment of hypertension, cardiac arrhythmias, etc , have two optical isomers and are marketed as racemic mixtures It is well known that the pharmacological activities of $S(-)$-enantiomers are considerably higher than those of $R(+)$-enantiomers [1] In the case of propranolol, the enantiospecific differences of the elimination [2, 3], the bioavailability [4] and the hepatic metabolism [5] have been reported Accordingly, to elucidate the behaviour of these agents in the body, the plasma concentration after the administration of the racemate should be measured stereoselectively There are few reports about the investigations of other β-blockers in such a way described above

Lavene et al [6] showed that the bioavailability of pindolol, another widely used β-blocker, was slightly diminished in hypertensive patients with chronic renal failure Evard et al. [7] also reported that the apparent distribution volume of racemic pindolol became smaller in malabsorption syndromes. In such studies, more valuable information would be given by the stereoselective determination of pindolol in plasma

We have examined the dispositions of both enantiomers after the administration of racemic pindolol in rats by using a stereoselective high-performance

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liquid chromatographic (HPLC) method reported previously [8, 9] We also studied the effect of bacterial endotoxin, which is known to change the behaviour of some drugs in blood [10, 11], on the disposition of both enantiomers By this stereoselective analysis, we elucidated the enantiospecific disposition of pindolol and also established the increase in the plasma protein binding of pindolol enantiomers induced by endotoxin

EXPERIMENTAL

Materials

Pindolol racemate was a kind gift from Nippon Sandoz 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocynate (GITC) was prepared from α-acetobromoglucose as described by Nimura et al [12] Bacterial endotoxin was extracted from *Escherichia coli* UKT-B strain and further purified by the ultracentrifugation according to Westphal and Jann [13] The preparation was free from protein and other macromolecular contaminants All other chemicals used were of analytical-reagent grade or HPLC grade (Wako, Osaka, Japan)

Animal treatment

Male Wistar rats (190–220 g) were used in all the present experiments Rats were divided into two groups control and endotoxin-pretreated The latter received an i v injection of endotoxin (10 μg/kg) dissolved in sterile saline, and the former an equal volume of sterile saline, through a tail vein They were fasted for 24 h with free access to water and anesthetized by pentobarbital (35 mg/kg i p) before the following experiments

In the first experiment, to measure the concentration of pindolol in plasma, racemic pindolol (5 mg/kg) was injected i v and blood samples were withdrawn into a heparinized syringe from an abdominal descending aorta at 5, 15, 30, 60 90, and 120 min, and were immediately centrifuged at 4°C to obtain plasma

In the protein binding measurements, blood samples were collected from control or endotoxin-pretreated rats under pentobarbital anesthesia without the injection of pindolol

To determine the concentrations of pindolol enantiomers in heart and lung, the tissues were quickly removed at 5 min after injection of the racemate, homogenized in 0 01 *M* HCl, and centrifuged at 1000 *g* for 15 min [2] The supernatants were kept frozen until analysis

Chemical analysis of pindolol

The concentrations of *R*(+)- and *S*(−)-pindolol were determined as follows [8], 0 1–2 ml of plasma or supernatant from heart or lung homogenate was alkalinized with 1 *M* NaOH to extract the enantiomers with 7 ml of diethyl ether Then, 2 ml of 0 1 *M* HCl were added to 6 ml of the ethereal layer for

back-extraction After the ethereal layer had been discarded, 1 8 ml of 0 1 *M* HCl layer were alkalinized with 1 *M* NaOH and re-extracted with 7 ml of diethyl ether Then 6 ml of the ethereal layer were evaporated to dryness under a stream of nitrogen, and the residue was treated at room temperature for 30 min with 50 μl of 2% GITC–acetonitrile containing 64 ng of 1-nitronaphthalene as an internal standard to form diastereoisomers of pindolol At the termination of the reaction, 10 μl of 0 2% hydrazine–acetonitrile were added to degrade the excess GITC

