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Piperine decreases binding of drugs to human plasma and increases uptake by brain microvascular endothelial cells

Dubey, Raghvendra K ; Leeners, Brigitte ; Imthurn, Bruno ; Merki-Feld, Gabriele Susanne ; Rosselli, Marinella

Abstract: We previously reported that piperine, an active alkaloidal principal of black and long peppers, enhances drug bioavailability by inhibiting drug metabolism. Another mechanism influencing drug availability/uptake is its free fraction. Since piperine is highly lipophilic, we hypothesize that it could also interact with drugs through binding displacement and influence their bioavailability. Accordingly, using equilibrium dialysis, we investigated whether piperine alters the binding of model drug ligands, that is flunitrazepam, diazepam, warfarin, salicylic acid, propranolol, lidocaine, and disopyramide to human plasma ($n = 4$). Since alterations in binding influence drug disposition, we also studied the effects of piperine on the uptake of plasma bound 3 H-propranolol and 14 C-warfarin by cultured bovine brain microvascular endothelial cells (BMECs). Piperine (1-1000 M) increased the free fraction (f_u) of both albumin and alpha-acid glycoprotein bound drugs in a concentration-dependent manner ($p < 0.01$). Moreover, piperine (10 M) increased the uptake of 3 H-propranolol and 14 C-warfarin by BMECs ($p < 0.01$). In conclusion, our findings provide the first evidence that piperine displaces plasma bound drugs from both albumin and alpha-acid glycoprotein and facilitates drug uptake across biological membranes (e.g. BMEC). Moreover, it is feasible that piperine may similarly facilitate the transport of drugs into tissues, in vivo, and alter both pharmacokinetics and pharmacodynamics of administered drugs.

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1 **Piperine Decreases Binding of Drugs to Human Plasma and Increases Uptake by**
2 **Brain Microvascular Endothelial Cells**

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5 Raghvendra K. Dubey,^{1,2,3,*} Brigitte Leeners¹, Bruno Imthurn,¹ Gabriele Susanne
6 Merki-Feld¹, Marinella Rosselli,¹

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9 ¹Department for Reproductive Endocrinology, ²Zurich Center for Integrative Human
10 Physiology (ZIHP), University of Zurich, Switzerland; ³Department of Pharmacology
11 and Chemical Biology, University of Pittsburgh School of Medicine, USA

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13 **Running Title :** Piperine Increases Drug Uptake

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15 **Corresponding Author :** Dr. Raghvendra K. Dubey
16 Clinic for Reproductive Endocrinology
17 University Hospital Zurich
18 Wagistrasse 14
19 CH-8952 Schlieren, Switzerland

20 **Tel:** +041-44-556-3070

21 **E-mail:** Raghvendra.dubey@usz.ch

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24 **Keywords:** drugs, bioavailability, piperine, protein binding, transport, endothelial cells

1 **Abstract**

2

3 We previously reported that piperine, an active alkaloidal principal of black and
4 long peppers, enhances drug bioavailability by inhibiting drug metabolism. Another
5 mechanism influencing drug availability/uptake is its free fraction. Since piperine is
6 highly lipophilic, we hypothesize that it could also interact with drugs through binding
7 displacement and influence their bioavailability. Accordingly, using equilibrium dialysis,
8 we investigated whether piperine alters the binding of model drug ligands i.e.
9 flunitrazepam, diazepam, warfarin, salicylic acid, propranolol, lidocaine and
10 disopyramide to human plasma (n=4). Since, alterations in binding influences drug
11 disposition, we also studied the effects of piperine on the uptake of plasma bound ³H-
12 propranolol and ¹⁴C-warfarin by cultured bovine brain microvascular endothelial cells
13 (BMECs). Piperine (1-1000μM) increased the free fraction (fu) of both albumin and
14 alpha-acid glycoprotein (alpha-AGP) bound drugs in a concentration-dependent manner
15 (P<0.01). Moreover, piperine (10μM) increased the uptake of ³H-propranolol and ¹⁴C-
16 warfarin by BMECs (P<.01). In conclusion, our findings provide the first evidence that
17 piperine displaces plasma bound drugs from both albumin and alpha-AGP, and facilitates
18 drug uptake across biological membranes (e.g. BMEC). Moreover, it is feasible, that
19 piperine may similarly facilitate the transport of drugs into tissues, in vivo, and alter both
20 pharmacokinetics and pharmacodynamics of administered drugs.

