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IMPAIRMENT OF UDP-GLUCOSE DEHYDROGENASE AND GLUCURONIDATION ACTIVITIES IN LIVER AND SMALL INTESTINE OF RAT AND GUINEA PIG *IN VITRO* BY PIPERINE

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Abstract—The effects of piperine, a major ingredient of black pepper, on UDP-glucose dehydrogenase (UDP-GDH) and glucuronidation potentials of rat and guinea pig liver and intestine were studied. Piperine caused a concentration-related strong inhibition of UDP-GDH (50% at 10 μM) reversibly and equipotently, in both tissues. Partially purified rat liver UDP-GDH was used to obtain the kinetic values at pH optima of 9.4 and 8.6. At pH 9.4: K_mUDP-glucose = 15 μM, V_{max} = 5.2 nmol NADH/min/mg protein, K_i = 6 μM. With NAD, a K_i of 16 μM was obtained. At pH 8.6: K_m = 35 μM, V_{max} = 7.5 nmol, K_i = 15 μM. In all of these cases, piperine caused non-competitive inhibition. Data from structure–activity comparisons of piperine analogs indicated that the presence of conjugated double bonds in the side chain of the molecule is a factor in piperine inhibition. However, the UDP-glucuronic acid (UDPGA) contents were decreased less effectively by piperine in isolated rat hepatocytes compared with enterocytes of guinea pig small intestine. Piperine at 50 μM caused a marginal decrease of UDPGA in hepatocytes when the rate of glucuronidation of 3-hydroxybenzo[*a*]pyrene (3-OH-BP) decreased by about 40%. The decrease obtained at 10 μM piperine in intestinal cells was comparable to that obtained at 50–100 μM in hepatocytes. UDP-glucuronosyltransferase (UGT) activities towards 3-OH-BP (UGT1A1) and 4-OH-biphenyl (UGT2B1) were also determined. Piperine did not affect the rate of glucuronidation of 4-OH-biphenyl in rat liver, whereas that of 3-OH-BP was impaired significantly. In guinea pig small intestine, both these activities were inhibited significantly requiring less than 25 μM piperine to produce a more than 50% inhibition of UGT(s). The results suggested that (i) piperine is a potent inhibitor of UDP-GDH, (ii) inhibition is offered exclusively by the conjugated double bonds of the molecule, and (iii) piperine exerts stronger effects on intestinal glucuronidation than in rat liver.

Black pepper (*Piper nigrum* Linn) and long pepper (*Piper longum* Linn) are used world-wide in various traditional systems of medicine [1] and as household spices. One of the major ingredients of the Piper species is piperine (1-piperoyl piperidine). Although piperine was discovered over a century ago,§ reports of its biochemical and pharmacological effects started recently. It has been reported to exhibit antifertility [2] and antiinflammatory [3] activities, stimulate brain serotonin synthesis [4] and intestinal γ-glutamyl transpeptidase activity [5], antagonize respiratory depression induced by morphine or pentobarbitone [6], and inhibit drug-metabolizing enzymes in hepatic [7] and pulmonary [8] tissues *in vitro* and *in vivo*.

Earlier studies showed that piperine increases significantly the plasma concentrations of test drugs, e.g. the naturally occurring compounds vasicine and sparteine in rat [9], phenytoin [10], and propranolol and theophylline [11] in healthy volunteers. We suggested that the enhancement of drug bioavailability by piperine might be a consequence of the observed reversible and non-competitive inhibition of NADPH-dependent cytochrome P450-mediated monooxygenases [7, 8]. In addition, we also observed that piperine lowers the rate of glucuronidation and UDP-glucuronic acid content in the epithelial cells of guinea pig small intestine *in vitro* [12]. The application of piperine, therefore, might be valuable in certain forms of drug therapy because its capacity to enhance drug bioavailability is combined with non-mutagenicity [13], high LD_{50} values [14], and a lack of other adverse effects [15].

Since the rate of glucuronidation *in situ* is influenced by the absolute amount of UDP-glucuronosyltransferase(s) (UGT‖) and the content of its cofactor, UDP-glucuronic acid (UDPGA) [12, 16], it is possible that the reduction in UDPGA content produced by piperine is a consequence of

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§ Cazeneuve C, *Bull Soc Chem* **27**: 291, 1877 [cf. *Merck Index*, 9th Edn, 7266 (1976)].

‖ Abbreviations: UGT, UDP-glucuronosyltransferase; UDPG, UDP-glucose; UDPGA, UDP-glucuronic acid; UDP-GDH, UDP-glucose dehydrogenase; 3-OH-BP, 3-hydroxybenzo[*a*]pyrene; and BP-3-glucuronide, benzo[*a*]-pyrene 3-glucuronide.