A 20μl volume of the reaction mixture was injected into an HPLC system (LC-6A, SPD-6A and C-R3A, Shimadzu, Japan) with a reversed-phase C_{18} column (TSK-gel ODS 120 A, 25 cm×4 6 mm I D , Tokyo Soda, Japan). The mobile phase was acetonitrile–10 m*M* phosphate buffer (pH 3 4) (1 1, v/v), the flow rate 0 9 ml/min, and the detection wavelength 258 nm Under these conditions, the retention times of the *R*(+)-and *S*(−)-pindolol diastereoisomers and the internal standard were 23 5, 19 3 and 25 7 min, respectively, and the racemic pindolol used was found to have equal peak areas for the two enantiomers The calibration curve was completely linear from 5 ng to 100 ng per 50 μl of reaction medium for each enantiomer ($r>0$ 999) To correct for the extraction loss and efficiency, 200 ng of racemic pindolol were added to drug-free plasma or supernatant from heart or lung homogenate, extracted and determined by the same method. The value determined at each experiment was used as the standard to calculate the concentrations of the enantiomers

Apparent distribution volume and clearance of pindolol

The pharmacokinetic parameters of each pindolol enantiomer were calculated according to the one-compartment open model [14] The half-life ($t_{1/2}$) and the zero-time concentration (C_0) were calculated from the concentration–time curves The concentration–time curves of both enantiomers, shown in Fig 1, were fit to the one-compartment model for elimination, and the apparent distribution volume (V_d) and the total clearance (Cl) of pindolol were calculated from the following equations·

$$V_d = \frac{\text{dose (i v)}}{C_0}$$

$$Cl = \frac{0\ 693 \times V_d}{t_{1/2}}$$

Protein binding of pindolol

Equilibrium dialysis was carried out using a multisample-dialysis apparatus with eight dialysis cells (Sanko Plastic) Each cell was divided into two parts by Visking cellulose membrane One side contained 1 ml of 0 15 *M* potassium phosphate buffer solution (pH 7 4) containing 6 μg of racemic pindolol, and

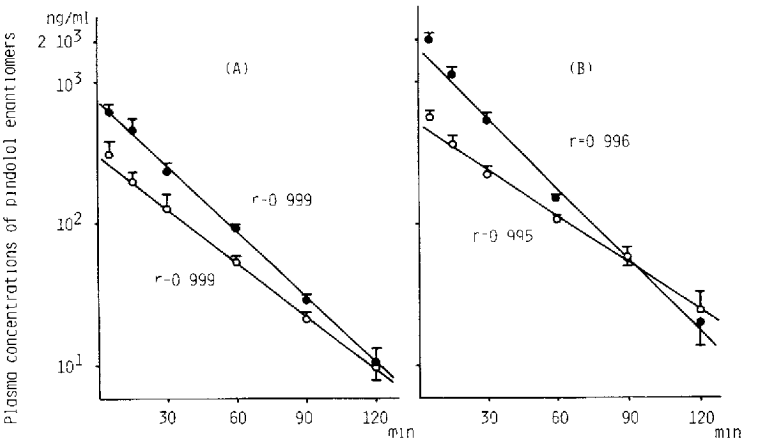

Fig 1 Plasma concentration-time profiles of $R(+)$-enantiomer (●) and $S(-)$-enantiomer (○) after racemic pindolol injection in (A) control and (B) endotoxin-pretreated rats Each point indicates the mean ± S D of four experiments

the other side 1 ml of plasma from control or endotoxin-pretreated rats Equilibrium dialysis was carried out at 37 °C for 4 h under shaking The concentrations of total, $R(+)$- and $S(-)$-pindolol in both sides were examined by the stereoselective determination described above

Determination of plasma α_1-acid glycoprotein

Measurement of plasma α_1-acid glycoprotein was carried out in duplicate by radial immunodiffusion using rabbit antirat α_1-acid glycoprotein antiserum [15] α_1-Acid glycoprotein was purified by the procedure of Charlwood et al [16] from terpentine oil-pretreated rats The purity of α_1-acid glycoprotein was checked by SDS gel electrophoresis, carbohydrate content determination and amino acid analysis Antibodies to α_1-acid glycoprotein were raised in rabbits by injecting antigen (1 mg/ml) emulsified with 1 ml of Freund's complete adjuvant once a month for 4 months before collecting serum.