21

1 **Introduction:**

2 Drug action in the body depends on the rate of metabolism, blood flow, and extent
3 of binding to plasma proteins (Dayton et al., 1973; Wilkinson and Shand, 1975;
4 Wilkinson, 1987; Rowland and Tozer, 1980). Alterations in either characteristics can
5 potentially effect the body distribution of the compound as well as its removal from the
6 body, thereby modulating its pharmacological action (Dayton et al., 1973; Wilkinson and
7 Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980). Moreover, dietary molecules
8 can influence the pharmacokinetics and pharmacodynamics of drugs by altering drug
9 binding as well as metabolism (Melander, 1978). Piperine (1-piperoyl piperidine) a major
10 active ingredient of black and long peppers (**Figure 1**), which is widely used in foods and
11 traditional systems of medicine has been shown to enhance bioavailability of drugs
12 (Srinivasan 2007). For example, piperine increases the plasma concentrations of test
13 drugs such as fexofenadine (Bedada and Boga, 2017; Jin and Han 2010), phenytoin (Bano
14 et al., 1987), propranolol (Bano et al., 1991), theophylline (Bano et al., 1991), rifampicin
15 (Zutshi et al., 1985), in humans. Earlier we suggested an explanation to this phenomenon
16 based on the compound's strong inhibitory effect on hepatic and intestinal drug
17 metabolising enzymes (Atal et al., 1985; Reen et al., 1996) both in-vivo and in-vitro and
18 offered it as the plausible explanation for the mechanism by which it could enhance drug
19 bioavailability and hence prolong drug action. Although inhibition of the monooxygenases
20 by certain compounds can enhance bioavailability of drugs (Sontaniemi, 1993) and alter
21 their pharmacokinetics and pharmacodynamics (Dayton et al., 1973; Wilkinson and
22 Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980), other mechanisms such as
23 alterations in plasma binding may also be involved.

1 Drug molecules circulate in blood in two rapidly interchangeable forms (Dayton
2 et al., 1973) bound to plasma proteins such as albumin, and/or α -acid glycoprotein
3 (Wilkinson and Shand, 1975) free or unbound form (Kwong, 1985). The extent of drug
4 binding to plasma proteins has important implications on drug disposition and drug action
5 (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; MacKichan, 1989).
6 Drug effect often depends on the free drug in plasma, because it is this fraction of drug
7 which can cross capillary and cell membranes and hence is an important determinant for
8 its ability to leave the vasculature and gain access to receptors, tissues, various sites of
9 elimination and metabolism (MacKichan, 1989). Alterations in plasma protein binding is
10 known to effect drug disposition (Wilkinson and Shand, 1975; Wilkinson 1987; Martin,
11 1965; Jusko and Gretch, 1976). Change in the free fraction alters hepatic elimination and
12 distribution (Wilkinson and Shand, 1975; Wilkinson, 1987) with resulting changes in
13 concentration at the site of action. Piperine, being highly lipophilic, could potentially
14 effect the binding interaction of drugs to plasma proteins and hence, influence the
15 disposition parameters of plasma bound drugs. Accordingly, we investigated the effects
16 of piperine on the binding interaction of a number of acidic and basic drugs to plasma
17 from healthy subjects. Additionally, we investigated the effects of piperine on the uptake
18 of propranolol and warfarin by cultured bovine brain microvascular endothelial cells.

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MATERIALS AND METHODS

Materials:

Diazepam, flunitrazepam, warfarin, salicylic acid, propranolol, lidocaine, antipyrine, disopyramide, phenazine and piperine were purchased from Sigma Chemical Co. (St. Louis, MO). Biodegradable scintillation fluid, acetonitrile, methyl-³H-diazepam (57 mCi/mmol), N-methyl-³H-flunitrazepam (57 mCi/mmol), 1-¹⁴C-warfarin (46 mCi/mmol), DL-³H-propranolol (18 mCi/mmol), carbonyl-¹⁴C-lidocaine hydrochloride (48 mCi/mmol), were obtained from Amersham Corp. (Arlington Heights, Ill.); 7-¹⁴C-salicylic acid (58 mCi/mmol) was from Du Pont Co. (New England Nuclear Research Products, Boston, Mass.); N-methyl-¹⁴C-antipyrine (50 mCi/mmol) from ICN Radiochemicals (Irvine, CA.); and 3-¹⁴C-disopyramide (4.3 mCi/mmol) from G.D. Searle and Co. (Chicago, Ill). All radiolabelled drugs were >98% pure as determined by thin layer chromatography. Dialysis microcells and semipermeable membrane (mol. wt. cut off 12,000 to 14,000 kd; Dianorm dialyzer with spectrapor membrane No.2) were procured from Spectrum Medical Industries (Los Angeles, CA.) and Ultrasphere column (5- μ m, octadecyl 25 x 4.6mm) from Beckman Instruments Inc. (Fullerton, CA). Tissue culture: dispase, deoxyribonuclease-I, rat tail collagen, fibronectin, endothelial cell growth supplement, penicillin-G, streptomycin, polymyxin-B, amphotericin-B, heparin, tris-base, were purchased from Sigma; collagenase/dispase from Boehringer-Mannheim biochemicals (Indianapolis, Ind.); Minimum essential medium (MEM), DMEM-F12 Ham nutrient mix HEPES, from Gibco laboratories (Grand Island, NY); fetal calf serum from Hyclone Laboratories (Logan, UT); pycarbonate membranes (13mm diameter, 12- μ m pore size) from Nucleopore Co., (Pleasanton, Calif.); Percoll from Pharmacia LKB

1 Biotechnology Inc. (Piscataway, NJ); and immunohistology kit for endothelial cell
2 characterization (Rabbit anti-factor VIII polyclonal antibodies) from Biogenex Labs.
3 (Dublin, CA). All other tissue culture and drug binding chemicals used were of the best
4 available grades.