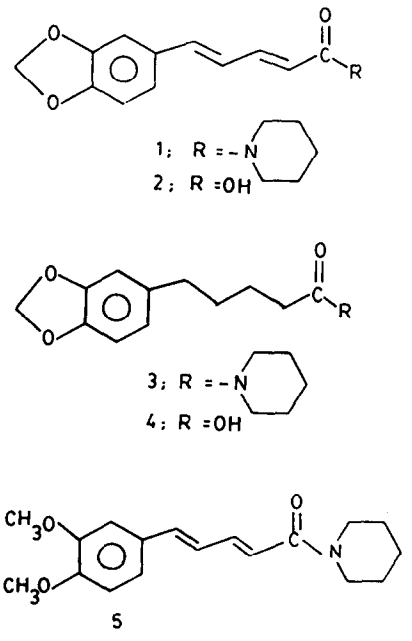


Fig. 1. Structures of piperine analogs.

piperine inhibiting UDP-glucose dehydrogenase (UDP-GDH), a key enzyme involved in UDPGA synthesis. Studies were thus undertaken to explore the effect of piperine primarily on UDP-GDH activity in liver and intestinal epithelium of the rat and guinea pig. Further attempts were made to study simultaneously the influence of piperine on the glucuronidation potentials of these tissues or their major cell types, because UDPGA is a cofactor of UGTs, which catalyze glucuronidation of a large number of compounds mainly in liver and also during intestinal "first-pass."

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: collagenase (lyophilysate from *Clostridium histolyticum*) and pyruvate from Boehringer, Mannheim (FRG); D-galactosamine, NADPH, NAD, UDPGA, UDP-glucose and ethylene-bis (oxyethylene nitrilo) tetracetic acid (EGTA) from the Sigma Chemical Co. (St. Louis, MO). 3-OH-Benzo[*a*]pyrene was a gift from Dr F. J. Wiebel, GSF, D-8042 Neuherberg.

Piperine analogs (Fig. 1). Piperine {**1**} was isolated from chloroform extract of black pepper (*P. nigrum* Linn) and crystallized in ethanol to yellowish white crystals, m.p. 130° (Lit. m.p. 130°; *Merck Index*, 7266, 1976). It was analysed for $C_{17}H_{19}NO_3$. The structure and homogeneity of {**1**} were confirmed by mixed melting point (m.m.p.), co-TLC and superimposable IR.

Piperic acid {**2**} was prepared by alkaline hydrolysis (15% KOH) of {**1**} in ethylene glycol at 198°. It was crystallized from methanol to yellowish crystals, m.p. 216–217° (Lit. m.p. 217°; *Merck Index*, 7260, 1976). It was analysed for $C_{12}H_{10}O_4$. Its structure and homogeneity were confirmed by m.m.p., co-TLC and superimposable IR.

Tetrahydropiperine {**3**} was prepared by hydrogenation of {**1**} in ethanol in the presence of a catalyst (10% Pd/C); it is a colorless viscous liquid and was analysed for $C_{17}H_{23}NO_3$ (M^+ at m/z 289). ^{1}H-NMR ($CDCl_3$) δ: 1.56 (10H, bs, 5x —C<u>H</u>$_2$), 2.40 (4H, m, 2x —C<u>H</u>$_2$), 3.40 (4H, m, 2x N—C<u>H</u>$_2$), 5.82 (2H, s, —OC<u>H</u>$_2$O—), 6.62 (3H, s, Ar—H).

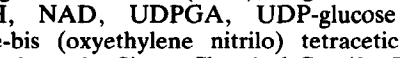
Tetrahydropiperic acid {**4**} was prepared by hydrogenation of {**2**} in ethanol in the presence of a catalyst (10%, Pd/C); it is a colorless crystalline solid, m.p. 96°. It was analysed for $C_{12}H_{14}O_4$. ^{1}H-NMR ($CDCl_3$) δ: 1.82 (4H, m, 2x CH_2), 2.60 (4H, m, 2x —C<u>H</u>$_2$), 5.88 (2H, s, —OC<u>H</u>$_2$O—), 6.78 (3H, s, Ar—H).

Synthesis of 5-(3,4-*dimethoxyphenyl*)-2(E), 4(E)-*pentadienoic acid piperidide* {**5**} (Scheme 1). 3,4-Dimethoxybenzaldehyde {**6**} (4.98 g) was made to react with methylmagnesium iodide in dry ether. After treatment with NH_4Cl solution, it produced {**7**} (5.1 g). The crude {**7**} was subjected to reaction with Vilsmeir reagent [17] and crude cinnamaldehyde derivative {**8**} was obtained. It was crystalized from methanol to light yellow crystals {**8**}, m.p. 85°. It was converted into pentadienoic acid {**9**} in two steps. Compound {**8**} (3 g) was first condensed with the ylide prepared from ethyl bromoacetate and triphenyl phosphine in the presence of sodium hydride in dry dimethoxy ethane. The ethyl ester thus obtained was hydrolysed in ethanolic potassium hydroxide to furnish 5-phenyl-2(*E*),4(*E*)-pentadienoic acid {**9**} (2.5 g), m.p. 166°. Conversion of the acid into the amide {**5**} was carried out through an acid chloride intermediate with thionyl chloride followed by reaction with piperidine to yield {**5**} (2.2 g), m.p. 116°. It was analysed for $C_{18}H_{23}O_3N$.

