Pergamon

Biochemical Pharmacology, Vol. 50, No. 2, pp. 221–227, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00124-7

REDUCTION OF DRUG KETONES BY DIHYDRODIOL DEHYDROGENASES, CARBONYL REDUCTASE AND ALDEHYDE REDUCTASE OF HUMAN LIVER

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(*Received* 15 *November* 1994; *accepted* 26 *January* 1995)

Abstract—In this study, we compared the enzymatic reduction of 10 drugs with a ketone group by homogeneous carbonyl reductase, aldehyde reductase and three dihydrodiol dehydrogenases of human liver cytosol. At least one and in some cases all of the three dihydrodiol dehydrogenases reduced each of the ten drugs. Among these naloxone, naltrexone, befunolol, ethacrynic acid and ketoprofen were substrates specific for the dehydrogenases. The other drugs—haloperidol, metyrapone, loxoprofen, daunorubicin and acetohexamide—were highly reduced by carbonyl reductase and/or aldehyde reductase. The dihydrodiol dehydrogenases also showed lower K_m values for haloperidol and loxoprofen than did carbonyl reductase. The results indicate that the three dihydrodiol dehydrogenases, as well as the two reductases, are implicated in the reduction of ketone-containing drugs in human liver cytosol.

Key words: dihydrodiol dehydrogenase; carbonyl reductase; aldehyde reductase; carbonyl reduction; drug ketone; human liver

Mammalian tissues contain cytosolic CR† (EC 1.1.1.184), which catalyzes the NADPH-linked reduction of various aldehydes and ketones to corresponding alcohols. CR differs from ALR (EC 1.1.1.2) and aldose reductase (EC 1.1.1.21) in its ability to reduce aromatic ketones and in its sensitivity to specific inhibitors [1]. Although most CRs purified from mammalian tissues are monomers with molecular masses of 28–38 kDa, they can be divided into two groups with respect to specificity for endogenous substrates of prostaglandins and steroids. CRs of human tissues [2, 4], pig kidney [4], and rat ovary [5] have been demonstrated or suggested to be identical to prostaglandin 9-ketoreductase or $NADP^+$-dependent 15-hydroxy-prostaglandin dehydrogenase. On the other hand, hepatic CRs of rats [6], rabbits [7, 8], guinea pigs [9] and mice [10] possess 3α- or 17β-hydroxy-steroid dehydrogenase and DD (EC 1.3.1.20) activities, and are thought to be identical to the hydroxy-steroid dehydrogenases and DD.

There have been many studies on the enzymatic reduction of ketone-containing drugs by human and animal tissues [11, 12]. Extensive studies on the reductases for several drugs have been done with rabbit liver, from which heterogeneous and drug-specific reductases with molecular masses around 35 kDa have been separated and partially characterized [13–15]. Subsequently, the major forms of the reductases for acetohexamide [16], befunolol [17] and loxoprofen [18] were isolated from rabbit liver and kidney, and are thought to be identical to CR with 3α-hydroxysteroid dehydrogenase activity [7]. The properties of the human reductases for several drug ketones have been studied with crude or partially purified enzyme preparations [19–23], except that two daunorubicin reductases isolated from liver have been identified with ALR and CR [24].

We previously purified four human liver DDs (DD1–DD4) with molecular masses around 36 kDa, of which DD3 was immunologically identified with ALR and the other DDs exhibited 3α- or 3(20)α-hydroxysteroid dehydrogenase activity [25]. Subsequently, DD2 and DD4 were demonstrated by their cDNA cloning to be members of the aldo-keto reductase family [26]. The sequence of DD4 is identical to that of human 3α-hydroxysteroid dehydrogenase [27], but differs from that of human CR [28, 29], a member of the short-chain alcohol dehydrogenase family. Since the DDs also exhibit CR activity [25, 30], there are at least two structurally distinct types of carbonyl-reducing enzymes, CR and DD, in human liver. However, the CR activity of the human enzymes has been studied mostly with model carbonyl compounds, and few attempts have been made to examine their specificity for the ketone-containing drugs that are administered therapeutically. Because of the pharmacological and pharmacokinetic importance of the drugs, it is necessary to elucidate the relationship of the drug reductases to CR, ALR and DDs in human liver. This paper compares reactivity toward ten ketone-containing drugs among CR, ALR and DDs purified

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† Abbreviations: CR, carbonyl reductase; ALR, aldehyde reductase; and DD, dihydrodiol dehydrogenase.

from human liver, and shows that DDs play a more important role than the other enzymes in the reductive metabolism of several drug ketones.