5

6 **Drug Binding Studies:**

7 Blood (50ml) was collected by venepuncture into EDTA-treated tubes from
8 healthy male subjects/volunteers (n=4) with an average age of 30 ± 2 years and following
9 the institutional ethical guidelines at that time. The subjects were fasted overnight and
10 had taken no medications for at least 1 week prior to obtaining the blood sample, and
11 were non-smokers. Plasma was obtained by centrifuging blood at 1000g for 30 min at
12 4°C . Drug standards were added to the freshly aspirated plasma in the absence or presence
13 of piperine (1, 10, 100 or 1000 μM) to achieve a concentrations typical of those observed
14 in clinical studies and as follows: diazepam, 0.1 $\mu\text{g/ml}$; flunitrazepam, 0.1 $\mu\text{g/ml}$;
15 warfarin, 1 $\mu\text{g/ml}$; salicylic acid, 1 $\mu\text{g/ml}$; propranolol, 0.1 $\mu\text{g/ml}$; lidocaine, 3 $\mu\text{g/ml}$;
16 antipyrine, 10 $\mu\text{g/ml}$ and disopyramide, 10 $\mu\text{g/ml}$. Each of the standard solutions contained
17 a tracer quantity of radiolabelled drug appropriately diluted with unlabelled compound to
18 produce the required total concentration.

19

20 Plasma binding was determined by equilibrium dialysis at 37°C using microcells
21 and a semipermeable membrane (Mol. wt. cut-off 12,000-14,000 kd). Plasma (0.2ml) was
22 dialysed against an equal volume of 0.067 mol/L phosphate buffer, pH 7.4 for 4 hours.
23 The percentage of drug present in plasma in unbound form calculated using following
24 equation:

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$$\% \text{ Unbound drug} = \frac{\text{Drug concentration in buffer}}{\text{Drug concentration in plasma}} \times 100$$

Drug concentrations of the radiolabelled ligands was determined by liquid scintillation counting with external standardization of 50 µl of plasma or buffer added to 15 ml counting scintillation fluid.

Isolation and Culture of Brain Microvascular Endothelial Cells:

Microvascular endothelial cells were isolated from bovine brain according to the method of Audus and Borchardt (Audus and Borchardt, 1987), with minor modifications. Briefly, the cortical tissue of two or three bovine brains obtained from the local abattoir was cleaned of meninges and all large vessels. The gray matter from the cortical region was dissected, minced, and passed through a sterile stainless steel wire mesh (1mm square), and digested for 20-30 min at 47°C with a solution of 12.5% dispase (4ml /50gm tissue) and deoxyribonuclease-I (20µg/ml) in MEM buffered with 50µM HEPES. Subsequent to this incubation, Dulbeccos' minimal essential medium (MEM) (equal to the weight of grey matter) buffered with TRIS-base (pH 9.5) was added and the mixture further incubated for 2.5-3 hours. After digestion with dispase the cell suspension was centrifuged at 3200 rpm for 10 min and the pellet suspended in 500 ml of dextran (13%) and centrifuged at 7750 rpm for 10 min. The crude capillaries were obtained as a pellet whereas, the floating cell debris, fat, myelin was discarded. Capillary pellet was further

1 digested for 4hrs at 37°C with a solution of collagenase/dispase (1mg/ml) and
2 deoxyribonuclease-I (20µg/ml). The digested suspension was centrifuged at 1000g for 10
3 min, and the pellet suspended in 8ml MEM and 2ml aliquots of it layered over 50ml of
4 pre-established percoll gradient and centrifuged at 3750 rpm for 10 min. Endothelial cells
5 were collected from the percoll and washed and suspended in complete culture medium
6 (1:1 mixture of MEM and DMEM-F12 ham, 10mM HEPES, 13mM NaHCO₃, 100 µg/ml
7 penicillin G, 100 µg/ml streptomycin, 50µg/ml polymyxin B, 2.5µg/ml amphotericin B,
8 100 µg/ml heparin, 25µg/ml endothelial cell growth supplement and 20% fetal calf
9 serum). Cell viability was determined by trypan blue exclusion technique and isolated
10 cells for later use were frozen at -70°C in complete culture medium supplemented with
11 10% dimethyl sulfoxide.