IR(KBr) cm^{-1}: 2930, 1635, 1565, 1513, 1452, 1440, 1312, 1250, 1131, 1005, 1020, 870, 808, 760. NMR ($CDCl_3$) δ: 1.66 (6H, bs, —CH_2—), 3.59 (4H, bs, $N(CH_2)_2$), 3.92 (6H, s, 2x OCH_3), 6.42

(1H, d, J = 15 Hz, —C(=O)—C<u>H</u>=CH—),

6.73–7.03 (5H, m, =CH and Ar—H), 7.2–7.6

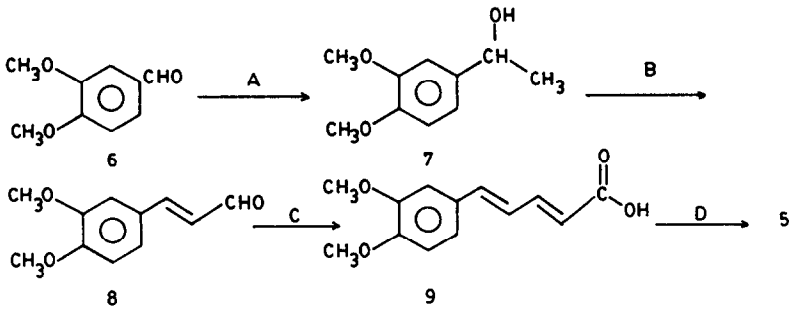


(1H, bm, —C(=O)—CH=C<u>H</u>).

MS: M^+ at m/z 301(49.5), 217(100), 185(47), 155(57), 155(14.5), 114(17.1), 83(15.5).

Animals. Adult male albino Charles Foster rats (180–200 g body wt) and male guinea pigs (strain albino and pigmented, 350–400 g body wt), maintained on pelleted food (Hindustan Lever, Bombay) and water *ad lib.*, were used. The animals were obtained from the breeding centre of this laboratory.

Isolation of rat hepatocytes. The cells were isolated by calcium-free perfusion of liver according to the modification [18] of Berry and Friend [19] and without the use of hyaluronidase in the perfusion medium [20]. The liver cells were dispersed in ice-cold medium C (132 mM NaCl, 5 mM glucose, 0.12 mM $CaCl_2$, 0.85 mM $MgCl_2$, 5.2 mM KCl, 15 mM Hepes, 3 mM phosphate buffer, pH 7.4) where $MgSO_4$ was replaced completely by $MgCl_2$.



A; (i) CH_3MgI/Ether (ii) NH_4Cl

B $POCl_3$/DMF

C (i) Ph_3P/$BrCH_2COOEt$/NaH (ii) KOH/EtOH

D (i) $SOCl_2$/Benzene (ii) Piperidine

Scheme 1.

Table 1. *In vitro* effect of piperine on UDP-GDH activities in liver and intestine of guinea pig and rat

Piperine (μM)	UDP-GDH (nmol/min/mg protein)			
	Guinea pig		Rat	
	Liver	Intestine	Liver	Intestine
0	2.33 ± 0.30	1.20 ± 0.10	3.30 ± 0.28	1.25 ± 0.21
10	1.15 ± 0.09*	0.67 ± 0.11*	1.05 ± 0.09*	0.73 ± 0.12*
25	0.70 ± 0.08*	0.40 ± 0.05*	0.33 ± 0.06*	0.41 ± 0.05*
50	0.22 ± 0.03*	0.21 ± 0.03*	0.22 ± 0.05*	0.13 ± 0.03*

Enzyme activity was determined as described in Materials and Methods. Values are means ± SD from four separate experiments.

* $P < 0.01$ vs untreated control (Dunnett's *t*-test).

Cell viability was routinely tested with 0.05% trypan blue and only preparations having trypan blue exclusion frequency of greater than 87% were used for experimental studies.

Isolation of intestinal epithelial cells and preparation of hepatic microsomes. Epithelial cells of small intestine and microsomes from male guinea pig liver were prepared according to procedures described earlier [12]. The microsomal fraction from male guinea pig liver contains negligible pyrophosphatase activity towards UDPGA, and the preparation was used as a source of UGT for enzymatic determination of UDPGA [21].

Cell incubation system. Cells from both liver and intestine (approx. 3.5 mg/mL) were incubated [12] in medium C containing 10 mM $NaHCO_3$ and 0.13 mM $CaCl_2$ at 37°, where $MgSO_4$ was replaced completely by $MgCl_2$ so as to keep low any sulfation of the substrate 3-hydroxybenzo[*a*]pyrene (3-OH-BP) to BP-3-sulfate which might interfere in the determination of benzo[*a*]pyrene 3-glucuronide (BP-3-glucuronide). After preincubation of cells for 10 min, different concentrations of piperine or D-galactosamine were added in 10 μL of dimethylsulfoxide or normal saline, respectively. Aliquots of 200 μL were taken at different time intervals for determination of UDPGA or BP-3-glucuronide formed after the addition of 3-OH-BP as substrate.

Determination of UDPGA concentration and glucuronidation activity of cells. Aliquots of incubated cells were immediately extracted and the supernatant obtained was used for the determination of UDPGA [21]. Piperine contents carried into the biological probes are diluted at least 15- to 50-fold in the final enzymatic assay system, which does not influence the quantitation of UDPGA. The glucuronidation activity in the intact isolated hepatocytes or intestinal cells towards the substrate 3-OH-BP was determined as described earlier [12].