Statistics

Statistical analysis was made by Student's t test

RESULTS AND DISCUSSION

The diastereoisomers were sufficiently separated, with a resolution factor of 4 3 ± 0 4 The good reproducible result in HPLC was obtained after the extraction of pindolol enantiomers from rat plasma and the derivatization to the diastereoisomers The coefficients of variation ($n=5$) for experiments using 200 ng of racemate in 1 ml rat plasma were 3.6% and 3 8% for each enantiomer

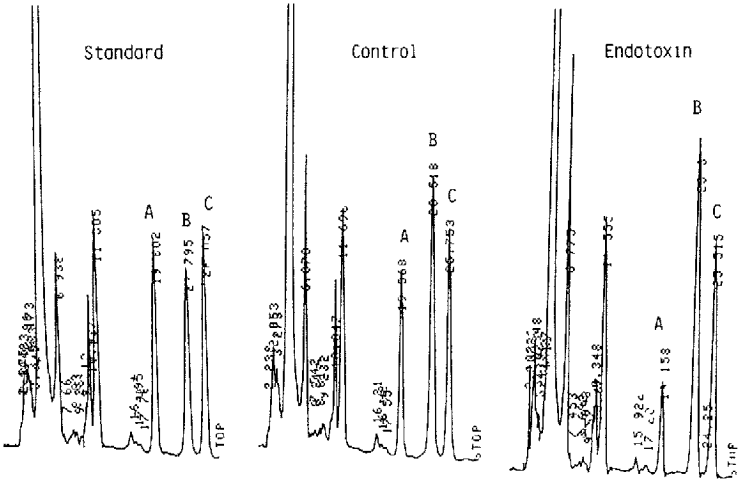

Fig 2 Typical chromatograms of normal plasma containing added racemic pindolol, and of control and endotoxin-pretreated plasma 5 min after racemic pindolol injection 0 3 ml of control plasma and 0 1 ml of endotoxin-pretreated plasma were used Peaks A = $S(-)$-pindolol, B = $R(+)$-pindolol, C = internal standard

TABLE I

EFFECT OF ENDOTOXIN ON PHARMACOKINETIC PARAMETERS OF PINDOLOL ENANTIOMERS

Values were calculated from data in Fig 1

	C_0 (ng/ml)	V_d (l/kg)	$t_{1/2}$ (min)	Cl (l/kg min)
R-Pindolol				
Control	749	3 34	19 5	0 119
Endotoxin	2055	1 22	17 5	0 048
S-Pindolol				
Control	329	7 60	23 1	0 228
Endotoxin	554	4 51	27 1	0 115

Fig 2 shows typical chromatograms from normal rat plasma containing racemic pindolol, and control and endotoxin-pretreated rat plasma 5 min after racemic pindolol injection From this stereoselective determination of pindolol in each sample, plasma concentration–time profiles of the enantiomers in control and endotoxin-pretreated rats were obtained (Fig 1) Table I shows the calculated values of pharmacokinetic parameters for each enantiomer in this experiment The values of the apparent distribution volume and the total drug clearance for $R(+)$-pindolol were half of those for $S(-)$-pindolol in control

rats These results are very similar to those for propranolol enantiomers reported by Kawashima et al [2] and Caccia et al [3] The apparent distribution volumes of $R(+)$- and $S(-)$-enantiomers in endotoxin-pretreated rats decreased to 37% and 59% of the control values, and the total clearances of both enantiomers were decreased to ca 50% of the control values It is thus clear that, with regard to the apparent distribution volume, $R(+)$-pindolol was more affected by endotoxin The biological half-lives of both enantiomers were not changed by endotoxin

