Purification and Characterization of Befunolol Reductase from Rabbit Liver

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An enzyme (befunolol reductase) which catalyzes the reduction of befunolol to dihydrobefunolol was purified from the cytosolic fraction of rabbit liver to homogeneity by various chromatographic techniques. Befunolol reductase had molecular weights of 29000 on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and 34000 on gel filtration. The enzyme required reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor and showed an optimal pH of 6.5. The apparent K_m and V_{max} values of the enzyme for the reduction of befunolol were 1.7 mM and 4.4 units/mg, respectively. Flavonoids, sulfhydryl reagents, heavy metals and coumarins strongly inhibited the enzyme. The enzyme catalyzed the reduction of a variety of aromatic ketones. In addition to befunolol, some ketone-containing drugs such as daunorubicin and levobunolol were efficiently reduced by the enzyme. On the basis of substrate specificities for steroids, befunolol reductase purified from the cytosolic fraction of rabbit liver appeared to be a 3α-hydroxysteroid dehydrogenase.

Keywords befunolol reductase; rabbit liver; enzyme purification; molecular weight; optimal pH; cofactor requirement; amino acid composition; substrate specificity; inhibitory effect

Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent carbonyl reductases are known to reduce ketone-containing drugs such as daunorubicin[1-4] and oxisuran[5] to the corresponding alcohol products. These enzymes have broad substrate specificities and are present in abundance in a variety of mammalian tissues.[6-11] The constitutive nature and ubiquitous distribution of these enzymes suggest their involvement in important physiological processes. Until recently, however, information concerning the purification and physiological role of enzymes which catalyze the reduction of ketone-containing drugs has been limited.

Befunolol is a typical ketone-containing drug and is widely used in the treatment of arrhythmia as a potent β-adrenoceptor blocker.[12,13] Tohno *et al.*[14] reported the *in vitro* reductive metabolism of befunolol to dihydrobefunolol in rabbit, rat and guinea pig. Since dihydrobefunolol is a pharmacologically active metabolite,[15] it is important to have an adequate knowledge of befunolol metabolism. Recently, we have examined in detail the reductive metabolism of befunolol, using the cytosolic fraction of rabbit liver.[16] The purpose of the present study was to purify befunolol reductase from the cytosolic fraction of rabbit liver, and to elucidate the properties of befunolol reductase.

Experimental

Chemicals Befunolol hydrochloride [2-acetyl-7-(2-hydroxy-3-isopropylaminopropoxy)benzofuran hydrochloride, racemate], (*R*)-(+)-befunolol hydrochloride, (*S*)-(−)-befunolol hydrochloride and dihydrobefunolol were supplied by Kaken Pharm. Co. (Tokyo, Japan). Indomethacin (Taisho Pharm., Tokyo, Japan), furosemide (Mochida Pharm., Tokyo, Japan), chlorpromazine, acetohexamide (Shionogi & Co., Osaka, Japan), warfarin (Eisai, Tokyo, Japan), levobunolol (Warner-Lambert, Ann Arbor, U.S.A.), daunorubicin (Meiji Seika, Tokyo, Japan), loxoprofen (Sankyo, Tokyo, Japan), ketoprofen (Hisamitsu Pharm., Saga, Japan), suprofen (Taiyo Yakuhin, Tokyo, Japan) and fenbufen (Lederle (Japan), Tokyo, Japan) were provided by the manufacturers. Steroids were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Other substrates and inhibitors of analytical reagent grade were obtained from Wako Pure Chemical Ind. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Tokyo Kasei Kogyo Co. (Tokyo, Japan). Pyridine nucleotides and proteins used as molecular weight markers for sodium dodecyl sulfate (SDS) polyacrylamide gel were purchased from Oriental Yeast Co. (Tokyo, Japan). Proteins used for Sephadex G-100 column calibration were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Diethylaminoethyl (DEAE)-Sephacel, Sephadex G-75 (superfine), G-100 and Blue sepharose CL-6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and hydroxylapatite was from Bio-Rad Laboratories (Richmond, U.S.A.).