Enzyme assays. UDP-GDH was determined as described by Pontis and Leloir [22]. Briefly, small intestine was rinsed with normal saline containing 1 mM dithiothreitol, followed by phosphate-buffered saline (PBS) containing 1.5 mM EDTA and 0.5 mM

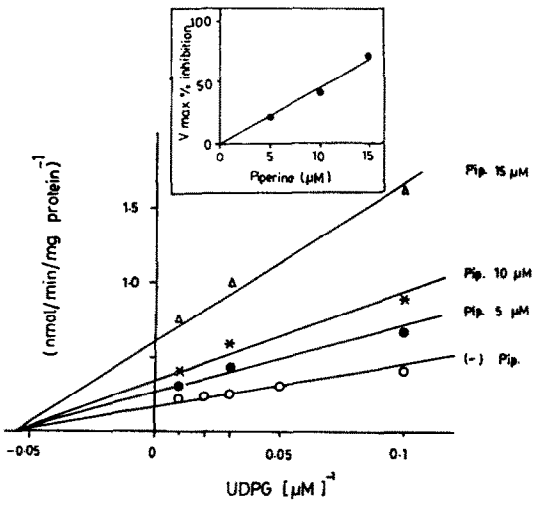


Fig. 2. Lineweaver–Burk plot of UDP-GDH towards UDPG. Partially purified enzyme from rat liver at pH 9.4 was used in the presence and absence of fixed concentrations of piperine. Piperine was introduced in 10 μL of dimethyl sulfoxide (DMSO) before the reaction was started with NAD. Other conditions were the same as described in Materials and Methods. Inset: Piperine-dependent lowering of the V_{max} of UDP-GDH. The values were derived from a double-reciprocal plot.

dithiothreitol at 37°. The epithelial layer was scraped off with a spatula. Rat liver and the intestinal epithelium were homogenized in 0.25 M sucrose (20%, w/v) at 0–4° and centrifuged at 20,000 *g* for 20 min. The enzyme activity was determined in the supernatant. The assay system in a total volume of 1 mL contained 45 μmol Tris–HCl, pH 8.6, 0.5 μmol UDP-glucose (UDPG), 2 μmol NAD and 10–50 μL of the biological probe (0.1 to 0.5 mg). The reaction was carried out at 25°. The increase in absorption at 340 nm per unit time as a function of the dehydrogenase activity was measured using an extinction coefficient of $6.22^6 \times 10^2$/cm/mol of NADH. For enzyme kinetics, rat liver UDP-GDH was partially purified. The post-mitochondrial supernatant of rat liver whole homogenate (10%, w/v) prepared in 0.25 M sucrose was subjected to isolation of the microsomal fraction [12]. The supernatant was used for partial purification [23] by 50% ammonium sulfate saturation, followed by dialysis and heat treatment (50°) of the dialysate at pH 6.2 for 2 min. The supernatant was stored at −20° in small proportions. UGT activities were assayed towards substrates 3-OH-BP [24] and 4-OH-biphenyl [25]. Protein was determined by the method of Lowry *et al.* [26].

RESULTS

In vitro *effect of piperine on the UDP-GDH activity of liver and small intestinal epithelium of guinea pig and rat.* Table 1 shows the interaction of piperine with UDP-GDH activity *in vitro*. Piperine caused a concentration-related inhibition of the dehydrogenase activity. The magnitude of inhibition with indicated concentrations of piperine was similar in both tissues of rat and guinea pig. Piperine at 10 and 50 μM caused about 50 and 90% inhibition, respectively.

Kinetics of inhibition of UDP-GDH. The enzyme was purified partially from rat liver to preclude interference by endogenous substrates and cofactors. The enzyme kinetics were determined in the presence and absence of piperine. Liver UDP-GDH is known to display different inhibition characteristics based on its pH optima of 8.6 and 9.4 [23]. Kinetics of dehydrogenation of UDPG determined at pH 9.4 gave an apparent K_m of 15 μM and a V_{max} of 5.2 nmol NADH formed/min/mg protein (Fig. 2). Increases in piperine concentrations decreased the V_{max} values linearily (inset, Fig. 2). A Dixon plot of the kinetic data indicated that the inhibition was non-competitive with a very low K_i of 6 μM (Fig. 3). When the hepatic enzyme activity was determined with variable enzyme contents against various piperine concentrations, the nature of inhibition was found to be reversible as observed for the intestinal enzyme

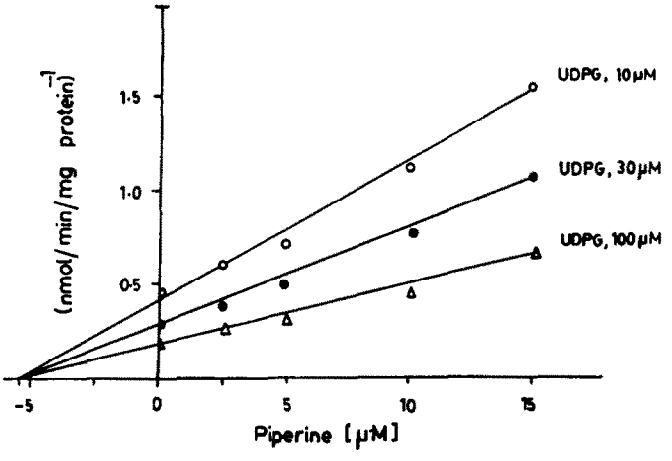


Fig. 3. Dixon plot of UDPG dehydrogenation in the presence of piperine. Other conditions were the same as described in the legend of Fig. 2.