As a reason for the decrease in the apparent distribution volume of pindolol enantiomers caused by endotoxin, it may be proposed that the plasma protein binding of pindolol enantiomers increased and/or the tissue binding decreased in endotoxin-pretreated rats First of all, the protein binding of pindolol racemate was examined using plasma from control or endotoxin-pretreated rats by the equilibrium dialysis Table II shows that the percentage of total pindolol bound to plasma protein increased by 45% in endotoxin-pretreated rats Stereoselective determination shows that, following pretreatment with endotoxin, the percentage of bound $R(+)$ —pindolol increased by 36% and that of $S(-)$-pindolol by 45% These results indicate that the increase in protein binding of both enantiomers might induce the observed decrease in the apparent distribution volume by endotoxin Consequently, compared with the control, the unbound fraction of $R(+)$- or $S(-)$-pindolol in plasma decreased from 41 4 to 20 4%, or from 76 8 to 66.3% in endotoxin-pretreated rats, respectively Therefore, it is suggested that the larger decrease of the unbound fraction ratio for $R(+)$-pindolol might bring about the higher response of $R(+)$-pindolol to endotoxin (Table I)

Secondly, the tissue concentrations of both enantiomers in control and endotoxin-pretreated rats were examined by the same stereoselective method Table III shows much higher concentrations of both enantiomers in heart and lung than in plasma 5 min after the injection of the racemate All the tissue concentrations were closely related not to the total plasma concentrations but

TABLE II

PROTEIN BINDING OF RACEMIC PINDOLOL TO CONTROL OR ENDOTOXIN-PRETREATED RAT PLASMA

Values are mean ± S D ($n=4$) Asterisks indicate significant differences from control values ($P<0.01$)

Plasma source	Total pindolol binding (%)	$R(+)$-Pindolol		$S(-)$-Pindolol	
		Binding (%)	Free (%)	Binding (%)	Free (%)
Control	43 1 ± 1 2	58 6 ± 1 0	41 4	23 2 ± 1 1	76 9
Endotoxin	62 7 ± 2 2*	79 6 ± 0 7*	20 4	33 7 ± 1 8*	66 3

TABLE III

CONCENTRATION OF PINDOLOL ENANTIOMERS IN HEART AND LUNG 5 MIN AFTER RACEMATE INJECTION

Values are mean ± S D ($n=4$), amount of racemate injected was 5 mg/kg

	Concentration of pindolol enantiomers				
	Unbound in plasma (ng/ml) (A)	Heart (ng/g) (B)	(B/A)	Lung (ng/g) (C)	(C/A)
R(+)-Pindolol					
Control	260 ± 34	2293 ± 77	8 8	3930 ± 579	15 1
Endotoxin	391 ± 86	3009 ± 584	7 7	6753 ± 1113	17 3
S(−)-Pindolol					
Control	214 ± 30	2618 ± 201	12 2	4478 ± 800	20 9
Endotoxin	384 ± 45	3898 ± 701	10 2	8743 ± 1065	22 8

to the concentrations of the unbound fraction in plasma, which may indicate that the affinities of both enantiomers were not affected by the pretreatment with endotoxin, at least in these tissues

The increased protein binding by endotoxin may well be due to an increase in the content of binding protein, because it is impossible that such a large increase of protein binding could result from increased binding affinity α_1-Acid glycoprotein is the most likely candidate, because endotoxin can induce messenger RNA of α_1-acid glycoprotein in liver [17], a number of β-blockers containing pindolol specifically bind to α_1-acid glycoprotein [18–21], and the plasma content of α_1-acid glycoprotein is increased five- to ten-fold by laparotomy [22] As no determination kit for rat α_1-acid glycoprotein is yet available, antibody was produced in rabbits with purified α_1-acid glycoprotein and showed sufficient specificity The contents of α_1-acid glycoprotein in control and endotoxin-pretreated rat plasma were 13 4 ± 1 5 and 75 3 ± 5 6 mg/dl ($n=5$), respectively These results strongly suggest that the increased protein binding of pindolol enantiomers by endotoxin might be due to the increased content of α_1-acid glycoprotein in blood It is also assumed that α_1-acid glycoprotein may have an enantiospecific binding affinity for pindolol, as in other cases [21]

The mechanism of the decreased total clearance of both pindolol enantiomers by endotoxin remains to be clarified From the present results obtained by using the stereoselective assay method, we demonstrated the enantiospecific binding of pindolol to plasma protein and concluded that one of the major effects of endotoxin on the disposition of pindolol enantiomers would come from the increase of the protein binding in blood for both enantiomers

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