Assay of Enzyme Activity Befunolol reductase activity was determined spectrophotometrically in a reaction mixture consisting of enzyme, 1.0 mM befunolol, 0.25 mM NADPH and 0.1 M Na,K-phosphate buffer (pH 6.5) in a final volume of 2.8 ml. In the case of determination of the optimal pH, the following 0.1 M buffers were used (pH 4.0 to 6.0, citrate–Na_2HPO_4 buffer; pH 6.0 to 8.0, Na, K-phosphate buffer; pH 8.0 to 9.0, Tris–HCl buffer). Although befunolol was soluble in the buffers, substrates which were not sufficiently soluble in the buffers were dissolved in methanol, and 2—20 μl of each was added to the standard reaction mixture. The reverse reaction was performed in 0.1 M glycine–NaOH buffer (pH 10.0) containing 0.25 mM $NADP^+$ and suitable amounts of substrate and enzyme. The reaction was started by the addition of coenzyme, and the decrease or increase in absorbance at 340 nm was monitored with a Shimadzu UV-240 spectrophotometer. One unit of enzyme activity was defined as the oxidation or reduction of 1 μmol of coenzyme/min at 30 °C.

Befunolol absorbs at 340 nm ($\varepsilon = 1.37 \times 10^3\ M^{-1}\ cm^{-1}$), and so it was necessary to determine a correction factor for the initial rates observed in the presence of this compound. This correction factor was calculated in the following way[17]:

$$\text{correction factor} = \varepsilon_{NADPH}/(\varepsilon_{NADPH} - \varepsilon_{dihydrobefunolol} + \varepsilon_{befunolol})$$

The K_m and V_{max} values were determined by means of Lineweaver–Burk plots.[18] Protein concentration was determined by a modification of the method of Lowry *et al.*[19] using bovine serum albumin as the standard.

Enzyme Purification For the purification of befunolol reductase from rabbit liver, all procedures were performed at 4 °C. Male rabbits (Japanese white, 2.5—3.0 kg body weight) were exsanguinated from the carotid artery and the livers (300 g) were homogenized in 2 volumes of 10 mM Na,K-phosphate buffer (pH 7.4) containing 0.15 M KCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM 2-mercaptoethanol with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $10000 \times g$ for 20 min and the resulting supernatant was centrifuged at $105000 \times g$ for 60 min. The $105000 \times g$ supernatant, defined as cytosol, was fractionated by adding ammonium sulfate. The proteins which precipitated between 30 and 70% saturation were collected by centrifugation, dissolved in a minimal volume of 10 mM Na,K-phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A) and dialyzed overnight against about 100 volumes of the same buffer. The dialysate was then clarified by centrifugation. The supernatant was applied to a DEAE-Sephacel column (2.5 × 23 cm) equilibrated with buffer A and washed with the same buffer until the eluate showed almost no absorbance at 280 nm. A linear salt gradient (900 ml) of 0 to 0.1 M NaCl in the above buffer was passed through the column to elute the bound proteins. The peak befunolol reductase activity appeared in the eluate at approximately 30 mM NaCl. The fractions containing high enzyme activity were combined, extensively dialyzed against 10 mM Na,K-phosphate buffer (pH 6.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer B), and applied to a Blue Sepharose CL-6B column (1.9 × 32 cm) previously equilibrated with the same buffer. The column was initially washed with

buffer B, followed by a 500 ml linear salt gradient of 0 to 1.0 M KCl to elute the bound proteins. The peak befunolol reductase activity was eluted at approximately 0.8 M KCl. The fractions containing high enzyme activity were combined, extensively dialyzed against buffer A without EDTA, and applied to a hydroxylapatite column (1.5 × 13 cm) previously equilibrated with the above buffer. The peak befunolol reductase activity was not adsorbed on the hydroxylapatite column and was recovered in the washings. The fractions containing high enzyme activity were combined and further purified by chromatography on a Sephadex G-75 (superfine) column (2.5 × 45 cm) and eluted with buffer A. The combined enzyme-active fractions were concentrated to about 2 mg/ml and stored at 0 °C in 20% (v/v) glycerol.