12
13 Frozen or freshly obtained endothelial cells were thawed, suspended in complete
14 culture medium and plated at a density of 2.5×10^6 cells/100mm in a culture dish
15 containing polycarbonate membranes (13mm diameter). The polycarbonate filters had
16 been fixed to the culture dish surface and coated with rat-tail collagen and fibronectin (40
17 µg/ml), cross linked with ammonia fumes, and sterilised under UV light as described
18 before (Audus and Borchardt, 1987). The medium was changed every day after four days
19 of plating and the cells reached confluence in 10-12 days as determined visually under a
20 light microscope. The purity of the cells was established morphologically and by
21 immunofluorescent staining the cells to test for the presence of anti-Von willebrand factor
22 VIII as described before (Audus and Borchardt, 1987) using monoclonal antibodies.

23

24 **Drug Uptake Studies:**

1 Brain microvascular endothelial cells (BMECs) play a key role in regulating
2 transport of drugs across the blood-brain barrier. Cultured BMECs have been widely used
3 as a model to assess the transport of drugs across BBB, in vitro, as well as uptake of drugs
4 (Audus and Borchardt, 1987). Since free fraction of drugs is postulated to cross BBB
5 (Dubey et al., 1989) we used BMECs to assess the effects of piperine on drug uptake.
6 Endothelial cells grown to confluence on polycarbonate filters were incubated with
7 plasma containing 0.1µg/ml propranolol or 1.0µg/ml warfarin (close to clinical
8 concentrations), and pulsed with 10.5×10^6 dpm/ml of ^3H -propranolol or 15×10^6
9 dpm/ml ^{14}C -warfarin, in the absence or presence of 10 µM, piperine, at 37°C for 5, 15,
10 30, 60, and 120 seconds. Immediately after incubation the filters were washed 4 times by
11 carefully suspending them in ice-cold phosphate buffered saline. The filters were placed
12 in scintillation vials and treated overnight with 0.5ml of 0.3N NaOH in 1%
13 sodiumdodecyl sulphate and drug concentrations determined by liquid scintillation
14 counting. In separate experiments conducted in parallel, the effect of piperine on cell
15 viability was determined by looking for trypan blue exclusion. Moreover, the loss of cells
16 during treatment were assessed by counting the cells after trypsinization and by looking
17 at monolayers under a light microscope after treatment.

18
19 **Statistical Analysis:** Differences in the plasma binding of drugs in piperine treated and
20 untreated groups were evaluated by the paired Student's t-test, whereas the differences in
21 drug uptake and cell viability in treated and untreated groups was evaluated by ANOVA,
22 utilising intergroup comparisons by Fisher's Least Significant Difference Test (Numbers
23 Cruncher Statistical System, Kaysville, UT). In both cases $p < 0.05$ was taken as the
24 minimum level of statistical significance.

25

RESULTS

Effect of Piperine on the Binding of Drugs to Human Plasma:

Piperine (**Figure 1**) being highly lipophilic in nature bound extensively to human plasma, with a free fraction of 0.015 ± 0.004 . Pre-treatment of plasma with different concentrations of piperine significantly ($p < 0.01$) altered the binding of both acidic and basic drugs in a concentration dependent manner (**Figure 2A and 2B**). In presence of piperine ($100 \mu\text{M}$) the increase in free fraction of various acidic drugs (mean \pm S.D., $n=4$) was: 2.74 ± 0.06 fold ($p < 0.01$) for flunitrazepam; 3 ± 0.16 fold ($p < 0.01$) for diazepam; 4.6 ± 0.08 ($p < 0.01$) warfarin; 2.4 ± 0.09 ($p < 0.01$) salicylic acid, and for basic drugs was: 1.9 ± 0.03 fold for propranolol ($p < 0.01$); 1.5 ± 0.02 fold ($p < 0.01$) for lidocaine; 1.5 ± 0.04 fold for disopyramide ($p < 0.01$).

There was no effect of piperine on the binding of antipyrine at any concentration. Since $100 \mu\text{M}$ piperine significantly increased the unbound fraction of all drugs, except antipyrine, we chose this concentration of piperine to assess the effects on bound to free ratio. The relative decrease in bound to free ratios of these drugs at $100 \mu\text{M}$ piperine concentration was in the order: warfarin > diazepam > flunitrazepam > salicylic acid > propranolol > lidocaine > disopyramide (**Figure 3**). In presence of $100 \mu\text{M}$ piperine the bound to free ratio (B/F) of albumin bound drugs (diazepam, flunitrazepam, warfarin, salicylic acid) decreased by 3.16 ± 0.8 fold, whereas, the bound to free ratio of α -AGP bound drugs (propranolol, lidocaine, disopyramide) decreased by 1.65 ± 0.05 fold (**Figure 3**). The decrease in B/F ratio of albumin bound drugs was significantly higher ($p < 0.01$) than those bound to α -AGP.