MATERIALS AND METHODS

Chemicals. NADP(H), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan); metyrapone and ethacrynic acid from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); and haloperidol and its reduced metabolite, dihydrohaloperidol [4-(4-chlorophenyl)-α-(4-fluorophenyl)-4-hydroxy-1-piperidinobutanol] from Research Biochemicals (Natick, MA, U.S.A.). Naltrexone, 6α-naloxol and 6α-naltrexol were supplied by the National Institute of Drug Abuse (Lexington, KY, U.S.A.); and befunolol and its reduced metabolite, dihydrobefunolol, by the Kaken Pharmaceuticals Co. (Tokyo, Japan). Acetohexamide and naloxone (Shionogi & Co., Osaka, Japan), daunorubicin (Meiji Seika, Tokyo, Japan), ketoprofen (Hisamitsu Pharmaceuticals, Saga, Japan), and loxoprofen (Sankyo, Tokyo, Japan) were provided by the manufacturers. Hydroxyhexamide and metyrapol were donated by Dr. Y. Imamura (Kumamoto University, Kumamoto, Japan) and Dr. E. Maser (Philipps University, Marburg, Germany), respectively. 6β-Naloxol and 6β-naltrexol were synthesized from naloxone and naltrexone, respectively, by the method of Ahmed *et al.* [14], and dihydroketoprofen, the reduced metabolite of ketoprofen, and *cis*- and *trans*-alcohols of loxoprofen were synthesized according to the method of Tanaka *et al.* [31]. Sephadex G-100 and Q-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used in this study were reagent grade.

Enzyme assay. The reductase activities for drugs and the model substrates, 4-benzoylpyridine and 4-nitrobenzaldehyde, were assayed spectrophotometrically by measuring the oxidation rate of NADPH at 340 nm. The standard reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 6.0, 1 mM substrate or drug, 0.1 mM NADPH, and enzyme to a final volume of 1.5 mL. The reaction, determined in duplicate, was started by the addition of the enzyme. The control reaction mixture contained all the reactants except the enzyme. The reverse reaction rate with the reduced metabolite as the substrate was assayed by recording the NADPH fluorescence at 450 nm (excitation at 340 nm) in 1.0 mL of 0.1 M glycine-NaOH buffer, pH 10.0, containing 0.25 mM $NADP^+$, alcohol metabolite and enzyme. DD activity was determined with 1.8 mM naphthalene dihydrodiol as the substrate [30]. One unit (U) of enzyme activity was defined as the oxidation and reduction of 1 μmol NADPH/min at 25°. To estimate the kinetic constants, the initial rate determination of each substrate was carried out at five concentrations. Initial rates in the asymptotic region, which follows Michaelis kinetics, were plotted in a double-reciprocal form, and the constants were calculated with a computer program for least-squares linear regression. All the assay and kinetic values are expressed as the means of duplicate experiments.

Identification of reaction products. The products formed from the reduction of the parent drugs by the human liver enzymes were identified by TLC. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.5, 1 mM drug, one of the enzymes (80 μg) and an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase), in a total volume of 2.0 mL. Incubation was carried out at 30°

Table 1. Reductase activities of human liver DDs, ALR and CR toward drugs with a ketone group

Substrate	Specific activity (mU/mg)				
	DD1	DD2	DD4	CR	ALR
Group I					
Naloxone	47	66	80	NS*	NS
Naltrexone	100	67	52	NS	NS
Befunolol	236	199	NS	NS	NS
Ethacrynic acid	77	NS	40	NS	NS
Ketoprofen	NS	17	43	NS	NS
Group II					
Haloperidol	39	23	NS	385	NS
Metyrapone	NS	NS	33	447	NS
Loxoprofen	NS	42	52	255	NS
Group III					
Daunorubicin	NS	97	NS	557	52
Acetohexamide	39	26	44	NS	66
Model substrates					
4-Benzoylpyridine	203	134	110	12,600	NS
4-Nitrobenzaldehyde	438	1,248	308	2,860	15,200

Activity was assayed at pH 6.0 with 1 mM substrate and 0.1 mM NADPH.
* NS, no significant activity was detected.