Polyacrylamide Gel Electrophoresis and Gel Filtration The purified enzyme was subjected to electrophoresis in 7.5% (w/v) acrylamide gels according to the method of Ornstein[20] and Davis.[21] SDS-gel electrophoresis was performed on 10% (w/v) gels by the procedure of Weber and Osborn.[22] Protein bands were stained with Coomassie brilliant blue. The molecular weight of the enzyme was estimated by gel filtration on a column (1.9 × 50 cm) of Sephadex G-100. Calibration proteins used and their molecular weights were: cytochrome c (12300), myoglobin (17800), chymotrypsinogen A (25000), ovalbumin (45000) and bovine serum albumin (67000). Blue dextran 2000 was used to determine the column void volume.

Amino Acid Analysis Amino acid analysis of befunolol reductase was performed by the method of Shoji *et al.*[23] The sample was hydrolyzed in 6 N HCl for 24 h at 110 °C *in vacuo* and derivatization of the resultant amino acids with phenyl isothiocyanate was carried out according to the method of Bidlingmeyer *et al.*[24]

Results

Enzyme Purification After ammonium sulfate fractionation of the rabbit liver cytosol, a befunolol reductase was purified by column chromatographies on DEAE-Sephacel, Blue sepharose CL-6B, hydroxylapatite and Sephadex G-75 (superfine). Since the enzyme activity appeared as one main peak and some minor peaks after the DEAE-Sephacel column chromatographic step, as shown in Fig. 1, the main peak was further purified by the subsequent column chromatographic steps. In a typical purification sequence, 6.5% of the initial enzyme activity was recovered, representing a 224-fold enrichment in specific activity (Table I). The purified befunolol reductase showed a single protein band on polyacrylamide gel electrophoresis with or without SDS (Fig. 2).

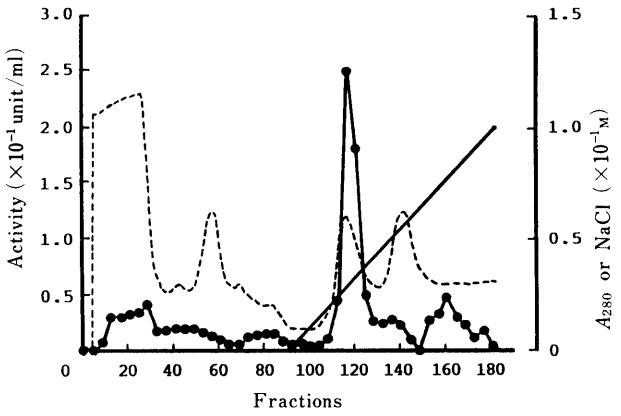

Fig. 1. DEAE-Sephacel Column Chromatography of Rabbit Liver Befunolol Reductase

The column was eluted with a linear salt gradient from 0 to 0.1 M NaCl (——), and 10 ml fractions were analyzed for protein (------) and for the activity of befunolol reductase (●).

Molecular Weight and Amino Acid Composition The molecular weight of the purified enzyme was estimated to be 29000 by SDS-polyacrylamide gel electrophoresis and 34000 by gel filtration. The amino acid composition of the enzyme is shown in Table II, and is similar to that of carbonyl reductase from human brain purified by Wermuth.[6]

Enzyme Stability When the purified enzyme was stored at 0 °C in 10 mM Na,K-phosphate buffer (pH 7.4) containing EDTA (0.5 mM), 2-mercaptoethanol (5 mM) and glycerol (20% (v/v)), it was stable for at least 1 month. The enzyme in 0.1 M Na,K-phosphate buffer (pH 6.5) was completely inactivated in 10 min at 55 °C.