1 Effect of Piperine on Cell Viability:

2 Since the uptake studies were conducted for 2 min, we chose a longer i.e. 15
3 minute period to study if the effects of piperine on drug uptake were not compromised by
4 cell toxicity. Treatment with the highest concentration (1mM) of piperine for 15 min did
5 not induce cell toxicity. The BMEC viability (trypan blue exclusion) in monolayers
6 treated with vehicle or 1 mM piperine for 15 min was $99 \pm 2 \%$ and $98.5 \pm 3 \%$,
7 respectively ($P > 0.05$ vs vehicle). No floating dead cells were detected in the medium of
8 BMEC monolayers treated for 15 min with vehicle and piperine 1mM, respectively.
9 Moreover, the dead cell count in monolayers grown on polycarbonate filters treated with
10 or without piperine did not differ.

12 Effect of Piperine on Uptake of Drugs:

13 Piperine significantly increased the free fraction of warfarin and propranolol at a
14 concentration of $10\mu\text{M}$. Since free fraction of drug governs its uptake, we selected this
15 concentration for the uptake studies using monolayers of BMECs (**Figure 4A**). Uptake
16 of propranolol and warfarin by BMECs was time dependent. In absence of piperine
17 warfarin uptake by BMECs was significantly lower than that observed for propranolol
18 ($p < 0.05$). However, in the presence of $10\ \mu\text{M}$ piperine the uptake of warfarin and
19 propranolol by BMECs was significantly increased ($p < 0.01$; **Figure 4B and 4C**,
20 respectively). The increase in uptake after 60 seconds of incubation was 3.8 ± 0.4 fold and
21 2.1 ± 0.04 fold, respectively. This increase in uptake was proportional to the increase in
22 free fraction of propranolol and warfarin which in the presence of $10\ \mu\text{M}$ piperine
23 increased, from 0.229 ± 0.05 to 0.411 ± 0.02 for propranolol ($P < .05$) and from 0.009 ± 0.003
24 to 0.022 ± 0.002 for warfarin ($P < .05$).

DISCUSSION

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Drug action in the body depends on the rate of absorption, distribution, metabolism and elimination (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980). The pharmacological response obtained is for a majority of drugs, related to the concentration of drug at receptor site(s) (MacKichan, 1989). Moreover, the binding of blood-borne endogenous or exogenous ligands to plasma constituents is an important determinant of the compounds distribution in and removal from the body (Wilkinson, 1987). The binding affinity of drugs to plasma proteins defines their ability to diffuse across the vasculature and gain access to receptors in various organs or tissues. Moreover, it governs the elimination and metabolism characteristic of a drug. Indeed, changes in a compound's binding to plasma proteins can result in altered drug distribution and unexpected pharmacological responses (Dayton et al., 1973; Svensson et al., 1986). Plasma albumin is quantitatively the most important protein to which a large number of drugs, particularly the neutral and acidic (anionic) drugs, bind in a reversible fashion, (Sjoholm et al., 1979). Albumin contains a number of sites with differing specificities. Warfarin for example, binds with high affinity to site-I, which is also the primary binding site for bilirubin, whereas benzodiazepines and tryptophan interact more specifically with site-II. By contrast, the albumin binding of salicylic acid involves both these sites. While acidic drugs bind exclusively to albumin, drugs with basic character bind to α -acid glycoprotein more extensively, than to plasma albumin (Kwong, 1985, MacKain, 1989; Matin, 1965). Moreover, as compared to albumin, alpha-AGP contains only one binding site to which basic and some typically site-I albumin binding drugs may gain access to.

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Drug binding, can be modified by the presence of other exogenous or endogenous compounds, which can compete for the same binding sites (drug displacement by competitive inhibition). Alternatively, two ligands can be bound to two separate sites and mutually effect each other's binding by free energy coupling between the two sites (Weber, 1975). Our observation that piperine increases the free fraction of drugs which bind to albumin (diazepam, flunitrazepam, warfarin, salicylic acid) and alpha-AGP (propranolol, lidocaine, and disopyramide), strongly suggests that piperine binds to both albumin and alpha-AGP. Comparison of the displacing effects of piperine on the binding of both acidic and basic drugs to human plasma demonstrates that the effect is more pronounced for acidic drugs. Moreover, it suggests that piperine is more effective in displacing albumin bound drugs. A two fold difference in the bound to free ratio of warfarin (site-I) and salicylic acid (site-II) suggests a higher affinity of piperine for site-I. Together, these findings suggest that piperine effectively displaces highly plasma bound drugs from both site-I and site-II of albumin and alpha-AGP.

For drugs that bind to more than one protein in plasma, significant displacement from one of the binding proteins may not result in a demonstrable change in unbound fraction and result in unaltered pharmacokinetics and pharmacodynamics. However, the observation that piperine displaces drugs, which are bound to both albumin and alpha-AGP (disopyramide, propranolol, and lidocaine) suggests that piperine may also be effective in altering the pharmacokinetics and pharmacodynamics of these drugs.