Table 2. TLC of drugs and their enzymatic metabolites

Drug and metabolite	R_f value		Enzymatic metabolite* by:					Solvent system
	Drug	Alcohol	DD1	DD2	DD4	CR	ALR	
Naloxone	0.61							A
6α-Naloxol		0.30	−	−	−	ND†	ND	
6β-Naloxol		0.25	+	+	+	ND	ND	
Naltrexone	0.64							A
6α-Naltrexol		0.35	−	−	−	ND	ND	
6β-Naltrexol		0.29	+	+	+	ND	ND	
Befunolol	0.46							A
Dihydrobefunolol		0.22	+	+	ND	ND	ND	
Ketoprofen	0.49							B
Dihydroketoprofen		0.43	ND	+	+	ND	ND	
Metyrapone	0.39							C
Metyrapol		0.10	ND	ND	+	+	ND	
Haloperidol		0.44						A
Dihydrohaloperidol		0.35	+	+	−	+	−	
Loxoprofen	0.45							B
cis-alcohol		0.40	ND	+	+	−	ND	
trans-alcohol		0.37	ND	+	+	+	ND	
Acetohexamide	0.47							B
Hydroxyhexamide		0.33	+	+	+	ND	+	

* The detection of the enzymatic products is expressed by a plus sign (+); a minus sign (−) represents non-detectable product.
† ND, not determined.

for 1 hr. For analyzing the enzymatic products from naltrexone, naloxone, befunolol, haloperidol and metyrapone, the reaction was terminated by the addition of 1.0 mL of 1 M sodium carbonate buffer, pH 10.0, and the products were extracted with 3 mL of ethyl acetate. The reaction products from ketoprofen and loxoprofen were extracted with ethyl acetate after the pH of the reaction mixtures was adjusted to 3.0 with 1 M HCl. The reduced products of acetohexamide were extracted using the method of Imamura *et al.* [32]. The extracts were concentrated under reduced pressure, and the residue was dissolved in methanol. The sample was subjected to TLC, in which the parent drugs and their authentic metabolites were cochromatographed on the same silica gel plate containing a fluorescence indicator (E. Merck, Darmstadt, Germany). The following solvent systems for TLC were used: (A) chloroform–methanol–ammonia (90:10:3, by vol.); (B) benzene–ethyl acetate–acetic acid (30:15:1, by vol.); and (C) benzene–acetone–ammonia (70:30:1, by vol.). These systems have been used for TLC separation of most of the drugs and their metabolites [14, 31].

Enzyme purification. Human liver DDs (DD1 with a pI of 9.1; DD2 and DD4 with a pI of 5.4), ALR [25] and CR with a pI of 8.3 [3] were purified to homogeneity from 81- and 28-year-old men undergoing legal medical autopsy. For co-purification of DD and drug reductase activities, a liver specimen (30 g) obtained from a 65-year-old man was used. The enzyme activities were purified by extraction, ammonium sulfate fractionation, Sephadex G-100 filtration, and Q-Sepharose chromatography as previous described [25].

RESULTS

Ten ketone-containing drugs were tested as substrates for homogeneous DDs, CR and ALR purified from human liver (Table 1). These enzymes reduced the drugs at different rates, although the reduction rates of the drugs were low compared with those of the model substrates, 4-benzoylpyridine and 4-nitrobenzaldehyde. The drugs can be divided into three groups based on the reactivities of the enzymes. Naloxone, naltrexone, befunolol, ethacrynic acid and ketoprofen (group I drugs) were reduced only by DDs. Haloperidol, metyrapone and loxoprofen (group II drugs) were accepted as substrates for DDs and CR. Daunorubicin and acetohexamide (group III drugs) were reduced by DDs, CR and ALR. In addition, the three DDs (DD1, DD2 and DD4) showed different specificity for the drugs, except for naloxone, naltrexone and acetohexamide.