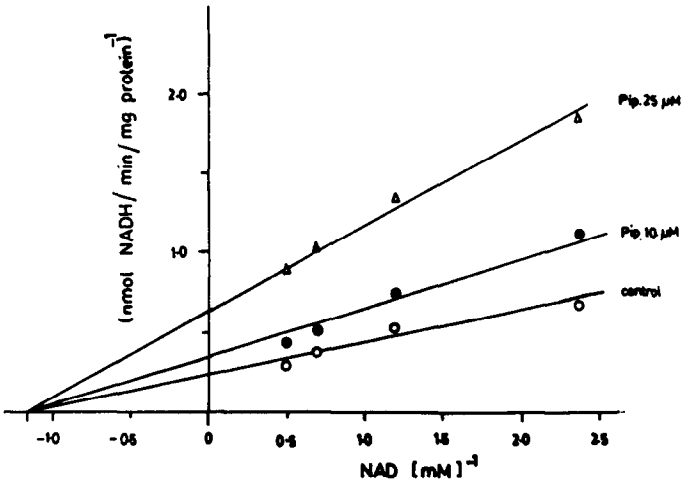


Fig. 4. Lineweaver–Burk plot of UDP-GDH towards the substrate NAD. Other conditions were the same as in Fig. 2.

(see Fig. 5). At pH 8.6, an apparent K_m of 35 μM with a V_{max} of 7.5 nmol was obtained. Piperine inhibited the enzyme activity with a K_i of about 15 μM. The nature of inhibition observed again was reversible and non-competitive (data not shown).

Piperine has a piperidine (hexahydropyridine) moiety in its structure, whereas NAD has a 3-amidopyridine moiety which upon reduction is converted to 3-amido, 4*H*-pyridine. Since UDP-GDH is an NAD-dependent dehydrogenase, one would expect that piperine might inhibit this enzyme competitively with respect to NAD. However, the enzyme kinetics determined with NAD indicated non-competitive inhibition with a K_i of 16 μM for piperine (not shown). The V_{max} value of 4.4 nmol obtained with NAD (Fig. 4) was close to the one obtained with UDPG, while the K_m value of 0.8 mM was quite high. Efforts to conduct kinetic studies on intestinal UDP-GDH were abandoned because the enzyme became inactive following the procedure of partial purification applied to liver. However, the enzyme activity determined in the 20,000 *g* supernatant showed reversible inhibition (Fig. 5). In general, piperine did not appear to discriminate between tissues of the same or different species in its inhibition of UDP-GDH *in vitro*.

Structure–activity relationship of piperine analogs with inhibition of UDP-GDH activity. In our attempt to elucidate the role of functional groups in piperine responsible for inhibition of the UDP-GDH, we used piperine analogs (see Fig. 1) with modification/substitution at different positions in piperine. Removal of the piperidine moiety, viz. piperic acid {2} or substitution of a 3,4-dimethoxyphyenyl group for the methylenedioxyphenyl ring, viz. the dimethoxy analog {5}, did not modulate the potency of inhibition that was comparable to that of piperine (Fig. 6), whereas saturation of both double bonds of piperine, i.e. analogs {3} and {4}, resulted in compounds without inhibitory influence on the enzyme activity. The presence of the hexahydropyridine (piperidine) ring was, therefore, not associated with inhibitory function (Fig. 6).

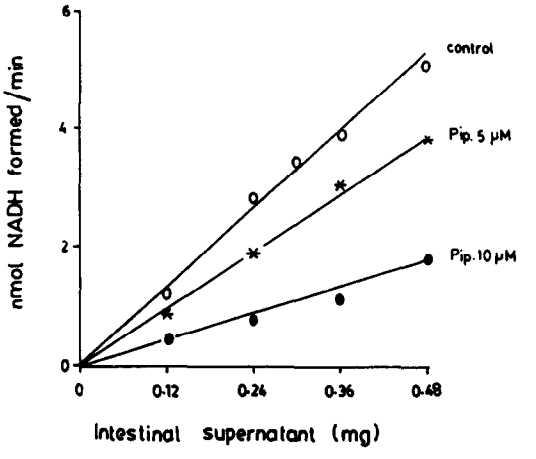


Fig. 5. Inhibition of rat intestinal UDP-GDH by piperine and the nature of its reversibility. The 20,000 *g* intestinal supernatant was used for enzyme assay at pH 8.6. Piperine was added in 10 μL DMSO, and the reaction was started with NAD at 25°.

Effect of piperine on UDPGA levels and the rate of glucuronidation in freshly isolated rat hepatocytes. The UDPGA content in the freshly isolated hepatocytes from rat liver after 10 min of equilibration at 37° ranged between 1.2 and 1.6 nmol/mg of cellular protein. The UDPGA content followed a linear increase with time of incubation so that after 20 min it registered an increase of about 50% above the zero min control (Fig. 7). A similar increase was also noticed earlier by others and one of us [16]. Piperine at concentrations of 10–100 μM caused an initial transient arrest of UDPGA increase at 5 min followed by poor recovery with increase in incubation time. With 100 μM piperine, the UDPGA content was 30–40% lower than the corresponding control value at 20 min, while a similar decrease of low magnitude (15–20%) was observed with 10–50 μM