Catalytic Properties The purified enzyme required

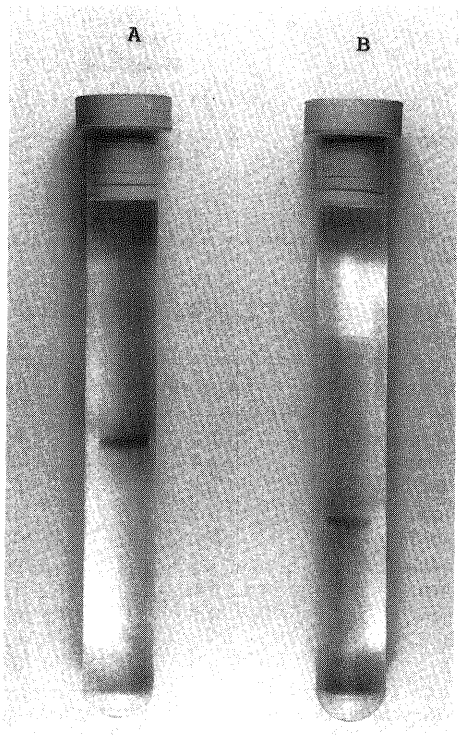

Fig. 2. Polyacrylamide Gel Electrophoresis of Rabbit Liver Befunolol Reductase

About 10 μg of the enzyme was run in the absence (A) and presence (B) of SDS, and the gels were stained for protein.

Table I. Purification of Befunolol Reductase from Rabbit Liver Cytosol

Purification step	Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (-fold)	Recovery (%)
Cytosol	17518	142	0.0081	1	100
$(NH_4)_2SO_4$ fraction (30—70%)	10920	102	0.0093	1.1	72
DEAE-Sephacel	235	32.2	0.137	16.9	23
Blue sepharose CL-6B	52.2	17.7	0.340	42.0	12
Hydroxylapatite	11.6	14.2	1.23	151	10
Sephadex G-75 (superfine)	5.11	9.28	1.82	224	6.5

NADPH as a cofactor. The apparent K_m value for the oxidation of NADPH by the enzyme in the presence of befunolol was 7 μM, whereas little enzyme activity was detected when NADPH was replaced by reduced nicotinamide adenine dinucleatide (NADH) at the same concentration. This implies that befunolol reductase can not be classified as an alcohol dehydrogenase which requires NADH as a cofactor. The optimum pH of the enzyme in the reduction of befunolol was 6.5. The apparent K_m and V_{max} values of the enzyme for the reduction of befunolol (racemate) were 1.7 mM and 4.4 units/mg, respectively. These values, interestingly, were somewhat different between (*R*)-(+)-befunolol and (*S*)-(−)-befunolol, as shown in Table III. This suggests that the enzyme exhibits substrate stereoselectivity in the reduction of befunolol. In addition to befunolol, some ketone-containing drugs such as daunorubicin and levobunolol were efficiently reduced by the enzyme, but ketoprofen, suprofen and fenbufen were little reduced (Table III). Furthermore, the substrate specificity pattern of befunolol reductase for various aldehydes and ketones is summarized in Table IV. A number of aromatic ketones served as efficient substrates. In the case of aliphatic aldehydes and ketones, cyclohexanone was an efficient substrate, whereas *n*-caprylaldehyde was a poor substrate and acetone was not reduced at all. Sugars were very poor substrates. Quinones are well known to contribute as oxidation-reduction catalysts in many biological processes. Befunolol reductase, however, could not catalyze the reduction of menadione (vitamin K_3), which is a representative quinone. Table V shows the substrate specificity of befunolol reductase for steroids. In particular, 3-ketosteroids with 5α-configuration appeared to be good substrates for the reduction. The enzyme also efficiently oxidized 3α-hydroxysteroid with 5α-configuration (5α-androstan-3α-ol-17-one).

Inhibition Studies Table VI compares the effects of various inhibitors on befunolol reductase. Flavonoids (quercetin and quercitrin), sulfhydryl reagents [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuri-

Table II. Amino Acid Composition of Rabbit Liver Befunolol Reductase

Amino acid	Number of residues	Amino acid	Number of residues
Lysine	14	Alanine	20
Histidine	5	Cysteine	4
Arginine	19	Valine	26
Aspartic acid	19	Methionine	7
Threonine	17	Isoleucine	12
Serine	12	Leucine	31
Glutamic acid	31	Tyrosine	4
Proline	13	Phenylalanine	9
Glycine	24		

The numbers of residue/mol enzyme were calculated by assuming a molecular weight of 29000.