The validity of monitoring NADPH oxidation at 340 nm as an indirect assay of carbonyl reduction by the enzymes was established by examining the reaction products by TLC. The enzymatic reductions of the drugs each yielded a product on TLC analysis of the extracts of the reaction mixtures. The reduced products from the drugs, except for ethacrynic acid and daunorubicin, were confirmed as the respective secondary alcohols of the drugs by comparing their R_f values with those of the corresponding authentic alcohols (Table 2). In the cases of the reactions with naloxone, naltrexone and loxoprofen as the substrates, the two isomers of the reduced products, 6α- and 6β-hydroxy derivatives for naloxone and naltrexone [33, 34], and *cis*- and *trans*-alcohols for loxoprofen [31], were expected. The R_f values of the enzymatic products of naloxone and naltrexone by all of the DDs were consistent with those of 6β-hydroxy derivatives of the drugs. On the other hand, both *cis*- and *trans*-alcohol products of loxoprofen were obtained from the reaction mixture containing

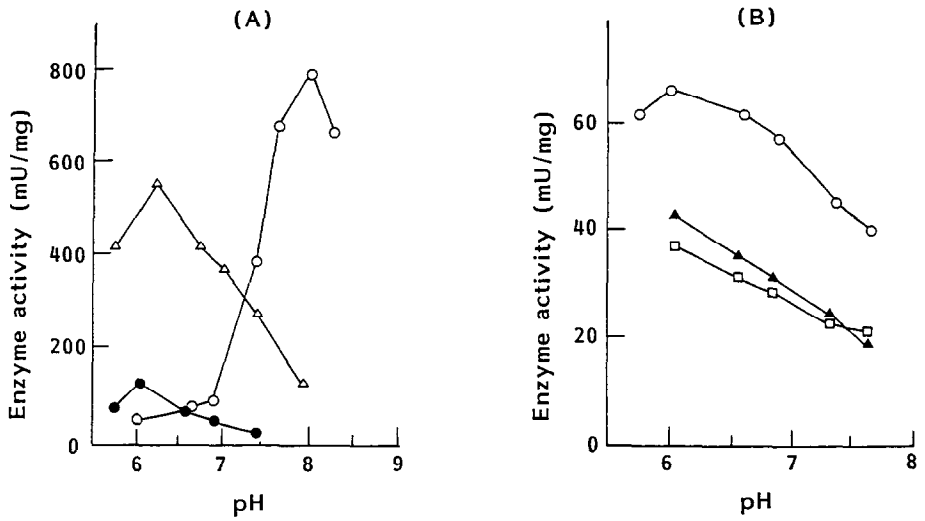

Fig. 1. The pH dependency of daunorubicin and acetohexamide reductase activities of DDs, CR and ALR. The activities of DD1 (□), DD2 (●), DD4 (▲), CR (△) and ALR (○) were determined with 1 mM daunorubicin (A) or acetohexamide (B) as the substrate.

Table 3. Apparent K_m and V_{max}/K_m values for drugs

	DD1		DD2		DD4		CR		ALR	
Drug	K_m (mM)	V_{max}/K_m (U/mg/M)	K_m (mM)	V_{max}/K_m (U/mg/M)	K_m (mM)	V_{max}/K_m (U/mg/M)	K_m (mM)	V_{max}/K_m (U/mg/M)	K_m (mM)	V_{max}/K_m (U/mg/M)
Naloxone	0.74	108	1.9	100	0.41	270	—*	—	—	—
Naltrexone	0.21	595	1.6	110	0.19	332	—	—	—	—
Befunolol	0.52	707	0.76	455	—	—	—	—	—	—
Ethacrynic acid	1.7	124	—	—	1.1	78	—	—	—	—
Ketoprofen	—	—	0.38	63	0.50	133	—	—	—	—
Haloperidol	0.19	232	0.62	61	—	—	1.2	736	—	—
Metyrapone	—	—	—	—	19	35	0.93	534	—	—
Loxoprofen	—	—	1.1	78	1.2	96	38	260	—	—
Daunorubicin	—	—	3.2	116	—	—	0.36	2140	0.26	312
Acetohexamide	1.3	70	3.9	59	0.5	133	—	—	0.22	467

* —, the constant was not determined.

DD2 and DD4, but CR produced only the *trans*-alcohol.

The pH optima of the reductase activities of DDs, CR and ALR for the drugs were around 6.0, except for the daunorubicin reductase activity of ALR, which was maximal at pH 8.0 (Fig. 1). Therefore, the apparent kinetic constants of the enzymes for the drugs were compared at identical conditions of pH 6.0 (Table 3). Of the three DDs, the highest catalytic efficiency (V_{max}/K_m value) for naloxone and ketoprofen was exhibited by DD4, and that for naltrexone, befunolol and ethacrynic acid by DD1. CR showed high catalytic efficiency for the group II drugs and daunorubicin, but its K_m values for haloperidol and loxoprofen were higher than those of the DDs. ALR efficiently reduced daunorubicin as well as acetohexamide, and its K_m and V_{max}/K_m values for daunorubicin at the pH optimum of 8.0 were 0.16 mM and 4520 U/mg/M, respectively.