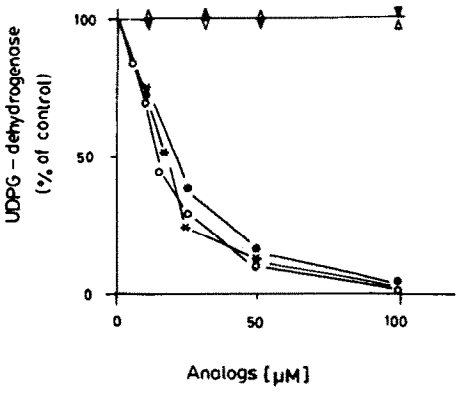


Fig. 6. Structure–activity relationship of piperine analogs with UDP-GDH inhibition. Shown are the effects of piperine analogs, at indicated concentrations, on enzyme activity in rat liver. Other conditions are given in Materials and Methods. Piperine analogs (see Fig. 1): {1} ○—○; {2} ●—●; {3} △—△; {4} ▼—▼; and {5} *—*.

piperine. On the contrary, a much more pronounced inhibition was observed earlier with intestinal cells at far lower concentrations, i.e. 0.5 μM [12] and examined again for comparison purposes in this study (Fig. 8). In contrast, D-galactosamine used as a positive control decreased UDPGA by 70% after 20 min of incubation (see Fig. 7). When the rate of

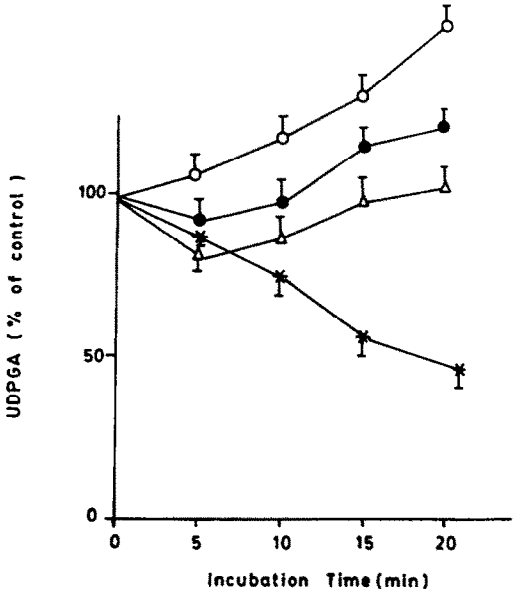


Fig. 7. Modulation of endogenous UDPGA content by piperine in freshly isolated rat hepatocytes. Cells were preincubated for 10 min. Piperine in 10 μL DMSO at 0 μM (○), 10 μM (●) and 100 μM (△), and D-galactosamine at 2 mM (*) were added. At indicated time intervals, aliquots were removed for the determination of UDPGA. Data are expressed as the percentage of UDPGA present in cells after a preincubation time of 10 min in the absence of piperine. One hundred percent = 1.44 ± 0.25 nmol UDPGA/mg protein. Values are means ± SD from three experiments.

3-OH-BP glucuronidation and UDPGA content were determined simultaneously in hepatocytes, piperine was found to cause a concentration-related decrease in glucuronidation of 3-OH-BP, while the decrease in UDPGA content was relatively much lower (Fig. 8). It required 100 μM piperine to cause a 45% decrease in the basal rate of glucuronidation and a <25% decrease in UDPGA content. However, in intestinal cells piperine exerted strong effects. At 10 and 100 μM piperine, glucuronide formation was decreased by 45 and 64%, whereas the UDPGA content decreased by 25 and 43%, respectively (Fig. 8).

Differential effect of piperine on the activities of two major forms of UGT. Recently a nomenclature for the UGT gene superfamily, which consists of two families and a total of three subfamilies, has been proposed [27]. Because of known differential substrate specificity and inducibility characteristics of UGTs, the effect of piperine on two major isoforms corresponding to the two UGT gene families was investigated (Fig. 9). Piperine appeared to affect differently the hepatic activities of two UGT isoforms *in vitro*, although substrate specificity studies have not been done for most of the rat liver UGTs. The hepatic UGT2B1 form towards 4-OH-biphenyl (cf. Ref. 27) was not inhibited by piperine at a concentration even as high as 50 μM, while the UGT1A1 form towards the phenolic metabolite of polycyclic aromatic hydrocarbons, such as 3-OH-BP [28], was inhibited by about 40%. On the contrary, piperine exerted a very strong and concentration-related inhibition on both the isoforms of UGT in the intestinal epithelial cells. It required as little as 25 μM piperine to produce almost 50% inhibition of both the forms in the intestinal cells compared with the hepatic microsomes (Fig. 9). The presence or absence of the detergent Brij-58 did not influence the degree of inhibition in the freshly prepared microsomal fraction.

Effect of microsomal metabolism of piperine on the activity of UGT1A1 in vitro. To determine whether the glucuronidation activity is influenced by the metabolites formed during NADPH-mediated microsomal metabolism of piperine, the UGT activity was measured in hepatic microsomes towards 3-OH-BP with different combinations (Table 2). The extent of transferase inhibition was found to be much lower when microsomes were incubated with piperine in the presence of NADPH than in its absence.