1 Distribution of drugs from the blood into the tissues occurs by the passage of drugs
2 with low molecular weights (<1000) through the layer of endothelial cells of the
3 capillaries, and binding of drugs to plasma proteins restricts the extravascular distribution
4 of drugs (MacKichan, 1989). The observation that the uptake of propranolol and warfarin
5 by endothelial cells was significantly increased in presence of piperine, and that there was
6 a parallel increase in the free fraction of these drugs, suggests that the enhanced uptake
7 of warfarin and propranolol was related to increase in free fraction of the drugs in
8 presence of piperine. It is plausible that similar effects could occur in-vivo and result in
9 enhanced transport, of drugs (displaced by piperine) across the biological membrane and
10 into the tissues. Accordingly, the displacement of alpha-AGP or albumin bound drugs by
11 piperine might result in altered pharmacokinetics and pharmacodynamics.

12
13 Since, plasma binding is also an important determinant of the systemic clearance
14 of a drug, with different effects depending on the free intrinsic clearance of the compound
15 (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; MacKichan, 1989).
16 It is conceivable that displacement of drugs by piperine may also result in altered systemic
17 clearance and therapeutic efficacy of the drug. For a poorly eliminated drug, clearance is
18 "restricted" to the unbound moiety and an increase in this fraction results in a proportional
19 enhancement in the clearance of total drug. Although, displacement of drugs by piperine
20 will not affect elimination half-life of displaced drugs with moderate to large volumes of
21 distribution, however, it may shorten the half-life of drugs with small volume of
22 distribution. Since piperine also inhibits hepatic and intestinal drug metabolizing enzymes
23 (Atal et al., 1985; Reen et al., 1996), it is possible that the intrinsic clearance of the drugs
24 will decrease, and this in combination with increased free concentration of the drug may

1 result in sustained increase in free concentrations of displaced drugs at the site of action.
2 Moreover, piperine induced displacement of drugs (propranolol, lidocaine) whose
3 clearance is not limited by binding to plasma proteins "non-restrictively cleared" could
4 result in immediate and sustained increase in unbound concentration. This may be of
5 clinical significance for drugs with narrow therapeutic indices and might minimally
6 prolong the half-life of drugs with a small volume of distribution, and greatly for drugs
7 with large volume of distribution.

8

9 The impact of alterations in protein binding on drug clearance depends on the
10 characteristics of the drug (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson,
11 1987; Rowland and Tozer, 1980). Clearance of restrictively cleared drugs is limited to
12 free fraction of the drug and clearance of highly bound and restrictively cleared drugs are
13 sensitive to alterations in protein binding. Hence, displacement of restrictively cleared
14 drugs (warfarin) by piperine may result in higher free fraction, which will lead to higher
15 clearance rates, shorter elimination half-lives and a larger fluctuation in peak and trough
16 levels. Furthermore, a transient increase in free drug can be of sufficient magnitude to
17 precipitate an adverse drug reaction if the drug has small volume of distribution and a
18 narrow therapeutic index. However, in case of non-restrictively cleared drugs
19 (propranolol, lidocaine), which are extracted by active mechanisms in both free and
20 bound forms by the liver or the kidney, the clearance is dependent on organ blood flow
21 and relatively independent of protein binding. Hence, alterations in free concentrations of
22 these drugs will not be of clinical significance.

23

1 In-vivo the efficacy of the drug not only depends on protein binding, but also
2 greatly influenced by its metabolism. Small concentrations of several drugs reach the
3 circulation when given orally, and are lost due metabolism "first pass effect". Piperine
4 enhances the bioavailability of theophylline, propranolol and phenytoin in healthy
5 volunteers (Melander, 1978; Bano et al., 1987; Bano et al., 1991) and increase the effects
6 of hexobarbital and zoxazolamine in a reversible fashion (Atal et al., 1985). Furthermore,
7 we previously demonstrated that the enhancement of drug bioavailability by piperine was
8 possibly due to reversible and non-competitive inhibition of NADPH-dependent
9 cytochrome P-450-mediated monooxygenases (Atal et al., 1985). In addition, we
10 observed that piperine also lowers the rate glucuronidation in intestine and liver (Singh
11 et al., 1986). Since piperine inhibits both monooxygenases and conjugases in the intestine
12 and liver, sites where the drugs are lost due to first pass effect, it is plausible that higher
13 concentrations of the drug will reach the circulation. However, upon reaching the
14 circulation, the drug is bound to proteins like albumin and alpha-AGP and the delivery of
15 the drug to the target tissue and receptor sites is restricted to the unbound fraction of the
16 drug. Since, piperine displaces drugs from both albumin and alpha-AGP this would result
17 in higher free concentrations of the drug and higher amounts of drug reaching the target
18 tissues and receptors or even increased elimination or clearance of the administered drug.
19 Hence, piperine could potentially alter the pharmacokinetics and pharmacodynamics of
20 drugs irrespective of the route of administration.