Table III. Apparent K_m and V_{max} of Befunolol Reductase for Ketone-Containing Drugs

Substrate	K_m (mM)	V_{max} (unit/mg)
Befunolol (racemate)	1.7	4.4
(*R*)-(+)-Befunolol	1.4	3.6
(*S*)-(−)-Befunolol	2.1	5.8
Levobunolol	0.30	5.0
Daunorubicin	4.1	64.1
Acetohexamide	1.2	4.4
Loxoprofen	0.41	1.0
Ketoprofen	0.57	0.20
Suprofen	—	(0.13)
Fenbufen	—	(0.13)

The value in parentheses indicates the activity with 1.0 mM substrate.

Table IV. Substrate Specificity of Rabbit Liver Befunolol Reductase for Aldehydes and Ketones

Substrate	K_m (mM)	V_{max} (unit/mg)
D-Xylose	—	(0)
D-Glucuronic acid	—	(0)
D-Glucuronolactone	—	(0.07)
DL-Glyceraldehyde	—	(0.05)
Pyridine-4-aldehyde	2.6	24.8
n-Caprylaldehyde	6.1	2.3
Acetone	—	(0)
2-Benzoylpyridine	0.004	13.4
3-Benzoylpyridine	1.6	8.4
4-Benzoylpyridine	0.04	14.3
o-Nitroacetophenone	—	(0.04)
m-Nitroacetophenone	4.6	5.6
p-Nitroacetophenone	0.79	4.0
Acetophenone	—	(0.05)
Benzophenone	0.25	0.51
Benzoylacetone	2.3	8.2
2-Acetylfuran	—	(0.03)
Hydrindantin	0.12	14.1
Cyclohexanone	0.36	10.3
Menadione	—	(0)

The value in parentheses indicates the activity with 1.0 mM substrate.

Table V. Substrate Specificity of Rabbit Liver Befunolol Reductase for Steroids

Steroid	Reduction		Oxidation	
	K_m (μM)	V_{max} (unit/mg)	K_m (μM)	V_{max} (unit/mg)
4-Androsten-17β-ol-3-one	1239	0.61	2159	5.6
5α-Androstan-17β-ol-3-one	213	22.8	98	0.44
5β-Androstan-17β-ol-3-one	63	6.1	—	(0)
5α-Androstan-3α-ol-17-one	22	0.19	638	3.6
5α-Androstan-3β-ol-17-one	1288	31.5	527	0.73
4-Androstene-3,17-dione	142	4.6	—	(0)
5α-Androstane-3,17-dione	100	16.0	—	(0)
5β-Androstane-3,17-dione	234	7.9	—	(0)

The value in parentheses indicates the activity with 0.1 mM substrate.

TABLE VI. Effect of Various Inhibitors on Befunolol Reductase Activity

Inhibitor	Concn. (mM)	Inhibition (%)	Inhibitor	Concn. (mM)	Inhibition (%)
Pyrazole	1.0	6	$AgNO_3$	0.01	99
Barbital	1.0	6	Indomethacin	0.1	33
Phenobarbital	1.0	9	Furosemide	1.0	84
Quercetin	0.05	55	Chlorpromazine	1.0	0
Quercitrin	0.05	93	Warfarin	0.01	7
DTNB	0.01	85	4-Hydroxycoumarin	0.01	26
*p*CMB	0.01	99	Phenprocoumon	0.01	43
N-Ethylmaleimide	1.0	4	Dicumarol	0.01	15
2,2′-Dipyridil	1.0	0	Coumetarol	0.01	48
o-Phenanthroline	1.0	0	Citrate	1.0	0
EDTA	1.0	0	Nicotinate	1.0	19
$HgCl_2$	0.001	56	Benzoate	1.0	32
$CuSO_4$	0.01	99	$NADP^+$	1.0	71
$FeSO_4$	0.01	32	NAD^+	1.0	3

Inhibition studies were carried out as described in the text using 1.0 mM befunolol as the substrate.

benzoic acid (*p*CMB)], heavy metals and coumarins exhibited strong inhibitory effects. Indomethacin and furosemide were also effective inhibitors. On the other hand, pyrazole, barbiturates (barbital and phenobarbital), metal-chelating reagents (2,2′-dipyridil, *o*-phenanthroline and EDTA) and chlorpromazine had little or no effect on the reduction of befunolol.