DISCUSSION

The results of the present study suggest that (i) piperine *in vitro* is a potent inhibitor of UDP-GDH activity, and (ii) it affects the glucuronidation potentials of rat liver and guinea pig small intestine at different levels. The alkaloid was found to inhibit UDP-GDH activity of liver and intestine equipotently. The dehydrogenase appeared to be highly sensitive to piperine inhibition with a K_i of about 6 and 15 μM at pH 9.4 and 8.6, respectively. The nature of inhibition appeared to be reversible and non-competitive. The liver enzyme is reported to be hexameric in nature [29], and its activity is

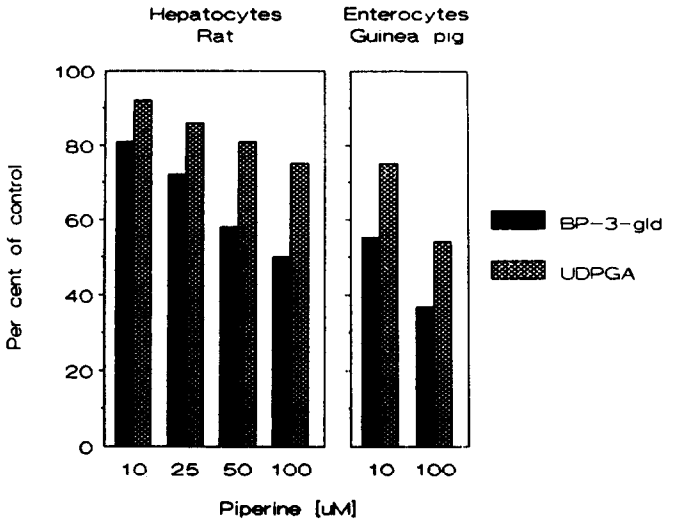


Fig. 8. Comparative effects of piperine on UDPGA content and the rate of 3-OH-BP glucuronidation in freshly isolated rat hepatocytes and enterocytes of guinea pig small intestine. Hepatocytes: Cells (3.5 mg/mL) were pre-equilibrated for 15 min followed by further incubation for 15 min with indicated concentrations of piperine. Aliquots were removed for UDPGA determination followed by addition of 3-OH-BP (50 μM) and again removal of aliquots at 30, 60 and 90 sec for assay of BP-3-glucuronide. Other details are given in Materials and Methods. Data are given as a percentage of UDPGA present or BP-3-glucuronide formed at the end of a 15-min incubation in the absence of piperine. One hundred percent values: UDPGA, 1.71 nmol/mg protein; and BP-3-glucuronide, 0.51 nmol/min/mg protein. Values are representative of two similar experiments. Enterocytes: Cells were pre-equilibrated for 10 min before a 15-min incubation with piperine. Aliquots were removed for assay of UDPGA and BP-3-glucuronide contents. Other conditions were the same as described earlier for the hepatocytes. One hundred percent values: UDPGA, 1.2 nmol/mg protein: and BP-3-glucuronide, 0.31 nmol/min/mg protein. Values are means from three similar experiments.

regulated by pH and various modifiers [23]. Further, it has been reported that the enzyme exists in some form of association–dissociation system; the dissociated form is active and occurs at pH > 6 [30]. We do not know whether the strong inhibition of UDP-GDH at a very low piperine concentration is a consequence of interference with the functionally dissociated state of the enzyme or involves some other interaction causing aggregation of isomers to the inactive state. Nevertheless, piperine appears to interact with sites other than the active site(s) of the enzyme and may thus render the enzyme functionally inactive. This is evidenced from the non-competitive inhibition of the enzyme when UDPG or NAD is used as a substrate. Data from the structure–activity comparisons using various analogs of piperine amply support the above observations that the piperidine part of the alkaloid does not compete with the nicotinamide part of the NAD. Rather the only functional group that appears responsible for eliciting inhibition seems to be the presence of conjugated double bonds in the side chain of the alkaloid. This side chain probably is important in furnishing electrons through the double bonds to the carbonyl part of the molecule which, in turn, may interfere in the dehydrogenation of the substrate UDPG. Nevertheless, the nature of inhibition, though non-competitive, was found to be reversible with enzyme extracts from both liver and intestine. Furthermore, the inhibition appeared similar at both pH 8.6 and 9.4, as evidenced from the kinetic studies.

UDP-GDH is the key soluble enzyme involved in the synthesis of UDPGA, and its inhibition may contribute to reduction of UDPGA content *in situ*. Moreover, endogenous concentration of UDPGA is rate limiting in the glucuronidation of various aglycons [12, 16, 31]. Cofactor availability is thus important for the aglycone disposition of the organism. UDPGA content has also been reported to decrease in hepatic tissue following administration of various drugs [32, 33] or exposure to anaesthetics [31]. However, the results of the present studies reflected species differences, which indicate that the decrease in UDPGA content by piperine appeared to be more pronounced in enterocytes of guinea pig small intestine than in rat hepatocytes in spite of the fact that UDP-GDH was inhibited to the same extent in the extracts of both tissues of the two species. It is also possible that *in situ* turnover of piperine and its interaction with subcellular components, notwithstanding modulation of UDP-GDH, in the intestinal epithelium and liver may be different. For instance, it was observed [34] that 28% of the orally administered piperine is recovered from stomach (22%) and small intestine (6%) in contrast with 0.1 to 0.25% from liver. It has also been shown [35] that piperine metabolism in the human body involves cleavage of the methylene dioxy group and reduction of both the unsaturated double bonds in the side chain. The authors identified four major metabolites from human urine, viz. 5-(3,4-dihydroxy phenyl) 2,4-pentadienoic acid piperidide and its 4-hydroxy-