Since DDs oxidize several alicyclic alcohols as well as *trans*-dihydrodiols of aromatic hydrocarbons [25, 30], their $NADP^+$-linked dehydrogenase activities were examined against several alcohols that are chemically produced by the reduction of the carbonyl group of the parent drugs. DD1 and DD2, but not DD4, showed low dehydrogenase activity for some alcohols at a pH optima of 10.0 (Table 4). The K_m values of DD1 and DD2 for dihydrobefunolol were 2.0 and 1.1 mM, respectively. No dehydrogenase activity for these alcohols by CR and ALR was observed at pH 8–10.

To test whether DDs are the major reductases toward naloxone and befunolol in human liver cytosol, we co-purified the drug reductase activities

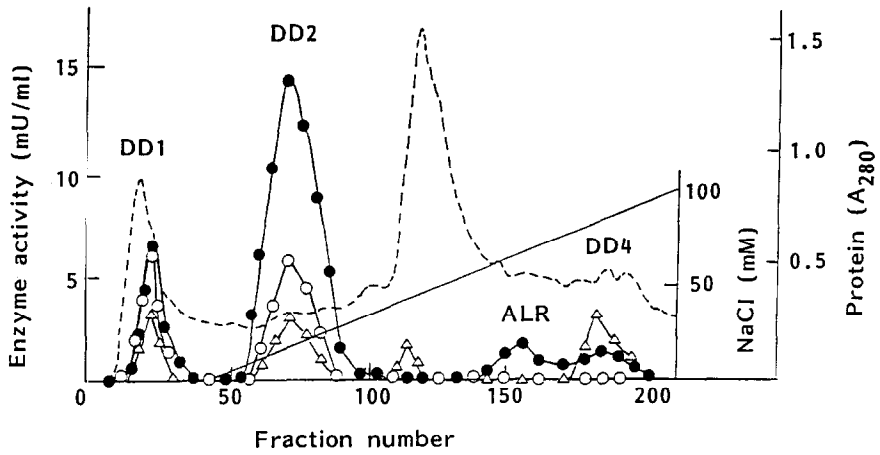

Fig. 2. Q-Sepharose chromatography of DD and drug reductase activities of human liver. The enzyme fraction obtained from Sephadex G-100 chromatography was applied to the Q-Sepharose column (2 × 30 cm), and then the adsorbed proteins were eluted with a linear 0 to 0.1 M NaCl gradient. The fractions (4 mL) were analyzed for protein (---) and enzyme activities. The activities of DD (●), naloxone reductase (△) and befunolol reductase (○) were assayed with 1.8 mM naphthalene dihydrodiol, 1.0 mM naloxone and 1.0 mM befunolol, respectively, as the substrate.

Table 4. Dehydrogenase activity for secondary alcohol metabolites of ketone-containing drugs

Alcohol	Specific activity		
	DD1 (mU/mg)	DD2 (mU/mg)	DD4 (mU/mg)
Dihydrobefunolol	18	77	NS*
Hydroxyhexamide	NS	15	NS
Dihydroketoprofen	NS	5	NS
6α-Naloxol	NS	NS	NS
6β-Naloxol	NS	NS	NS
Metyrapol	NS	NS	NS
Dihydrohaloperidol	NS	NS	NS
(*S*)-1-Indanol	400	1330	1810

Enzyme activities were assayed with 1 mM substrate, except for dihydrohaloperidol at 0.2 mM.

* NS, no significant activity was observed.

with DDs. The activities of DDs and drug reductases were eluted as a single broad peak around 33 kDa on Sephadex G-100 chromatography of the 30–75% ammonium sulfate fraction of the human liver extract. In the subsequent Q-Sepharose chromatography, most peaks of the drug reductase activities were co-eluted with DD1, DD2 and/or DD4 (Fig. 2).