Discussion

Carbonyl reductases, which catalyze the reduction of xenobiotic and endogenous aldehydes and ketones in the presence of NADPH, have been found in animal tissues.[4,25-27] For example, Felsted *et al.*[4] have purified carbonyl reductases from rabbit liver and examined the properties of the enzymes. In the present study, we purified befunolol reductase from the cytosolic fraction of rabbit liver. The purified enzyme had a molecular weight of 29000 and an optimal pH of 6.5, and required NADPH as a cofactor. Pyrazole (a classical inhibitor of alcohol dehydrogenase) and barbiturates (potent inhibitors of aldehyde reductases had little effect on the enzyme activity. Sulfhydryl reagents markedly inhibited the reduction of befunolol, but metal chelating reagents did not. In addition, the purified enzyme efficiently catalyzed the reduction of a variety of aromatic ketones. These properties are in fairly good agreement with those of well-known carbonyl reductases.[28] Thus, befunolol reductase can be classified as a carbonyl reductase.

Furthermore, on the basis of substrate specificities for steroids, it is possible to assume that befunolol reductase is a 3α-hydroxysteroid dehydrogenase. In fact, this hypothesis is supported by the observation that the main peak of the befunolol reduction activity coincided with that of the oxidation activity with 5α-androstane-3α-ol-17-one in all the purification steps (data not shown). Recently, Sawada *et al.*[29] purified four reductases (F_1, F_2, F_3 and F_4) from rabbit liver and the F_3 enzyme was identified as a 3α-hydroxysteroid dehydrogenase. Tanaka *et al.*[30] reported that loxoprofen reductase purified from rabbit liver corresponded to the F_3 enzyme. However, befunolol reductase, unlike the F_3 enzyme, was weakly active for oxidoreduction of 17β-hydroxysteroid and 17-ketosteroids with 5α-configuration, suggesting that befunolol reductase is different from the F_3 enzyme.

Befunolol reductase was capable of reducing some ketone-containing drugs such as daunorubicin, levobunolol, acetohexamide and loxoprofen in addition to befunolol. In contrast, ketoprofen, suprofen and fenbufen, although they have a ketone group, were poor substrates for this enzyme. Interestingly, these three drugs are all nonsteroidal anti-inflammatory drugs. Our previous paper has shown that most nonsteroidal anti-inflammatory drugs markedly inhibit befunolol reduction in the cytosol of rabbit liver.[16] Thus, the fact that ketoprofen, suprofen and fenbufen are poor substrates for befunolol reductase may be explained by the inhibitory effect of these three nonsteroidal anti-inflammatory drugs on this enzyme, although further investigations should be conducted.

All benzoylpyridines were very good substrates for befunolol reductase. However, the apparent K_m value of the enzyme for the reduction of benzoylpyridines was found to be greatly dependent on the position of the benzoyl group. Also, *p*-nitroacetophenone was a good substrate, while *o*-nitroacetophenone was little reduced. It is interesting that the position of substituents plays an important role in the reduction of aromatic ketones by befunolol reductase. Coumarins, which are potent inhibitors of quinone reductase (DT-diaphorase),[31-33] inhibited befunolol reductase. Nevertheless, befunolol reductase was unable to reduce menadione, which is a representative quinone. Although furosemide, an inhibitor of aldehyde reductase from sheep heart,[34] was effective in the inhibition of befunolol reductase, chlorpromazine, which is an inhibitor of aldehyde reductase from human[35] and rat brains[36] and of carbonyl reductase from human brain,[6] was not. Sawada *et al.* have shown that nicotinate and benzoate inhibit the F_3 enzyme (3α-hydroxysteroid dehydrogenase) from rabbit liver.[29] A similar result was observed in the effect of such organic acids with an aromatic ring on befunolol reductase.

Several investigators have demonstrated the properties of carbonyl reductases from rabbit liver. However, it has not been adequately clarified whether each carbonyl reductase displays broad substrate specificities for ketone-containing drugs such as daunorubicin, levobunolol and acetohexamide. In the present study, we provide evidence that the purified befunolol reductase from rabbit liver is a carbonyl reductase, and has the ability to reduce some ketone-

containing drugs other than befunolol.

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