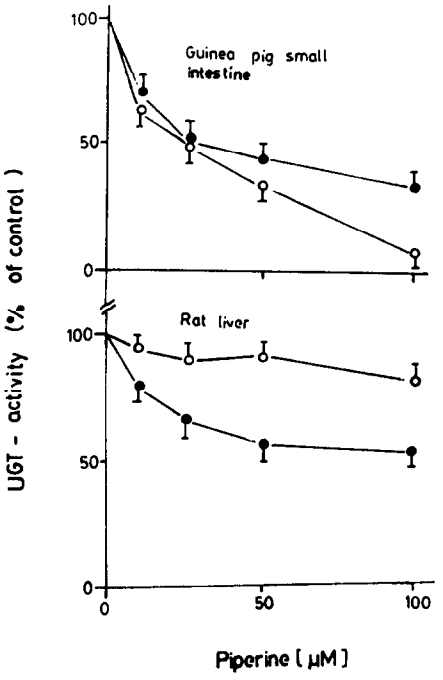


Fig. 9. Differential effect of piperine on the activities of two major forms of UGT from epithelial cells of guinea pig small intestine and rat liver microsomes. UGT activities towards the substrates 3-OH-BP (●—●) and 4-OH-biphenyl (○—○) were determined in duplicate from frozen–thawed samples in the presence of 0.015% Brij-58. Data are means ± SD from three separate experiments. Other conditions were the same as described in Materials and Methods. The specific activities of UGTs, expressed as nmol glucuronide formed per min per mg protein, taken as 100% were: rat liver microsomes: UGT1A1, 2.96 ± 0.17, and UGT2B1, 2.08 ± 0.21; guinea pig enterocytes: UGT1A1, 0.57 ± 0.10, and UGT2B1, 0.78 ± 0.11.

piperidine analog, and their respective tetrahydro-analogs. Only 10% of the individuals exhibited polymorphism where the unsaturated double bonds were not further reduced. Thus, the tetrahydro-derivatives are the major metabolites, and the differential effects of piperine, qualitatively and quantitatively, in intact rat hepatocytes and guinea pig small intestinal enterocytes appear to reflect primarily species differences or differences in their drug-metabolizing capacity as well as of the capability to reduce the side chain. This might account for the reasons of stronger UDPGA-lowering potential of piperine in intestine than in liver.

Table 2. Influence of NADPH on piperine-mediated inhibition of UGT1A1 towards 3-OH-BP of rat liver microsomes

Experimental regimens	BP-3-glucuronide (nmol/min/mg protein)
+ NADPH − Piperine	0.595
− NADPH + Piperine	0.302
+ NADPH + Piperine	0.460

Microsomal protein (0.25 mg) from untreated control rat liver was incubated with 75 μM piperine, 5 mM $MgCl_2$, 0.6 mM NADPH and 50 mM Tris–HCl buffer, pH 7.4, for 15 min in a final volume of 1 mL. Aliquots of 140 μL were taken and further incubated in a volume of 200 μL containing 0.015% Brij-58, 3 mM UDPGA and 50 μM 3-OH-BP. The BP-3-glucuronide formed was measured as described in Materials and Methods. Data are mean values from replicate assays of two separate experiments with the coefficient of variation less than 5%.

Piperine also inhibited UGT activities differently in liver and intestine. In liver it inhibited UGT1A1 activity weakly, whereas UGT2B1 activity was not affected significantly. In contrast, piperine strongly inhibited the activities of these isoforms in the intestine. Based on substrate specificity and inducibility characteristics of UGTs [36], it appears that these forms are quite prevalent in both liver and intestine, which is evidenced by their expression of enzymic activities towards 3-OH-BP and 4-OH-biphenyl as substrates. In determining the effect of piperine, the activities of these enzymes in liver exhibited differential sensitivity, i.e. UGT2B1 activity was not affected, whereas UGT1A1 activity showed significant inhibition; both of these activities were inhibited almost equipotently and strongly in intestine but not in liver. The reasons for this differential effect are not understood at present. However, the functional regulation of UGTs in the biomembranes is influenced by its microenvironment offered largely by phospholipids and lipid–protein interactions [37] and other factors [38, 39]. This might be attributed to a certain extent to the differential inhibition of UGT isoforms in liver and intestine, or differences in inhibition of these two isoforms of UGT activities could conceivably be due to species differences. These studies, nevertheless, point out that piperine exerts much stronger effects on intestinal glucuronidation than in liver. In addition, poor sensitivity of hepatic glucuronidation could also arise from the aforementioned tissue-specific biotransformation of piperine. This is also evidenced from the fact that fortification of the microsomal incubation system containing piperine with NADPH reduced the extent of inhibition of UGT1A1 towards 3-OH-BP. This indicated that NADPH-dependent metabolism of piperine did not augment the inhibition. Moreover, the *in vitro* inhibition of UGT1A1 appeared inevitably due to piperine alone as the assays were performed in the absence of cofactors responsible for piperine biotransformation.

The small intestine is very rich in glucuronidation potentials [36], and various drugs possessing phenolic hydroxyl groups as the pharmacologically active substituents are likely to be attractive substrates for glucuronidation during intestinal "first-pass." Piperine might, therefore, prove to be very useful clinically against intestinal conjugation of such drugs, though further *in vivo* studies are required in this direction. The poor inhibition of hepatic glucuronidation by piperine at high concentrations (50–100 μM), which is at least 75–150 times the average daily intake [40], seems desirable as it is not likely to hamper the disposition of such drugs and endogeneous compounds that require glucuronidation *a priori* to excretion.

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