21

22 Although, our findings provide evidence that piperine reduces drug binding to
23 plasma proteins and increases their uptake by BMECs in vitro, it has limitations. First,
24 whether piperine influences drug binding in vivo and whether this is associated with

1 enhanced drug bioavailability is unknown. Second, piperine binds reversibly to serum
2 albumin by hydrophobic interactions with a binding constant of 10^4 Mol/L and a
3 stoichiometry of 1:1 preferably at the binding site of subdomain I-B. Even though binding
4 constant of piperine with serum albumin is moderate, major portion of the piperine in
5 blood circulation may still be in the bound form because of very high physiological
6 concentration of albumin (0.6 mM). Based on these characteristics, whether piperine can
7 compete with other drugs for the binding site on serum albumins and consequently make
8 the drugs available in the un-bound form remains unknown and can only be speculated.
9 Third, the serum levels of piperine following oral or intraperitoneal administration are
10 0.15% of the dose administered, moreover significant presence of piperine metabolite in
11 serum have been reported (Srinivasan 2007; Suresh and Srinivasan 2010; Bajad et al.,
12 2003). Whether circulating concentrations of piperine and its metabolite can influence
13 drug binding in vivo remains unknown. Finally, the fact that piperine increases the uptake
14 in BMECs suggests that it may facilitate transport of drugs across the blood-brain barrier.
15 However, in vivo brain-uptake index studies are required to confirm this possibility.

16
17 In conclusion, piperine significantly decreases the binding of both acidic and basic
18 drugs to human plasma and increases the uptake of warfarin and propranolol by BMECs.
19 The findings that piperine inhibits drug metabolism as well as increases the free fraction
20 of plasma bound drugs, suggests that piperine can potentially alter the pharmacokinetics
21 and pharmacodynamics of drugs irrespective of the route of administration. Moreover,
22 piperine may be useful in modulating the effects of clinically used drugs and this
23 possibility needs further investigation.

24

1 **Abbreviations:** Brain microvascular endothelial cells, BMECs; alpha-acid glycoprotein,
2 alpha-AGP; free fraction, fu; Dulbeccos' minimum essential medium, MEM

3

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9

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24

1 **Figure Legends:**

2

3 **Figure 1:** Chemical structure of piperine.

4

5 **Figure 2:** Bar graph depicting the concentration-dependent effects of piperine (1, 10,
6 100, 1000 μM) on the binding of: (A) salicylic acid, diazepam, warfarin and lidocaine;
7 and (B) propranolol, flunitrazepam, disopyramide and antipyrine, to human plasma.
8 Results (mean \pm S.D.) express changes in the unbound free fraction (f_u) of the drug in
9 presence or absence of piperine (n=4). * represents $P < 0.05$ versus samples treated with
10 vehicle alone.

11

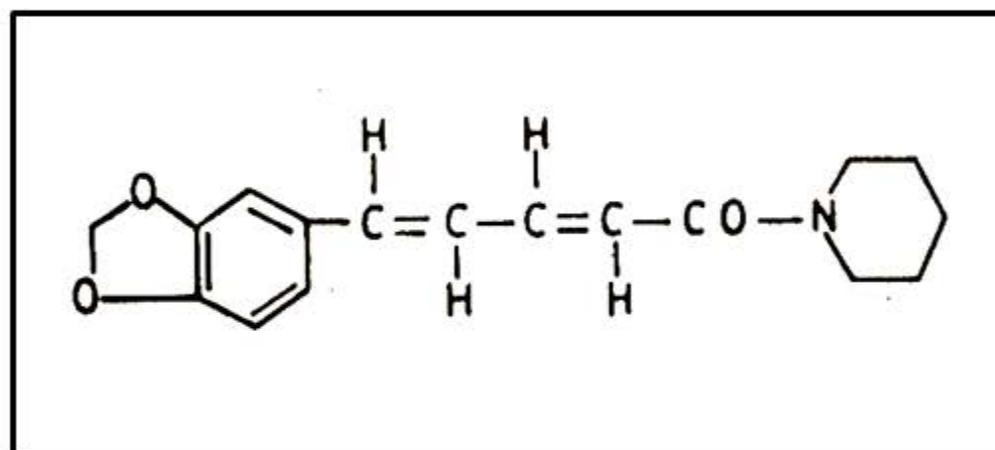
12 **Figure 3:** Bar graph comparing the effects of piperine (100 μM) on the bound/free ratio
13 of (A) alpha-AGP bound drugs (propranolol [PRL], lidocaine [LDC], disopyramide
14 [DPY]) and (B) albumin bound drugs (diazepam [DZP], flunitrazepam [FNZ], warfarin
15 [WRF], salicylic acid [SA], to human plasma. Results are expressed as mean \pm S.D.(n=4).
16 * represents $P < 0.05$ versus samples treated with vehicle alone.