DISCUSSION

The present study revealed that human liver DD, CR and ALR showed distinct specificity for the ten ketone-containing drugs employed therapeutically. Although the specificity for the drugs was also different among the three DDs of human liver, it was an outstanding feature of DD that the group I drugs were reduced only by this enzyme. The co-purification of DD activity and reductase activity for naloxone or befunolol strongly suggests that the DDs are major reductases for the two drugs. In addition, the stereospecific reduction of naloxone and naltrexone by the three DDs is consistent with the *in vivo* study of naltrexone metabolism with human subjects, in which only 6β-naltrexol is excreted as the urinary reduced metabolite [35]. Furthermore, DDs may be predominant reductases toward ethacrynic acid, whose activity has been detected in human liver cytosol [14].

The second characteristic of DD was a broad specificity for the drugs, compared with the relatively narrow specificity of CR and ALR. Since the catalytic efficiencies of DDs for drugs of groups II and III were lower than those of CR and ALR, DDs seem to be minor reductases for these drugs in human liver. However, the contribution of DDs to the hepatic reduction of haloperidol and loxoprofen may be significant because their K_m values for the two drugs were lower than those of CR.

DD has been shown to be important for the oxidative metabolism of *trans*-dihydrodiols derived from polycyclic aromatic hydrocarbons [36–38]. The isolation and characterization of the enzyme from several mammalian tissues have revealed that DDs are simply additional activities of 3α- and/or 17β-hydroxysteroid dehydrogenases, which are also associated with CR activity. The identity of the hydroxysteroid dehydrogenases with major forms of DD and CR has been demonstrated in liver cytosols of rats [6], guinea pigs [9] and mice [10]. Although this is not the case in human liver because of the existence of a distinct CR without DD activity, the present data clearly indicate that human liver DDs with 3α-hydroxysteroid dehydrogenase activity play

an important role in the reduction of several ketone-containing drugs. Of the human DDs, DD2 and DD4 are structurally related to rat liver 3α-hydroxysteroid dehydrogenase [26, 39]. These findings suggest that reductases for befunolol [17], loxoprofen [18] and acetohexamide [16], previously purified from rabbit tissues, are enzymes similar to rat and human dehydrogenases, because the rabbit reductases show high 3α-hydroxysteroid dehydrogenase activity and broad specificity for several drug ketones tested in this study. In addition, DDs purified from rabbit liver exhibit 3α- and/or 17β-hydroxysteroid dehydrogenase and CR activities [8].

In contrast to the broad specificity of DDs, CR and ALR accepted a limited range of drugs as substrates. CR reduced loxoprofen in addition to previously known drug substrates, metyrapone [3], daunorubicin [24] and haloperidol [40]. The comparative kinetic data for the drugs among CR, DD, and ALR suggest that CR is a predominant reductase only for metyrapone and daunorubicin in human liver cytosol. ALR has been shown to reduce daunorubicin at an alkaline pH optimum [24]. The present study not only confirmed the ability of the enzyme, but also demonstrated that the enzyme reduced acetohexamide to hydroxyhexamide. The high catalytic efficiency of ALR compared with those of DDs, together with its high amount in human liver [3, 25], suggests that ALR is the predominant acetohexamide reductase in human liver.

There have been several studies on drug reductases with crude or partially purified preparations of human tissues [19–23]. The substrate specificities of human liver DDs, CR and ALR suggest that some of the enzymes are identical to the human reductases for, at least, haloperidol [19], acetohexamide [20] and metyrapone [21], although metyrapone is also reduced by a microsomal enzyme [21]. Furthermore, DDs and ALR may be related to the expression of the pharmacological potency of befunolol [41, 42], loxoprofen [43] and acetohexamide [44] because the reduction of these drugs is a major metabolic pathway in humans, and their reduced metabolites are also pharmacologically active. Therefore, the elucidation of the inter-individual variability of the hepatic contents of DD, CR and ALR is an important topic for future study from the viewpoint of the pharmacology and pharmacodynamics of the drugs. Although such a study on ALR has not been reported, the inter-individual variation of CR activity [45] and DD activity [46] in human liver cytosol has been described. With respect to DD, the enzyme exists in multiple forms in human liver [25], and the three DDs showed distinct specificity for the ten ketone-containing drugs. The inter-individual differences in the hepatic contents of the respective DDs is a subject of great physiological and pharmacological importance. The previous purification of human liver DDs [25, 30] suggested the inter-individual difference in activity ratios of the three DDs in different liver samples.

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