17

18 **Figure 4:** Panel A depicts a representative photomicrograph of a confluent monolayer of
19 brain microvascular endothelial cells used for drug uptake studies. Panel B depicts a line
20 graph showing the effects of piperine (10 μM) on the time-dependent uptake of plasma
21 bound ^{14}C -warfarin by bovine brain microvascular endothelial cells. Values at each point
22 represents mean \pm S.D. (n=4) and expressed as disintegration per minute (dpm)/ 17.7×10^4
23 endothelial cells. * represents $P < 0.05$ versus samples treated with vehicle alone. Panel C
24 depicts a line graph showing the effects of piperine (10 μM) on the time-dependent

1 uptake of plasma bound ³H-propranolol by bovine brain microvascular endothelial cells.
2 Values at each point represents mean ± S.D. (n=4) and expressed as disintegration per
3 minute (dpm)/17.7x10⁴ endothelial cells. * represents P<0.05 versus samples treated with
4 vehicle alone.
5

Figure 1



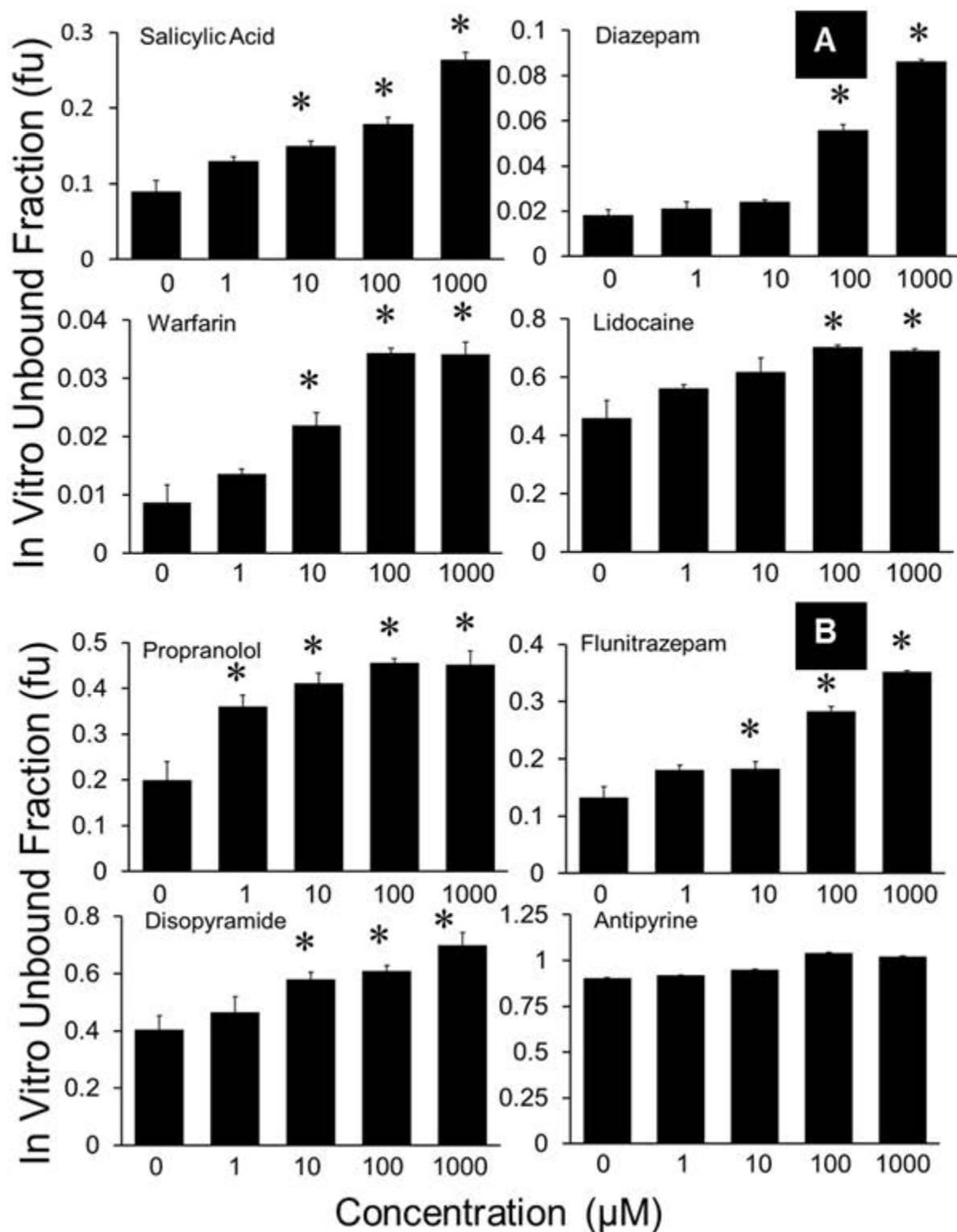


Figure 3

